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# Regulatory T cells in autoimmune diseases

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## Abstract

During the last years, our understanding of regulatory T cell ($T_{reg}$ cell) biology has greatly expanded. Key observations have challenged the traditional definition of $T_{reg}$ cell and have provided insight into the underlying mechanisms responsible for the development of autoimmune diseases with new therapeutic strategies that improve disease outcome. This review summarizes the newer concepts of $T_{reg}$ cell instability, plasticity and tissue-specific $T_{reg}$ cells and their relation to autoimmunity. These three major concepts have changed our understanding of $T_{reg}$ cell biology: how they interact with other immune and non-immune cells, their functions in specific tissues, and the implications for the pathogenesis of autoimmune diseases.

More than 20 years after their ‘re-discovery’, regulatory T cells ($T_{reg}$ cells) have emerged as an important component in our understanding of the immune response to pathogens and the mechanisms of peripheral tolerance that control the development of allergies and autoimmune diseases. In mice and humans, $T_{reg}$ cells are characterized by the high expression of the IL-2 receptor alpha chain (IL-2Rα, CD25) and the expression of the transcription factor Foxp3, which is required for their development, function and stability[1, 2, 3, 4]. In humans, as CD25 is also expressed by activated $CD4^+$ T cells, the absence of the IL-7 receptor alpha chain (IL-7Rα, CD127) is used as a complementary marker to CD25 expression to more precisely identify human $T_{reg}$ cells[5]. Moreover, numerous surface receptors have been described that are variably specific for defined $T_{reg}$ cell subsets, arguing for the heterogeneity of this population[6]. $T_{reg}$ cells can be broadly classified into two groups based on their developmental origin. Thymic $T_{reg}$ cells (tTregs) – also known as natural $T_{reg}$ ($nT_{reg}$) cells – are generated in the thymus as a separate lineage at the stage of CD4 single-positive thymocytes, and are thought to be enriched for T cell receptors (TCR) with high affinity for self-peptides[7]. Although their detailed mechanisms of suppression are still not completely understood and are most probably dependent on the microenvironment and the target population to be controlled, in general they perform their function by both cell-contact mechanisms that involve specific cell surface receptors, and the secretion of inhibitory cytokines such as IL-10, TGF-β and IL-35[7]. Induced $T_{reg}$ cells (iTregs) develop from conventional $CD4^+$ T cells in the periphery after antigen encounter and in the presence of specific factors such as TGF-β and IL-2[8]. To date, we lack a definitive protein marker(s) that distinguishes between these two $T_{reg}$ cell populations in vitro or in

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vivo, although there are important differences in their epigenetic signature, and particularly in the *Foxp3* locus, making $iT_{reg}$ cells intrinsically unstable to inflammatory and/or stress conditions[8, 9].

From a historical perspective, the initial observations made in the 1970s that led to the definition of $T_{reg}$ cells in the 1990s are tightly linked to autoimmune diseases. Seminal works by Nishizuka and Sakakura back in 1969 demonstrated that neonatal thymectomy in healthy mice led to inflammation and severe organ-specific autoimmune pathology, implicating a thymic-derived population in control of self-tolerance[10]. This observation was further confirmed in adult rats that were thymectomized and subjected to sublethal irradiation[11]. Most importantly, inoculation of $CD4^+$ T cells from healthy syngeneic mice inhibited disease in both systems, and once autoimmunity was established, $CD4^+$ T cells isolated from these mice and adoptively transferred to T cell deficient mice were able to induce disease[12]. These experiments demonstrated that T cells were able to not only serve as inducers of autoimmune diseases, but also to inhibit them. This led to the hypothesis that in the periphery of normal mice there existed two populations of $CD4^+$ T cells, i.e., one potentially capable of inducing autoimmunity, and a second population of 'suppressor' cells, different from helper T cells, that would inhibit autoreactive T cells. $T_{reg}$ cell research during those years floundered, in part due to the failure in finding specific cell markers that could define this population and the ambiguity observed in their mechanisms of suppression. The identification of a population of $CD4^+$ T cells as responsible for controlling autoreactive responses[13], and the search for a cell surface marker that would define this population, indicated that a subset of $CD4^+$ T cells present in the periphery of normal mice expressing CD25 was responsible for the inhibition of autoimmunity. Transfer of cell suspensions from spleen of BALB/c mice depleted in $CD25^+$ cells into athymic nude mice induced autoimmunity which afflicted several organs, and co-transfer of $CD25^+CD4^+$ T cells inhibited disease[4]. In 2001, human $T_{reg}$ cells were identified in the thymus and peripheral blood of healthy individuals as those $CD4^+$ T cells expressing very high CD25 and being very similar in phenotype and function to their rodent counterpart[14, 15]. Although we still lack a definitive marker for human $T_{reg}$ cell isolation, these studies, together with the discovery of Foxp3 as the master transcription factor of $T_{reg}$ cells[1, 3, 16], laid the groundwork for the beginning of in depth analysis of $T_{reg}$ cell biology in health and disease (Box 1).

With regards to $T_{reg}$ cell function in human autoimmune diseases, early studies with patients revealed that in a number of autoimmune disorders there is either a defect in the numbers or in the function of $T_{reg}$ cells isolated from peripheral blood, with these data being supported by in vivo models of disease (for a comprehensive list of autoimmune diseases, [17]). Although some of these early data are confounded due to the identification of human $T_{reg}$ cells based on the positive expression of CD25 as the only marker for $T_{reg}$ cell identification, subsequent works have clearly demonstrated that most autoimmune diseases display defects in either the number and/or the function of $tT_{reg}$ cells measured in peripheral blood, for example Type 1 diabetes[18, 19, 20], multiple sclerosis[21, 22], systemic lupus erythematosus (SLE)[23], myasthenia gravis[24], rheumatoid arthritis[25] and others[17].

Investigations during the past two decades have advanced our knowledge regarding the mechanisms that underlie $tT_{reg}$ cell development in the thymus, their gene expression

signature, their role in controlling both immune and non-immune cell responses, their impact in immune-mediated diseases (reviewed in [7, 26]); and finally identifying the tissues where they exert functions beyond suppression[27, 28]. Moreover, the traditional view that $T_{reg}$ cells are a terminally differentiated population not capable of secreting pro-inflammatory cytokines and whose only function is to suppress T cell responses has been challenged based on more recent data. Specifically, $T_{reg}$ cells possess some degree of plasticity and instability, although we still do not understand the molecular mechanisms that drive these two states in depth or the relationship between each other in disease settings.

