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Review

FoxP3$^+$ CD4$^+$ T cells in systemic autoimmune diseases: the delicate balance between true regulatory T cells and effector Th-17 cells

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Abstract

Breakdown of tolerance is a hallmark of autoimmune diseases. Over the past 10 years, there has been increased interest in the role of *FoxP3*$^+$ regulatory T cells (T_{Regs}) in maintaining peripheral tolerance. Dysfunction of these cells is considered to play a major role in the development of autoimmune diseases. Besides their suppressive function, a fraction of these cells has the capacity to differentiate into IL-17-producing cells (Th-17), a phenomenon associated with autoimmune inflammation. The revealed plasticity of T_{Regs}, therefore, has obvious implications when designing therapeutic strategies for restoring tolerance in autoimmune diseases using T_{Regs}. In this review, we discuss development, classification, molecular characterization and mechanisms of suppression by T_{Regs}. In addition, we describe recent data on their potential conversion into Th-17 cells in human systemic autoimmune diseases. We also outline a new strategy for T_{Reg}-based therapy via isolation, expansion and re-infusion of highly pure *FoxP3*$^+$ T_{Regs} free of contaminating effector T cells.

Key words: T cells, Regulatory T cells, T-helper-17 cells, Systemic autoimmune diseases.

Introduction

One of the major challenges in immunology is the understanding of cellular and molecular mechanisms involved in the discrimination between pathogens and autoantigens. Thymic clonal deletion and induction of anergy or apoptosis of self-reactive T cells upon exposure to self-antigen have been considered as major mechanisms of maintaining self-tolerance. Nevertheless, autoreactive T cells may escape these mechanisms and can be detected in the peripheral blood of most individuals [1, 2]. Autoimmunity, however, occurs in only 5% of the general population, suggesting the existence of other control mechanisms to prevent autoimmune responses.

T cells suppressing immune responses were first described in the early 1970s by Gershon and Kondo [3, 4]. In the mid-1990s, Sakaguchi *et al.* [5] reintroduced the paradigm of T-cell-mediated self-tolerance by identifying a subset of peripheral CD4$^+$ T cells expressing the IL-2 receptor α-chain (CD25), which were found critical for preventing autoimmunity. They showed that CD4$^+$ T cells depleted of CD25$^+$ T cells from normal mice, when transferred into syngeneic athymic nude mice induced the development of multi-organ autoimmune disease in the recipients, whereas disease development was prevented by co-transfer of CD25$^+$CD4$^+$ T cells together with CD25$^-$ T cells. This observation re-evoked interest in T-suppressor cells, and defined the primary phenotype of these cells. Subsequently, *in vitro* studies showed that human suppressor CD4$^+$ T cells, also termed regulatory T cells (T_{Regs}), constitute only those CD4$^+$ T cells with the highest level of CD25 expression [6]. A decrease in frequency or impaired function of T_{Regs} has been observed in several autoimmune diseases in humans, suggesting a role of these cells in the control of autoimmunity [7]. Modulation of T_{Reg} function and number may thus present an option for immunotherapy of autoimmune diseases.

Development and classification of T_{Regs}

Several lines of evidence, based on studies in experimental animals, support the hypothesis that T_{Regs} originate in the thymus. It has been observed that 5% of