This review will provide an update on recent discoveries in $T_{reg}$ cell biology in relation to autoimmune diseases. From our perspective, three major observations have changed our understanding of what $T_{reg}$ cells are, how they function in peripheral lymphoid organs and non-immune tissues, how they relate to other immune and non-immune cells, and how their phenotype and function can be modulated, with clear consequences for the development of new therapeutic strategies for a number of autoimmune diseases. These are: 1) instability of $T_{reg}$ cells and acquisition of an effector phenotype after losing Foxp3 expression under inflammatory conditions, 2) plasticity of the $T_{reg}$ cell phenotype, with acquisition of effector-like properties while maintaining Foxp3 expression, and 3) the discovery of tissue-specific $T_{reg}$ cells, demonstrating again that $T_{reg}$ cells, as other immune cells, are influenced by their environment. These observations arise at a time where the development of high throughput genomic, epigenetic and proteomic technologies allow the analysis of rare cell populations to a single cell level, which will undoubtedly improve our knowledge of basic $T_{reg}$ cell biology in general, and human $T_{reg}$ cell biology in particular.

## Foxp3 as a master regulator of $T_{reg}$ cell phenotype and function.

The discovery of Foxp3 as the master regulator of $T_{reg}$ cell development and function was critical for our understanding of $T_{reg}$ cell biology[1, 2, 3]. Inactivating mutations in the *Foxp3* gene causes spontaneous development of severe autoimmunity with a scurfy phenotype in mice[29] and Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans[30, 31]. Foxp3 is necessary for t$T_{reg}$ cell development, maintenance and function[1, 2, 3], although alone it is not sufficient to fully recapitulate the $T_{reg}$ cell phenotype[9, 32]. Besides Foxp3, the second requirement necessary for the $T_{reg}$ cell functional program to be established is the generation of a specific epigenetic signature acquired during development and finalized in the periphery[9, 33]. Both play essential roles in maintaining $T_{reg}$ cell function, and alteration of Foxp3 or epigenetic modifications during auto-inflammatory conditions are likely the cause of $T_{reg}$ cell instability and aberrant plasticity observed in several autoimmune settings (Fig. 1). Due to the importance of Foxp3 in $T_{reg}$ cell maintenance and prevention of autoimmunity, regulation of Foxp3 expression is a matter of active research. Foxp3 is subjected to two major layers of regulation, i.e. transcriptional and post-translational, both of which are responsive to positive and negative regulation by factors in the tissue environment, including cytokines, metabolic mediators and inflammatory factors.

*Foxp3* gene expression is a tightly regulated process, and while the mechanisms of *Foxp3* transcriptional regulation by transcription factors during t$T_{reg}$ cell development and in

mature $T_{reg}$ cells have been clearly established (reviewed in[34]), data obtained in rodents have shown that *Foxp3* gene transcription is also epigenetically controlled. The *Foxp3* locus contains several conserved non-coding sequences (CNS) that are critical for the initiation and maintenance of *Foxp3* transcription [35]. From the three CNS described so far, only CNS2 has been demonstrated to prevent autoimmunity. It is a TCR-responsive enhancer with binding sites for Runx1-CBFβ complexes that is important in maintaining Foxp3 stability. CNS2-deficient mice develop spontaneous autoimmunity, underscoring the importance of CNS2 for $T_{reg}$ cell stability and function[36, 37]. Furthermore, CNS2 contains a conserved CpG island (also called the TSDR region) that is specifically hypomethylated, and thus, transcriptionally active, in tTregs, but hypermethylated in naive or effector T cells[38, 39]. This region has been widely used to distinguish *bona fide* $tT_{reg}$ cells from those conventional T cells transiently upregulating *Foxp3* expression and iTregs[38, 39].

Three major post-translational modifications have been described for the Foxp3 protein: acetylation, phosphorylation and ubiquitination. These modifications affect Foxp3 protein stability and DNA binding capacities, thus modulating $T_{reg}$ cell function and the development of autoimmunity. Acetylation in specific lysine (Lys) residues by Lys-acetyltransferases globally stabilizes Foxp3 expression and promotes $T_{reg}$ cell function by favoring Foxp3 binding to its transcriptional targets[40] and avoiding proteasomal degradation[40]. Several acetylases and deacetylases have been shown to interact with Foxp3 and control its acetylation, such as TIP60[41] and p300[40, 42]. Other post-translational processes include phosphorylation at Serine (Ser) and Threonine (Thr) residues by several kinases, including PIM-1, PIM-2 and CDK2[43, 44, 45, 46], and ubiquitination at Lys residues, that targets Foxp3 for proteasomal degradation[47]. Inflammatory stimuli result in proteasomal-dependent degradation of Foxp3 mediated by the ubiquitinase Stub1, which binds to Foxp3 and promotes its K48-linked ubiquitination[48]. Overexpression of Stub1 abrogates $T_{reg}$ cell suppressive capacity in vitro and in vivo and confers $T_{reg}$ cells with a $T_H1$-like phenotype, posing the question of whether the T effector-like $T_{reg}$ cell phenotype in this setting is an intermediate stage towards instability and loss of Foxp3 protein expression. In contrast, the deubiquitinating enzyme USP7 is highly expressed in $T_{reg}$ cells and is associated with Foxp3 in the nucleus, regulating Foxp3 turnover. Under inflammatory conditions this enzyme is downregulated, facilitating Foxp3 degradation[40]. Furthermore, conditional deletion of USP7 on $T_{reg}$ cells leads to lethal autoimmunity, with decreased numbers of $T_{reg}$ cells in the periphery that display an aberrant $T_H1$-like phenotype in vitro and in vivo[49]. USP7 targeting decreased recruitment of acetyltransferase TIP60 to the CNS2 region of Foxp3. TIP60 promotes acetylation-dependent dimerization of Foxp3[50, 51] and in the absence of TIP60 there is lethal autoimmunity[52]. Other factors have also been shown to control Foxp3 expression, such as HIF-1α, which is induced by IL-6 and TCR stimulation and inhibits Foxp3 through ubiquitination[53].

Both ubiquitination and acetylation target Lys residues, so they may compete for regulating Foxp3 expression. In fact, hyper-acetylation of Foxp3 prevents polyubiquitination and proteasomal degradation, increasing Foxp3 stability[40]. These data suggest that post-translational modifications of Foxp3 play a crucial role in modulating $T_{reg}$ cell plasticity or instability, adding a new layer of complexity to the regulation of the $T_{reg}$ cell functional program with potential consequences for the development of autoimmunity.

Foxp3 works in concert with other transcription factors and proteins, forming multiprotein complexes that determine the transcriptional signature and effector functions of $T_{reg}$ cells. Hundreds of protein partners have been described, including transcription factors Gata-3, NFAT, Runx1, Eos and others[54]. Global analysis of the Foxp3 interaction network suggests a model in which Foxp3 and its binding partners form multi-protein complexes that bind to pre-existing DNA enhancers[55] and regulate transcription positively or negatively depending on the recruited interaction proteins[56]. As previously mentioned, Foxp3 also associates with proteins that mediate epigenetic modifications, such as TIP60, Sirtuin 1, or HDAC7[41, 57], which alter the acetylation state of partner loci and Foxp3 itself, with consequences for transcription factor binding, and histone modifications. In this regard, disruption of Foxp3 interactions with specific proteins diminishes $T_{reg}$ cell function and leads to autoimmune responses due to increased Foxp3 polyubiquitylation[58, 59]. Other evidence that supports the importance of Foxp3 interaction partners for $T_{reg}$ cell function comes from the observation that different Foxp3 mutations result in a wide range of IPEX disease severity, reflecting the relative importance of the affected residues in the integrity of the Foxp3 protein and the protein partners that form the DNA-binding complexes. For example, the most common mutation in IPEX, p.A384T, which disrupts the sequence specificity of Foxp3 DNA binding and alters Foxp3 interactions with specific targets genes[31], inhibits $T_{reg}$ cell function but preserves the capacity of $T_{reg}$ cells to repress inflammatory cytokine production, due in part to a specific inhibition of Foxp3 interaction with TIP60 (ref. [60]). Furthermore, experiments in mice that contained this mutation specifically in $T_{reg}$ cells perturbed Foxp3 binding to specific target genes including *Batf*, which was partly responsible for the induction of a unique pattern of tissue-restricted inflammation in certain non-lymphoid tissues due to defective function of these $T_{reg}$ cells[61]. Additionally, a Foxp3 reporter mouse that expresses an $NH_2$-terminal EGFP-Foxp3 fusion protein, which disrupts the interaction of Foxp3 with many cofactors including TIP60, p300 and Eos, does not experience apparent autoimmunity, but its $T_{reg}$ cells display alterations in function in vivo, with autoimmune-prone NOD mice developing diabetes faster than their wild-type counterparts[58]. Interestingly, these same $T_{reg}$ cells are more potent suppressors of antibody-mediated arthritis due to a preferential interaction of EGFP-Foxp3 with IRF4[62]. These findings demonstrate that not only are certain cofactors crucial for $T_{reg}$ cell-mediated function, but they suggest that $T_{reg}$ cells might be 'tuned' to control particular types of inflammation by modulating the constituents of the Foxp3 protein complexes under specific environmental conditions. Furthermore, it is tempting to speculate that the variety of functions that $T_{reg}$ cells perform in different tissue environments could be accompanied by the formation of specific multi-protein complexes with tissue-specific proteins that would cooperate with Foxp3 in performing $T_{reg}$ cell function in specific environments.

## $T_{reg}$ cell instability.

Both the expression and stability of Foxp3 play crucial roles in the maintenance of $T_{reg}$ cell function[63]. Thus, genetic deletion of a conditional *Foxp3* allele in mature $T_{reg}$ cells results in effector T cells that are capable of causing inflammatory tissue lesions[64]. Although the instability of Foxp3 in $iT_{reg}$ cells has been widely observed and it is intrinsic to their developmental origin[8, 9], $tT_{reg}$ cells have been investigated to determine how instability of

Foxp3 expression under basal or inflammatory conditions in specific tissues impacts the development and resolution of autoimmunity[65, 66, 67]. Loss of Foxp3 expression by $tT_{reg}$ cells had been previously observed in vitro[68, 69], in adoptive transfers into lymphopenic hosts[70], in infection settings[71] and graft-versus-host disease[72]. A fate-mapping mouse model where the YFP reporter marks all cells that at any time expressed Foxp3 in both homeostatic and autoimmune inflammatory conditions has been generated. A small percentage of apparently stable $T_{reg}$ cells lost Foxp3 expression and acquired an effector-memory phenotype with secretion of different levels of the pro-inflammatory cytokines IFN-γ and IL-17[67]. These 'exFoxp3' cells were able to induce autoimmunity in an adoptive transfer model in the NOD background and consisted of a mixed population based on the level of TSDR demethylation, suggesting that not all exFoxp3 in this model might have once been de facto tTregs. More recent data suggests that this fate-mapping mouse model was detecting a percentage of Foxp3 $tT_{reg}$ cells that were either upregulating Foxp3 transiently, or were not fully committed to the $tT_{reg}$ cell lineage[73]. Further data supporting $tT_{reg}$ cell instability shows that Foxp3 expression is lost on $MOG_{38–49}$-specific $tT_{reg}$ cells during experimental autoimmune encephalitis (EAE) development, with increased frequency of exFoxp3 in the central nervous system at the preclinical and peak stages of EAE that decreases during EAE resolution. These exFoxp3 $T_{reg}$ cells expressed IFN-γ and were able to transfer EAE[74]. It remains to be determined whether the decrease in exFoxp3 during disease resolution was due to re-acquisition of Foxp3 expression by exFoxp3 cells.