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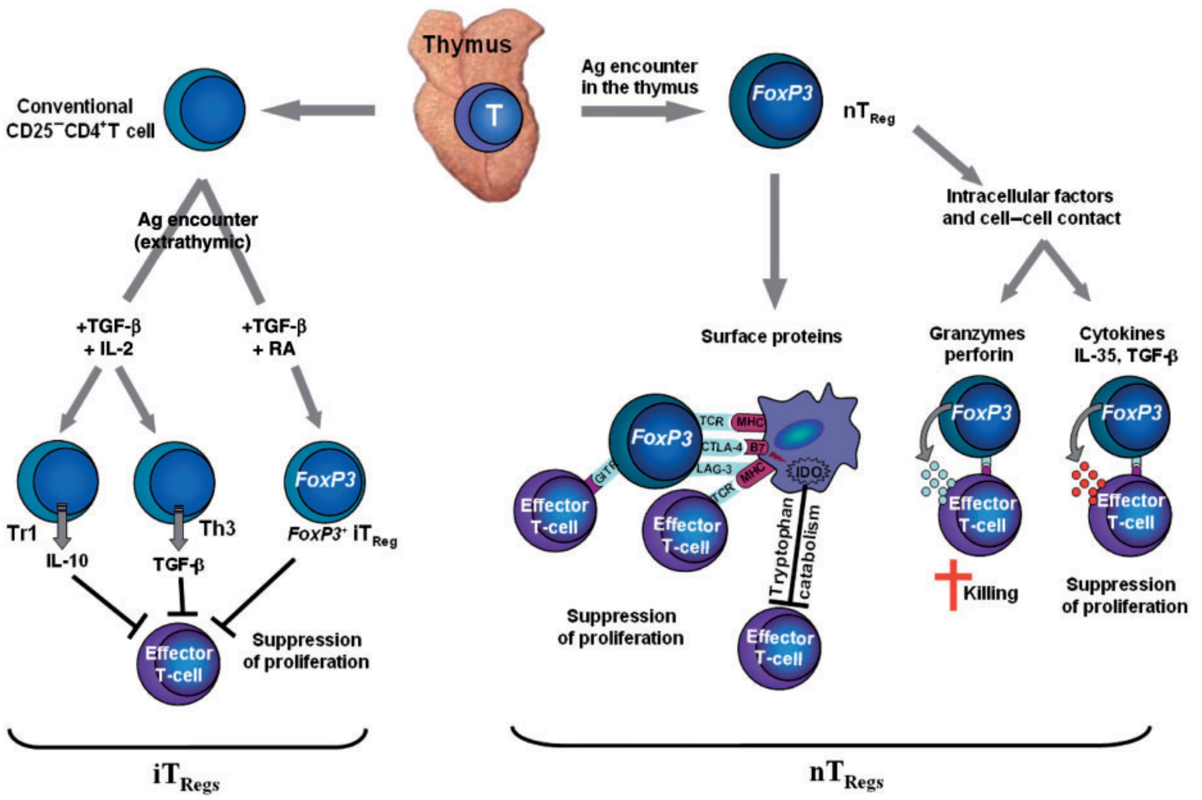
CD4$^+$CD8$^-$ thymocytes express CD25. The frequency and functional characteristics of these CD25$^+$CD4$^+$CD8$^-$ thymocytes are similar to cells with the same phenotype found in the peripheral blood [8]. As CD4$^+$CD8$^-$ thymocytes depleted of CD25$^+$ cells from mature mice produce a spectrum of autoimmune pathology when transferred into syngeneic athymic nude recipient mice, one might conclude that the normal thymus produces functionally mature CD25$^+$CD4$^+$ T cells capable of controlling autoimmune pathogenic T cells [8]. Mice deficient for MHC Class II on cortical thymic epithelial cells fail to develop CD4$^+$CD25$^+$T$_{\text{Reg}}$ cells, suggesting that generation of T$_{\text{Regs}}$ is an early (cortical) event during thymocyte development [9]. In contrast, a recent study by Aschenbrenner *et al.* [10] provides unambiguous evidence that generation of T$_{\text{Regs}}$ in the thymus is a late (medulla) event, which is mediated by medullary thymic epithelial cells.

Once generated, thymic T$_{\text{Regs}}$ are released into the circulation to control auto-reactive responses. These T$_{\text{Regs}}$ are referred to as naturally occurring regulatory T cells (nT$_{\text{Regs}}$).