Another genetic fate-mapping mouse model provided evidence that the majority of mature $tT_{reg}$ cells in spleen and lymph nodes are relatively stable under homeostatic conditions[66]. This model, based on inducible labeling of $Foxp3^+$ cells upon tamoxifen treatment, marks all those cells that express Foxp3 at the moment of tamoxifen administration, and, in contrast to continuous labeling[67], prevents cells transiently expressing Foxp3 to be detected. Although stable under homeostatic conditions, with growth factor deprivation or IL-2 receptor blockade which induces autoimmunity[75], mature $tT_{reg}$ cells significantly decreased the level of Foxp3 expression per cell, and a small population lost Foxp3 expression completely. However, they did not produce pro-inflammatory cytokines[66], suggesting some degree of instability under specific environmental settings. This apparent discrepancy might arise from the different fate-mapping mouse models and the type of labeling of Foxp3 cells, which could lead to the labeling of non-committed $T_{reg}$ cells[67], or the absence of labelling of exFoxp3 appearing before tamoxifen administration[66]. Discordant results could also depend on the inflammatory stimuli utilized to test Foxp3 stability. Regardless, in both fate-mapping models there is a small population of $tT_{reg}$ cells that loses Foxp3 expression, and those $T_{reg}$ cells that remain 'stable' display diminished Foxp3 expression at the single cell level[66]. As decreased levels of Foxp3 in $T_{reg}$ cells isolated from inflammatory sites have been observed in mouse models of autoimmunity[63, 76] and in patients with autoimmune diseases[24, 77, 78, 79, 80, 81], further works with these models are warranted in order to examine the mechanisms and consequences of long-term decreases in $T_{reg}$ cell Foxp3 expression. Further data has confirmed the observation that most mature $tT_{reg}$ cells are stable under steady-state conditions with a new fate-mapping mouse model where Foxp3 lacks CNS1, but they become unstable when stimulated in vitro and in vivo in a model of EAE, losing Foxp3 expression and acquiring $T_H1$- and $T_{FH}$ cell-like features[73]. While epigenetic

changes such as re-methylation of the CNS2 region could account for the loss of Foxp3 expression in these settings[67, 73], the molecular mechanisms that are responsible for the decrease in Foxp3 protein and the potential contribution of post-translational modifications of Foxp3 protein on exFoxp3 cell generation remain to be explored.

Finally, depletion of specific Foxp3 protein partners can also precipitate the appearance of exFoxp3 cells. For instance, $T_{reg}$ cell-specific deletion of the chaperone GP96 in the NOD background led to lethal autoimmunity due to defective $T_{reg}$ cell suppressive capacities in models of diabetes and colitis. In this system $T_{reg}$ cells progressively lose Foxp3 expression and gain IFN-γ secretion, although they maintain their specific TSDR demethylation pattern[59]. Mice with a $T_{reg}$ cell-specific deletion of the transcription factor Helios develop systemic autoimmune pathology, characterized by increased germinal center formation, lymphocytic infiltration into non-lymphoid organs and glomerulonephritis[82]. Although Helios does not form protein complexes with Foxp3 nor does it bind to the Foxp3 locus[82], Helios-deficient $T_{reg}$ cells express increased IFN-γ and IL-17 and are unstable, with decreased expression of Foxp3 and a tendency to completely lose its expression [82, 83]. $T_{reg}$ cells deficient in another transcription factor – Eos – exhibit increased expression of IL-2 and IFN-γ along with reduced suppressive capacity, while forced overexpression of Eos in $T_{reg}$ cells prevents $T_{reg}$ cell instability, even in inflammatory environments. $Eos^{lo}Foxp3^{+}$ $tT_{reg}$ cells are detectable in vivo and have regulatory function, with the ability to acquire $T_H$-like effector characteristics while maintaining Foxp3 expression, however such cells exhibit specific changes in the global DNA methylation pattern[84].

## Regulatory T cell plasticity.

Plasticity is a property inherent to most, if not all, immune cells, which allows them to adapt their phenotype and function to the changing environment and extracellular ‘danger’ signals. Thus it is not surprising that $T_{reg}$ cells possess some degree of plasticity. $T_{reg}$ cells have the capacity to acquire features specific to the type of immune response they control, mostly driven by ‘master’ transcription factors and regulated by environmental signals. Thus, $T_{reg}$ cells acquire T-bet expression to restrain type 1 inflammation during infection[85, 86], and utilize IRF4 and STAT3 to inhibit $T_H2$ (Ref.[87]) and $T_H17$ (Ref. [88]) responses, respectively. While this modality of plasticity seems to be advantageous for the host and beneficial for the outcome of the immune response, aberrant plasticity of $T_{reg}$ cells is also observed in several autoimmune diseases, with $T_{reg}$ cells expressing pro-inflammatory cytokines, acquiring T helper-like phenotypes and displaying diminished function in most cases but maintaining Foxp3 expression levels[21, 83, 89, 90, 91, 92, 93, 94, 95]. Paradoxically, these $T_H$-like $T_{reg}$ cells utilize the same transcription factors used by $T_{reg}$ cells to inhibit specific types of immune responses. Therefore, IFN-γ secretion by $T_H1$-like $T_{reg}$ cells requires T-bet expression[21, 91, 92, 93], while IL-6-driven Th17-like $T_{reg}$ cells require STAT3 for IL-17 secretion[72], and IL-4-driven $T_H2$-like $T_{reg}$ cells upregulate IRF4 and Gata-3[95, 96]. In most instances, $T_H$-like $T_{reg}$ cells have a demethylated TSDR Foxp3 locus even though they share effector features, suggesting that their phenotype might be reversible[21, 90, 93]. They display alterations in the epigenetic signature characteristic of $T_{reg}$ cells[9, 32, 34, 97], which may be the underlying mechanism that allows for pro-inflammatory cytokine secretion. Current efforts focus on understanding the signaling pathways that drive this plasticity in specific

autoimmune diseases, to harness this flexibility on the treatment of human disease[92] and the role of $T_{reg}$ cell plasticity in autoimmune-related tissues.

## $T_H1$-like $T_{reg}$ cells

Perhaps the best characterized $tT_{reg}$ cell plasticity event is the acquisition of $T_H1$-like features. In mouse models and patients with autoimmune diseases such as type 1 diabetes[93], multiple sclerosis[21, 91], autoimmune hepatitis[98] and Sjogren syndrome[99], there is an increased frequency of IFN-$\gamma^+$Foxp3$^+$ $tT_{reg}$ cells in the periphery that display reduced suppressive capacities as compared to $T_{reg}$ cells from healthy age-matched subjects. $T_H1$-like $T_{reg}$ cells upregulate the transcription factor T-bet and other $T_H1$ markers, such as CCR5 and CXCR3. Furthermore, in the *Apoe*$^{-/-}$ mouse model of atherosclerosis, which shares a number of pathogenic similarities with autoimmune disorders, there is accumulation of IFN-$\gamma$-producing $T_H1$-like $T_{reg}$ cells in the aorta that display altered suppressive capacities in vitro and in vivo[90]. Using an in vitro model of $T_H1$-like $T_{reg}$ cell generation using IL-12, it has been demonstrated that $T_H1$-like $T_{reg}$ cells possess an activated PI(3)K-Akt-FoxO pathway, which is partly responsible for the secretion of IFN-$\gamma$ and decreased suppressive capacity[91]. Interestingly, $T_{reg}$ cells isolated from patients with relapsing-remitting multiple sclerosis (RRMS) also display an activated PI(3)K-AKT-FoxO pathway *ex vivo* and their suppressive capacity is corrected upon PI(3)K pathway blockade[94]. In vivo, PI(3)K-Akt activation by $T_{reg}$ cell-specific deletion of the PI(3)K phosphatase PTEN provokes a type 1 autoimmune disorder, with $T_{reg}$ cells downregulating the expression of CD25 and Foxp3 and displaying reduced functionality[100, 101]. Moreover, FoxO itself has been implicated in the regulation of $T_{reg}$ cell plasticity. Mice with a $T_{reg}$ cell-specific deletion of FoxO succumb to lethal autoimmunity similar to that observed in scurfy mice[94], with $T_{reg}$ cells displaying a $T_H1$-like $T_{reg}$ cell phenotype and being unable to prevent disease in a colitis model. IFN-$\gamma$ seemed to be involved in $T_{reg}$ cell defective function, as *Foxo1*$^{-/-}$*Ifng*$^{-/-}$ mice partially recovered from the wasting syndrome[94].

Transcriptomic analysis of IFN-$\gamma^+$ $T_H1$-like $T_{reg}$ cells at the population[91] or single cell[90] levels demonstrates that they exhibit reduced expression of immunosuppressive genes as compared to $T_{reg}$ cells and have altered expression of costimulatory molecules, migratory properties and specific signaling pathways. It is unknown whether $T_H1$-like $T_{reg}$ cells play a role in disease pathogenesis/protection in specific tissues. The presence of $T_H1$-like $T_{reg}$ cells has been observed in MOG-specific $T_{reg}$ cells infiltrating the central nervous system of mice during EAE development. These $T_{reg}$ cells were not capable of suppressing central nervous system-infiltrating MOG-specific effector T cells and prevent disease onset, secreting IFN-$\gamma$ at the onset and peak of disease but with a reduced frequency during the recovery phase[102]. In contrast, T-bet$^+$Foxp3$^+$ cells in pancreatic tissue seem to be protective in a mouse model of type 1 diabetes[103]. Lastly, while most work on $T_H1$-like $T_{reg}$ cells suggest that IL-12 and/or type 1 cytokines are inducing the phenotype, the fact that the PI(3)K-Akt is involved in their generation, which is a major pathway integrating diverse environmental signals into cell function, suggests that other environmental cues could have the ability to induce $T_{reg}$ cell plasticity, as it has been observed, for example, with increased dietary salt concentrations[104].

### $T_H17$-like $T_{reg}$ cells

A small proportion of human peripheral $T_{reg}$ cells produce IL-17 in healthy individuals and upregulate *RORC2* ($T_H17$-like $T_{reg}$ cells) ex vivo[105] while conserving suppressive capacities[105, 106]. Considering the well-established developmental relationship between $T_{reg}$ cells and $T_H17$ cells, it remains to be determined whether $T_H17$-like $T_{reg}$ cells are a transient stage in the de-differentiation of $tT_{reg}$ cells into Th17 cells as it has been suggested[69, 107]. In support of this, conversion of $Foxp3^+$ $T_{reg}$ cells into $T_H17$ cells has a crucial role in the pathogenesis of autoimmune arthritis in a collagen-induced arthritis mouse model. This conversion is driven by IL-1β[109] and IL-6, and $Foxp3^+IL\text{-}17^+$ $T_{reg}$ cells are observed in the synovium of subjects with active rheumatoid arthritis[108]. Moreover, conversion of $T_{reg}$ cells into $T_H17$ cells has also been reported in the $CD18^{hypo}$ PL/J mouse model of psoriasis[109], and $T_H17$-like $T_{reg}$ cells have been observed in skin tissue of psoriatic patients[89].

Perhaps the tissue where $T_H17$-like $T_{reg}$ cells have been best identified is the gastrointestinal tract. Lamina propria appears enriched in $iT_{reg}$ cells that express high levels of $T_H17$-defining transcription factor RORγt. These $T_H17$-like $T_{reg}$ cells appear to have a positive function, as their absence exacerbates pathogenesis in several models of mucosal autoimmunity[110, 111]. Furthermore, recent studies have suggested that $Foxp3^+ROR\gamma t^+$ $T_{reg}$ cells control glomerulonephritis[112], and lack of $T_H17$-like $T_{reg}$ cells results in increased SLE-associated mortality and organ pathology[113]. Besides the importance of IL-1β and IL-6 on promoting IL-17 secretion by $T_{reg}$ cells[114, 115], other environmental factors have been shown to modulate $T_H17$-like $T_{reg}$ cell conversion either indirectly or directly, including indoleamine 2,3-dioxygenase (IDO)[116], Toll-like receptor 2 (TLR2) ligation[117], and certain infections[118].