In addition to nT$_{\text{Regs}}$, accumulating evidence suggests that another type of T$_{\text{Reg}}$ arises from conventional naïve CD4$^+$ T cells upon encountering extrathymic antigens (Fig. 1). These T$_{\text{Regs}}$ that develop extrathymically are termed adaptive/induced T$_{\text{Regs}}$ (iT$_{\text{Regs}}$).

iT$_{\text{Regs}}$ were originally identified in studies on mechanisms associated with oral tolerance. Weiner and colleagues [11] were the first to discover a population of antigen-reactive TGF-β-secreting CD4$^+$ T cells with regulatory function in mice that were orally tolerized to myelin basic protein (MBP). This distinct lineage of T cells was termed regulatory Th3 cells [11]. Interestingly, Sundstedt *et al*. [12] have shown that intranasal administration of MBP induces the appearance of IL-10-secreting CD4$^+$ T cells with suppressor capacity. This subset of regulatory T cells was described earlier by Groux *et al.* [13] and termed regulatory type-1 cells (Tr1). Both Th3 and Tr1 cells lack the transcription factor *FoxP3*, which was originally thought to be uniquely expressed by nT$_{\text{Regs}}$ (see below); however, their suppressive properties resemble those of *FoxP3*$^+$ T$_{\text{Regs}}$. In addition to *FoxP3*$^-$ iT$_{\text{Regs}}$, there also appears to be a population

Fig. 1 Overview of T$_{\text{Reg}}$-cell subsets and their proposed mechanisms of suppression. nT$_{\text{Regs}}$ and conventional naïve CD4$^+$CD25$^-$ T cells are generated from the thymus. nT$_{\text{Regs}}$ arise following intrathymic antigen encounter, can suppress effector cells by either reducing the antigen-presenting capacity of APC or by triggering IDO activity in APC, resulting in the generation of suppressive metabolites. In addition, nT$_{\text{Reg}}$ cells can interact directly with effector cells to inhibit their activation or induce death of effector cells by release of cytotoxic factors. On the other hand, iT$_{\text{Regs}}$ arise from naïve T cells, upon encounter with extrathymic antigens, and include *FoxP3*$^-$ iT$_{\text{Reg}}$ (T-regulatory-1/T-helper-3) and *FoxP3*$^+$ iT$_{\text{Reg}}$ cells. The suppressor effect induced by iT$_{\text{Reg}}$ cells is mainly mediated by cytokine expression.



of *FoxP3*$^{+}$ iT$_{\text{Regs}}$. Recent studies demonstrate that a population of gut dendritic cells (DCs), particularly lamina propria CD103^{+} DCs, can promote the conversion of naïve CD4^{+} T cells into *FoxP3*$^{+}$ iT$_{\text{Regs}}$ through the secretion of retinoic acid (RA) in conjunction with TGF-β [14, 15]. Human *FoxP3*$^{+}$ T$_{\text{Regs}}$ can also be classified into resting and activated T$_{\text{Regs}}$ according to a recent report by Miyara *et al.* (see below) [16].

Collectively, there are at least two distinct types of T$_{\text{Reg}}$: the nT$_{\text{Regs}}$, which are generated in the thymus upon intrathymic antigen encounter; and the iT$_{\text{Regs}}$, which are generated in the periphery upon extrathymic antigen encounter. Whereas the role of iT$_{\text{Regs}}$ in controlling pathological immune responses has been described in mouse models, data relating to their role in human systemic autoimmune diseases are currently lacking. Therefore, we will focus on nT$_{\text{Regs}}$ in this review.