### $T_H2$-like Tregs

Patients with systemic sclerosis display an increased frequency of $T_H2$-like $T_{reg}$ cells in skin but not in peripheral blood, characterized by the secretion of IL-4 and IL-13 and upregulation of Gata-3 and IRF-4. Peripheral $T_{reg}$ cells from these patients express high levels of ST2, the receptor for the alarmin IL-33. This cytokine is enriched in the skin, suggesting that it might play a role in the reprogramming of $T_{reg}$ cells into a $T_H2$-like phenotype[95]. $T_H2$-like $T_{reg}$ cells have also been observed in allergy-susceptible mutant mice (*Il4ra*$^{F709}$), and in peripheral blood $T_{reg}$ cells from food-allergic patients. $T_H2$-like $T_{reg}$ cells secrete IL-4 and/or IL-13 and upregulate transcription factors IRF-4 and Gata-3[96]. Moreover, mice with $T_{reg}$ cell-restricted deletion of the ubiquitin-ligase Itch show autoimmune features and $T_{reg}$ cells are not able to control $T_H2$ inflammation. This defect is associated with the acquisition of a $T_H2$-like phenotype by the $T_{reg}$ cells, with increased Gata-3 expression, STAT6 activation and secretion of IL-4[119].

Many open questions remain to be answered in regards to $T_{reg}$ cell plasticity. Does $T_{reg}$ cell plasticity reflect initial heterogeneity of the $T_{reg}$ cell population, with only a specific subset of $T_{reg}$ cells being able to acquire effector-like properties[65]? Do $T_H$-like $T_{reg}$ cells represent a transient stage on the path to becoming exFoxp3? Can we harness $T_{reg}$ cell plasticity to design new therapeutic strategies aimed at modulating $T_{reg}$ cell function in diverse disease

settings? What is the role of $T_H$-like $T_{reg}$ cells in autoimmune tissues during disease development and progression?

## Tissue-resident $T_{reg}$ cells

One of the major recent advances in the $T_{reg}$ cell field is the discovery that $T_{reg}$ cells populate specific tissues in the body during physiological and stress conditions. The characterization of these populations and their mechanisms of action will have important implications to our understanding of the development, maintenance and resolution of autoimmunity in specific organs. Tissue-resident $T_{reg}$ cells actively perform non-immunological functions and work at maintaining tissue homeostasis and wound repair, roles that could be important for tissue homeostasis in autoimmunity settings. A number of studies have begun to define the specific phenotype of tissue-resident $T_{reg}$ cells in muscle, skin, lung, gastrointestinal tract, liver and adipose tissue among others (reviewed in [28]). In most cases, these $T_{reg}$ cells appear to be of thymic origin[120, 121, 122], with considerable TCR repertoire oligoclonality, indicating that particular antigens in the tissue may be responsible for the accumulation of $T_{reg}$ cells in specific niches[120, 122, 123]. They are characterized by the expression of tissue-specific transcription factors and mediators that drive the function of other cells in that tissue, supporting the notion that the microenvironment exerts important effects on the phenotype of $T_{reg}$ cells in a given location. The molecular mechanisms by which tissue-resident $T_{reg}$ cells acquire their tissue-specific program have only begun to be explored. Epigenetic analysis of mouse $T_{reg}$ cells isolated from different tissues and compared to lymph node $T_{reg}$ cells have demonstrated that tissue $T_{reg}$ cells undergo extensive epigenetic reprograming that is globally tissue-specific, but there is a common tissue $T_{reg}$ cell population characterized by the expression of KLRG1 and ST2[124].

The best studied tissue-specific $T_{reg}$ cell population is that resident in visceral adipose tissue (VAT). VAT $T_{reg}$ cells exert important roles in defending against associated metabolic disorders. In mice, lean fat tissue is populated by T cells with a $T_H2$ phenotype and enriched in $T_{reg}$ cells, that maintain the predominance of resident anti-inflammatory macrophages. $T_{reg}$ cells accumulate over time and acquire a $T_H2$-like phenotype, with expression of Gata-3, BATF, IRF4, CCR4 and IL-10[121], and PPAR$\gamma$, a transcription factor that controls adipocyte differentiation and mediates VAT $T_{reg}$ cell accumulation, phenotype and function[121, 125]. $T_{reg}$ cell-specific depletion of PPAR$\gamma$ results in a specific loss of VAT $T_{reg}$ cells, demonstrating an important role of this transcription factor in the development and/or maintenance of VAT $T_{reg}$ cells[121]. VAT $T_{reg}$ cell frequency diminishes with age in obese mice, suggesting their role in modulating obesity-associated fat tissue inflammation[123]. Given that obesity is an established risk factor in autoimmune diseases, it will be important to define the features and function of VAT $T_{reg}$ cells in the development of autoimmunity.

Similarly, lung and muscle $T_{reg}$ cells express the epidermal growth factor receptor (EGFR) ligand amphiregulin to promote tissue repair. Muscle $T_{reg}$ cells accumulate in acutely injured skeletal muscle in mouse models of muscle injury and muscular dystrophy, where they control muscle inflammation upon injury and promote tissue repair by acting on immune and non-immune cells[27, 120]. Data in a zebrafish model further supports the dual role of $T_{reg}$ cells in immune regulation and tissue regeneration observed in mice. Zebrafish $T_{reg}$-like

cells[126] rapidly migrate to damaged organs in models or spinal cord, heart and retina regeneration and their function is dependent on the secretion of organ-specific regenerative factors. Conditional ablation of $T_{reg}$ cells inhibited organ regeneration[127].

$T_{reg}$ cells populate healthy skin and help maintain homeostasis. In healthy individuals and rodents, skin $T_{reg}$ cells possess an effector-memory phenotype, with expression of $T_{reg}$ cell-specific surface markers and higher proliferative capacity ex vivo as compared to their blood counterparts. They express IL-17 and IL-10 and display a demethylated TSDR region, suggesting that they are tTregs[122]. Interestingly, $T_{reg}$ cells reside in close apposition to hair follicles, where they contribute to hair follicle stem cell activation by promoting their proliferation and differentiation through the Notch pathway. Ablation of $T_{reg}$ cells completely abrogates the ability of the hair follicle to regenerate, implicating $T_{reg}$ cells as a key component of the skin stem cell niche[128].

Maintenance of local tissue homeostasis is of particular importance in the intestinal mucosa, where the immune system must be able to effectively discriminate between pathogens and dietary factors or commensal flora. Accordingly, deregulated immune responses against luminal flora in genetically susceptible individuals are generally recognized as key factors in the pathogenesis of Inflammatory Bowel Disease (IBD). While the role of commensal flora in inducing $T_{reg}$ cells has been well characterized, the dynamics and characterization of $T_{reg}$ cells in the mucosal tissue has only begun to be appreciated. Thus, lamina propria appears enriched in $T_{reg}$ cells that express high levels of ROR$\gamma$t[110, 129], and although most of them are not tTregs, they seem to have a positive function in maintaining tissue homestasis, and their absence exacerbates pathogenesis in several models of mucosal autoimmunity.

How do $T_{reg}$ cells maintain tissue integrity and what is the involvement of tissue-specific $T_{reg}$ cells in autoimmune diseases? One could speculate that defects in specific tissue-resident $T_{reg}$ cells would be involved in either the development, maintenance of resolution of autoimmune disorders affecting that specific tissue, e.g. Is there a specific role for muscle-resident $T_{reg}$ cells in the pathology of myasthenia gravis, or for VAT $T_{reg}$ cells in autoimmune disorders where obesity is an important risk factor, or for skin $T_{reg}$ cells in psoriasis? Lastly, while most studies performed so far have been done in mice, and although there are intrinsic difficulties with translating these observations to humans, it will be crucial to examine the phenotype and function of tissue-specific $T_{reg}$ cells in healthy individuals and patients with autoimmune diseases, to understand the role of tissue-resident $T_{reg}$ cells on the development and maintenance of human autoimmunity.

## Conclusions.

Intense research in the $T_{reg}$ cell field has improved our knowledge of their biology and has provided evidence of the existence of instability and plasticity within the $T_{reg}$ cell compartment. Accumulating data have shown the presence of exFoxp3 cells and $T_H$-like $T_{reg}$ cells in a number of autoimmune pathologies over the last years. Current efforts focus on determining the molecular mechanisms responsible for the induction of each of these functional states in the periphery and autoimmune organs as well as the environmental triggers that drive them. The ultimate goal of these studies is to harness $T_{reg}$ cell instability

and plasticity mechanisms to modulate $T_{reg}$ cell function not only in autoimmune pathologies, but also in other diseases such as cancer. With regards to tissue-resident $T_{reg}$ cells, although we have begun to understand the mechanisms and factors that control their function in tissues, many questions remain to be answered mostly related to the mechanisms that tissue $T_{reg}$ cells employ to maintain tissue integrity in autoimmune diseases and the characterization of tissue-resident $T_{reg}$ cells in humans (Box 2). Answers to these questions will undoubtedly improve the design of $T_{reg}$ cell-specific therapeutic options for patients with autoimmune diseases.

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**Box 1.**

### How are $T_{reg}$ cells studied in autoimmunity?

$T_{reg}$ cell studies in autoimmunity have been facilitated by the existence of mouse models for many autoimmune diseases[130]. While these models of autoimmune disease allow fate mapping and gene modification experiments that cannot be performed in humans, they do not generally reflect the genetic architecture that underlies human autoimmune diseases. That is, the models often reflect the efferent aspects of disease pathogenesis but not the underlying mechanisms that cause the activation of autoreactive T cells.

Human $T_{reg}$ cell studies are hindered by the lack of a definitive surface marker that uniquely isolates $T_{reg}$ cells from other T cell populations. Thus, many early works examining the frequency of $T_{reg}$ cells in peripheral blood of patients need to be considered with caution, as $T_{reg}$ cells were defined solely by the positive expression of CD25 and the results can be misleading as human conventional T cells can also express CD25 upon activation. Moreover, human studies are limited to *ex vivo* characterizations or short *in vitro* experiments and depend on the availability of samples from patients with autoimmunity.

Recent advances in high throughput technologies that require small cell numbers will considerably impact human $T_{reg}$ cell autoimmunity research, allowing the analysis of rare $T_{reg}$ cell populations, from small tissue samples, etc. Current available technologies are able to determine the epigenetic and transcriptional signature at the single cell level. The combination of these datasets and system biology approaches to analyze serial samples obtained from individuals at different time points, before and after therapeutic intervention and from different anatomical locations is delivering enormous amounts of information about response to treatment in patients, disease course, and mechanistic insights.

**Box 2.**

### $T_{reg}$ cell-specific therapies for autoimmune diseases.

One of the many therapeutic strategies being investigated in the autoimmunity field is the restoration of peripheral tolerance, and one explored avenue has been the development of $T_{reg}$ cell-based therapeutic strategies. Interesting results have resulted from immune therapies based on the adoptive transfer of polyclonally expanded $T_{reg}$ cells in patients with autoimmune diseases such as type 1 diabetes, cutaneous lupus erythematosus and Crohn's disease[131, 132, 133, 134]. Furthermore, with the development of technologies to engineer specific TCR to immune cells such as chimeric antigen-receptors (CARs[135]), future approaches will potentially achieve optimal numbers of $T_{reg}$ cells with TCR specific for disease-relevant antigens for adoptive transfers to patients with autoimmunity in those cases where the autoantigens are known. Although it is still early to know the long-term consequences of these therapies, the first results are encouraging and promise to adopt these technologies for other autoimmune diseases.

Another potential therapeutic approach to peripheral tolerance restoration is targeting specific molecules and/or pathways that are dysfunctional in the existing pool of $T_{reg}$ cells in patients. For this approach, it will be of importance to determine the physiological relevance and molecular mechanisms driving plasticity and instability of $T_{reg}$ cells in patients, as well as to improve our knowledge on how dysfunction of tissue-specific $T_{reg}$ cells affect the development of certain autoimmune diseases. In this respect, there are many questions still unsolved (see main text and Box 3) that will undoubtedly improve the design of $T_{reg}$ cell-specific therapeutic options for patients with autoimmune diseases.