Molecular characterization of T$_{\text{Regs}}$

A major advance in the understanding of nT$_{\text{Reg}}$ function was the discovery of the uniquely expressed transcription factor *FoxP3*, which controls the development and function of T$_{\text{Regs}}$ [17, 18]. The gene *FoxP3*, encoding the so-called scurfin protein, was first identified as a mutated gene in the scurfy mouse strain, which is an X-linked recessive mouse mutant. This mutation leads to lethality of hemizygous males 16–25 days after birth in association with lymphoproliferation and multi-organ infiltration of CD4^{+} T cells with overproduction of cytokines by these cells [19]. Similarly, the mutated *FoxP3* gene was identified in humans as a causative gene for the X-linked syndrome IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) [20–22]. Further analysis revealed that both in scurfy mice and IPEX patients, T$_{\text{Regs}}$ expressing CD4 and CD25 are lacking. Furthermore, retroviral transduction of naïve CD25^{-}CD4^{+} T cells with *FoxP3* can convert them into T$_{\text{Regs}}$, both phenotypically and functionally [18]. These findings demonstrate the importance of *FoxP3* as a master regulator of nT$_{\text{Reg}}$ development and function. However, *FoxP3* expression in human T cells was not directly correlated with their suppressive capabilities, and *FoxP3* was also shown to be expressed in activated non-T$_{\text{Regs}}$, indicating that *FoxP3* is not a unique marker to identify human T$_{\text{Regs}}$ [23, 24]. In accordance, Miyara *et al*. [16] have shown recently that human *FoxP3*$^{+}$ T cells are functionally heterogeneous, and can be classified into three phenotypically distinct subpopulations based on the expression level of *FoxP3* and the naïve T-cell marker (CD45RA). These three subpopulations can be defined as: activated suppressor T$_{\text{Regs}}$ (*FoxP3*$^{\text{High}}$CD45RA^{-}), resting suppressor T$_{\text{Regs}}$ (*FoxP3*$^{\text{Low}}$CD45RA^{+}) and non-suppressor T$_{\text{Regs}}$ (*FoxP3*$^{\text{Low}}$CD45RA^{-}).

Concerning the surface characteristics of T$_{\text{Regs}}$, a number of molecules have been reported to be constitutively expressed on T$_{\text{Regs}}$, which include, in addition to CD25 and CD45RA, the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) [25], lymphocyte activation gene-3 (LAG-3) [26], glucocorticoid-induced TNF receptor (GITR) [27], L-selectin (CD62L) [28], integrin $\alpha_E\beta_7$ (CD103) [29], C-C chemokine receptor 7 (CCR7) [28], CCR4 [30], CCR8 [30] and neuropilin-1 [31]. No surface marker was found to definitively distinguish T$_{\text{Regs}}$ from conventional, activated CD4^{+} T cells since the majority of the aforementioned surface markers are also expressed on activated T cells. Finally, IL-7R (CD127) has been identified as a new biomarker to distinguish regulatory from activated effector T cells [32, 33]. T$_{\text{Regs}}$ down-regulate the expression of CD127 and its expression is inversely correlated with *FoxP3* expression and suppressive function. Thus, this marker may be used to isolate a highly purified population of T$_{\text{Regs}}$ via cell sorting. In addition, recent reports have identified folate receptor-4 (FR4) [34] and ectonucleotidase CD39 [35] as unique cell-surface markers that distinguish nT$_{\text{Regs}}$ from effector T cells. Moreover, a recent publication has claimed that latency-associated peptide (LAP) and IL-1 receptor Type I (CD121a) and Type II (CD121b) are selectively expressed on activated FoxP3^{+} T$_{\text{Regs}}$ but not on activated FoxP3^{+} non-T$_{\text{Regs}}$ [36]. These studies may add to the design of strategies for sorting of functionally active nT$_{\text{Regs}}$.

Taken together, despite increasing interest in defining the phenotype of nT$_{\text{Regs}}$, the subset-specific surface marker(s) that are instrumental in the contact-dependent suppressive mechanisms of nT$_{\text{Regs}}$ have as yet not firmly been established and await further studies (Table 1).