**Box 3.**

### Unanswered questions.

- Discovery of markers to distinguish $tT_{reg}$ cells from iTregs.
- What are the molecular mechanisms and environmental triggers responsible for exFoxp3 cell generation in autoimmunity?
- What are the detailed molecular mechanisms and environmental cues that drive $T_{reg}$ cell plasticity into $T_H1$-, $T_H22$- and $T_H17$-like $T_{reg}$ cell phenotypes in specific autoimmune settings?
- What is the contribution of the $T_{reg}$ cell epigenetic signature to $T_{reg}$ cell instability and plasticity in autoimmunity?
- What is the epigenetic and phenotypic signature of human $T_{reg}$ cells in specific tissues, and how do they contribute to disease pathology?
- Does $T_{reg}$ cell plasticity reflect the initial heterogeneity of the $T_{reg}$ cell population, with only a specific subset of $T_{reg}$ cells being able to acquire effector-like properties?
- What is the role of $T_{reg}$ cell plasticity and instability in autoimmune tissues during disease development, progression and resolution of disease?
- What are the mechanisms that $T_{reg}$ cells utilize to maintain tissue integrity and what is the involvement of tissue-specific $T_{reg}$ cells in tissue damage in autoimmunity?

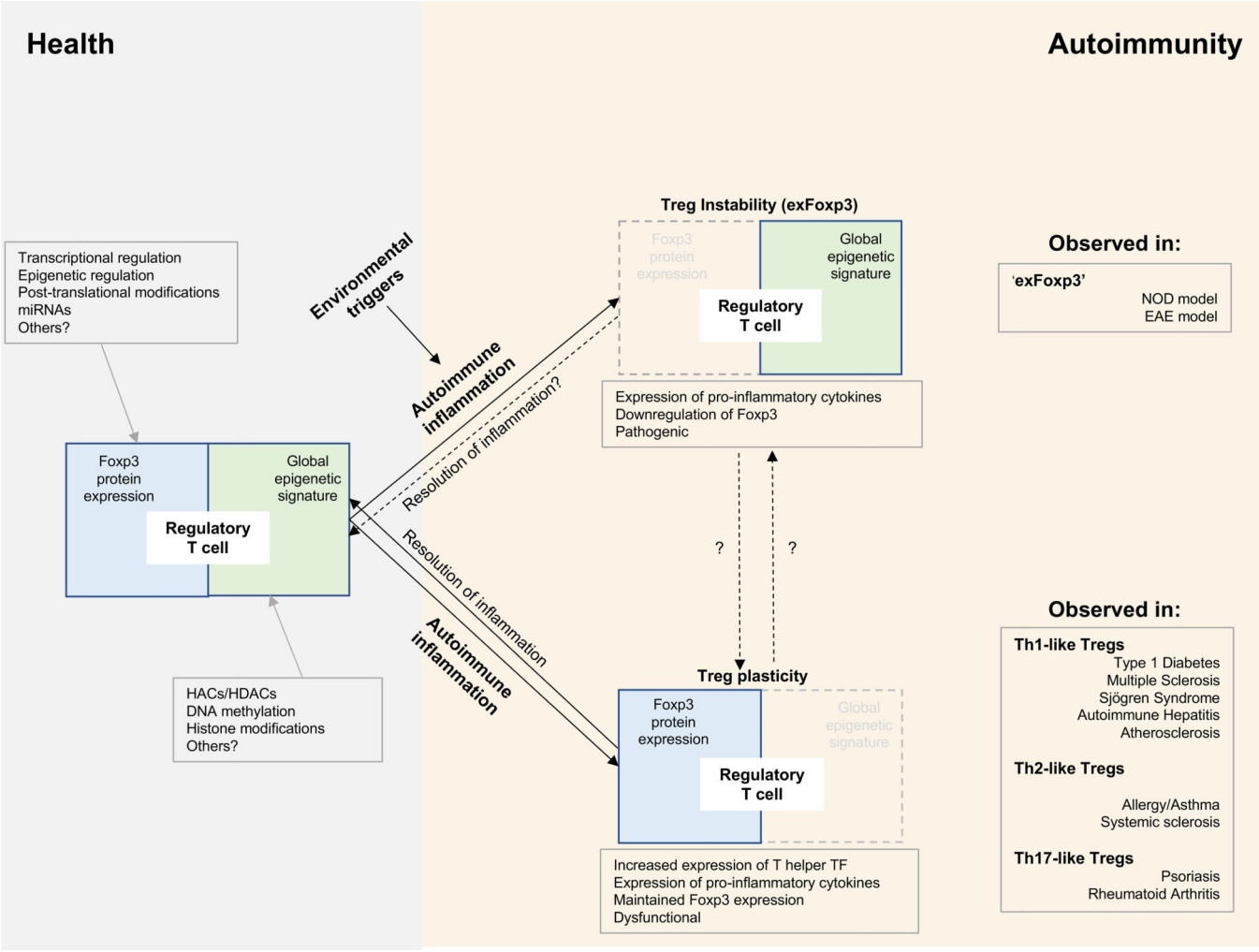


**Figure 1. $T_{reg}$ cell functional program in health and autoimmunity.**

The $T_{reg}$ cell functional program depends on two major axes: Foxp3 expression and a global epigenetic signature, both of which are regulated by a number of factors (gray boxes) and maintained under healthy conditions. In genetically susceptible individuals, environmental triggers induce inflammation, and a percentage of $T_{reg}$ cells can lose Foxp3 expression and become unstable ($T_{reg}$ cell instability, exFoxp3), or can maintain Foxp3 expression but alter their global epigenetic signature ($T_{reg}$ cell plasticity), secrete pro-inflammatory cytokines and display reduced function. $T_{reg}$ cell instability leads to the so-called 'exFoxp3' cells, which have been observed in mouse models of diabetes (NOD) and multiple sclerosis (EAE). $T_{reg}$ cell plasticity favors the acquisition of $T_H1$-like, $T_H2$-like or $T_H17$-like properties by the $T_{reg}$ cells, each of which have been observed in a number of autoimmune diseases in mice and humans. While the mechanistic connection between $T_{reg}$ cell instability and plasticity is not known, plastic $T_{reg}$ cells can become 'normal' $T_{reg}$ cells after resolution of inflammation. It remains to be determined whether this is also the case for $T_{reg}$ cell instability. Abbreviations: miRNA, microRNA; HACs, histone acetylases; HDACs, histone

deacetylases; TF, transcription factors; NOD, non-obese diabetic; EAE, experimental autoimmune encephalomyelitis.