Mechanisms of suppression by T$_{\text{Regs}}$

Numerous *in vitro* studies have shown that T$_{\text{Regs}}$ control the expansion of naïve T cells, suppress the activation and cytokine production of effector T cells [25, 37], and inhibit B-cell proliferation, immunoglobulin production and class switch [38, 39]. Multiple modes of action have been proposed for the suppressive function of T$_{\text{Regs}}$ (Fig. 1).

Soluble factors such as IL-10 and TGF-β were found to play a key role in the suppression mediated by iT$_{\text{Regs}}$. *In vitro* findings strongly suggest a role for these cytokines in preventing autoimmune reactions. It has been observed

Table 1 Characteristics of natural and induced human T$_{\text{Reg}}$ cells

Phenotype/feature	nT$_{\text{Reg}}$	Tr1	Th3
IL-2Rα (CD25)	++	+/−	+
FoxP3 (activated/resting)	+High/+Low	−	−
CD45RA (activated/resting)	−/+	?	?
LAG-3	+	?	?
GITR	+	−	?
CTLA-4	+	+	+
IL-10	−	++	+/−
TGF-β	+/−	+/−	++
FR4	++	?	?
CD39	+	?	?
LAP	+	?	?
IL-1R I/II	+	?	?
Place of origin	Thymus	Periphery	Periphery

?: not known yet.

that T_{Regs} isolated from IL-10 knockout mice lack the intrinsic capacity to protect immunodeficient mice from colitis [40–42]. In addition, treatment with anti-IL-10 receptor antibodies accelerated graft rejection [43]. Similarly, TGF-β-deficient mice manifest a spontaneous autoimmune syndrome, while neutralizing antibodies to TGF-β abrogate T_{Reg}-mediated suppression of IBD [44–46]. In addition to its effects in a soluble form, TGF-β is also operative as a surface-bound protein on nT_{Regs}. It has been shown that surface-bound TGF-β on nT_{Regs} induces suppression through TGF-β-R on autoaggressive cells [39]. In agreement, blockade of cell-surface TGF-β disrupts the suppressive function of nT_{Regs} [39]. In addition to TGF-β, recent data show that suppression by nT_{Regs} is potentiated by soluble IL-35 and IL-10 [47–49]. Thus, the production of soluble factors appears to be crucial for both iT_{Reg}- and nT_{Reg}-mediated suppression.

Another model of T_{Reg}-mediated suppression suggests a cytotoxic mechanism by which T_{Regs} induce death of effector T cells (T_{eff}) via release of granzymes in a perforin- and Fas-independent way [50, 51]. Accordingly, T_{Regs} from granzyme-B-deficient mice showed a reduced capacity to suppress T_{eff} proliferation. Otherwise, activated T_{Regs} were recently reported to suppress B-cell proliferation *in vitro* by inducing apoptosis of these cells via a granzyme-B-mediated cytotoxic mechanism in a perforin-dependent way [52]. This suggests involvement of granzyme-B in a cell contact-dependent kill mechanism.

In addition to the aforementioned mechanisms, *in vivo* and *in vitro* studies have demonstrated that nT_{Regs} can suppress the immune response by modulating the function of antigen-presenting cells (APCs). This is mediated via interaction between CTLA-4 or LAG3 (also called CD223) on nT_{Regs} and CD80/CD86 or MHC Class II molecules on APCs, respectively. Ligation of CD80/CD86 on APC by CTLA-4 on T_{Regs} triggers the induction of the enzyme indoleamine 2,3-dioxygenase (IDO) in APCs, which converts tryptophan into pro-apoptotic metabolites that suppress effector T cells [53]. On the other hand, engagement of MHC Class II molecules on APCs by LAG3 on T_{Regs} suppresses APC maturation and reduces their ability to activate T cells [26, 54]. Moreover, T_{Regs} can mediate suppression via a metabolic disruption through CD39 and CD73. These two ectoenzymes hydrolyse ATP or ADP to extracellular adenosine monophosphate, which inhibits effector T-cell functions through activating the adenosine A2A receptor [55–57].

In summary, many data have been collected on the suppressive mechanisms involved in the function of T_{Regs}, but the precise mechanism of action of immune suppression requires further studies.

Human T_{Regs} are not terminally differentiated but may convert to Th-17 cells

Besides the key role of T_{Regs} in the prevention of autoimmune diseases, a recent breakthrough has revealed that IL-17-secreting cells (Th-17) are the main pathogenic effector subset involved in the induction of inflammation and autoimmunity [58, 59]. During the past 2 years, multiple reports indicate a link between T_{Regs} and Th17 cells. It has been demonstrated that, *in vitro* and *in vivo*, activation of T cells in the presence of TGF-β results in the generation of *FoxP3*$^+$ T_{Regs}; however, the combination of IL-6 and TGF-β promotes the generation of Th17 cells, suggesting that both T-cell subsets may differentiate from the same precursor T cell [60]. Indeed, a reciprocal relationship between T_{Regs} and Th-17 cells has been shown recently at a molecular level [61]. It was found that full-length *Foxp3* directly binds the Th-17-specific transcription factor RORγt and inhibits the expression of genes that define Th-17 cells [61]. Collectively, these findings suggest that the balance of TGF-β and IL-6 might determine the differentiation of T_{Reg}/Th-17 cells through antagonistic competition of *FoxP3* and RORγt, and may underlie the propensity of T_{Regs} to convert to Th-17 cells in the context of pro-inflammatory stimuli. This phenomenon has only recently been recognized in man [62–64]. It has been shown that a subset of circulating human *FoxP3*$^+$CD4$^+$ T cells can express the Th-17 lineage-specific transcription factor RORγt and has the capacity to produce IL-17 upon activation [62–65]. Importantly, the production of IL-17 by this T_{Reg} subset was associated with concomitant loss of its suppressive function. However, others have demonstrated that IL-17-secreting *FoxP3*$^+$ T_{Regs} still maintain their suppressive function [64, 65]. Although *FoxP3* is critical for the suppressive function, IL-17 has been implicated in mediating inflammation and autoimmune diseases. Thus, production of IL-17 by a subset of *FoxP3*$^+$ T_{Regs} could place this subset within the category of effector T cells instead of regulatory cells.

The functional duality of this T-cell lineage appears to be generated in the periphery, as the human thymus does not contain IL-17-producing *FoxP3*$^+$CD4$^+$ T cells [63]. The latter cells may originate from circulating *FoxP3*$^+$ iT_{Regs} or circulating *FoxP3*$^+$ nT_{Regs} or from *FoxP3*$^+$ iT_{Regs}.

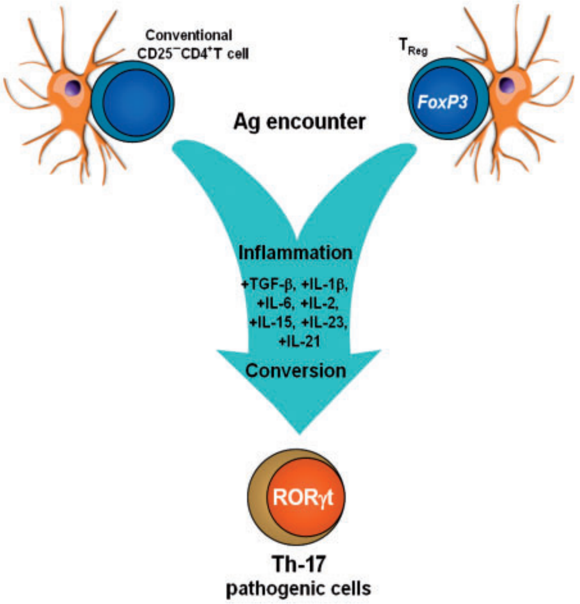
The conversion of human *FoxP3*$^+$ T_{Regs} into IL-17-producing cells can be enhanced in the context of an inflammatory cytokine milieu. Recent reports have shown that IL-1β alone or in combination with IL-23 or IL-6 induces this conversion [62, 65, 66]. Also other cytokines such as IL-2, IL-21 and IL-6 may act cooperatively to induce *FoxP3*$^+$ T_{Reg} differentiation into IL-17-producing cells [64]. Of note, IL-1β is critically involved in switching of *FoxP3*$^+$ T_{Regs} towards IL-17-producing cells and IL-1 receptor (IL-1R) counteracts this process, suggesting that IL-1R expression is involved [62]. Currently, Lee *et al.* [67] have further substantiated this notion and shown that the effect of IL-1β on promoting IL-17 production was dynamically regulated via IL-1R type I, a receptor that was found to be up-regulated on activated CD4$^+$ T-cells upon IL-15 treatment. It is also notable that monocytes differentiated with IL-15 produce inflammatory mediators that may influence the T_{Reg}/Th-17 axis [68]. It seems that IL-15 acts as an important factor in the generation of IL-17-producing T cells [69]. As such, the role of IL-15 in the T_{Reg}/Th-17 balance in autoimmunity awaits to be assessed.

Based on the aforementioned *in vitro* data and the reciprocal relationship of T_{Reg}/Th-17 cells, it can be hypothesized that reduced numbers of *FoxP3*$^{+}$ T_{Regs} in patients with inflammatory autoimmune diseases may occur due to enhanced conversion of these cells into IL-17-secreting cells in the context of an inflammatory milieu (Fig. 2).

Do T_{Regs} convert to pathogenic Th-17 cells in human systemic autoimmune diseases?

Alteration in cell number or function of T_{Regs} is associated with several autoimmune diseases [7]. Studies on peripheral tolerance in systemic autoimmune diseases have focused predominantly on abnormalities in *FoxP3*$^{+}$ T_{Reg} frequency or function, but did not accurately address the stability of *FoxP3* expression in T_{Regs} or the co-expression of other lineage transcription factors such as RORγt and the possible differentiation into IL-17-producing cells. Evidence for enhanced Th-17 responses in systemic autoimmune disease, however, is available and appears to support the hypothesis of conversion of T_{Regs} into pathogenic cells. Here, we summarize data concerning the involvement of *FoxP3*$^{+}$ T_{Regs} as well as Th-17 cells in several human systemic autoimmune diseases and discuss the inflammatory condition that may induce skewing of *FoxP3*$^{+}$ T_{Regs} towards IL-17-producing cells.

Fig. 2 Conversion of CD4^{+} T cells into IL-17-producing cells (Th-17). Priming of naïve T cells (CD4^{+}CD25^{-}) and *FoxP3*$^{+}$ T_{Reg} cells by antigen (Ag) presented on DCs, in the context of inflammatory cytokines, can convert them into pro-inflammatory IL-17-producing cells (Th-17) that may contribute to disease progression.



SLE

Although the aetiology of SLE is unknown, recent data suggest a role for T_{Regs} in disease pathogenesis. Studies in lupus mice models have demonstrated that depletion of CD25^{+}CD4^{+} T cells induces an increase in anti-dsDNA antibodies and accelerates the development of GN [70, 71]. In line with this observation, Lee *et al*. [72] reported an inverse relationship between the percentage of circulating CD4^{+}CD25^{+} T cells and serum levels of anti-dsDNA antibodies in paediatric patients with SLE. Other studies evaluating levels of circulating CD4^{+}CD25^{High} T cells in SLE patients are consistent in their results demonstrating a decrease in absolute numbers as well as in the proportion of CD4^{+}CD25^{High} T cells in active SLE patients as compared with healthy controls [73–75]. Analysis of *FoxP3* expression reveals decreased frequencies of CD4^{+}CD25^{High}*FoxP3*$^{+}$ T cells and increased frequencies of CD4^{+}CD25^{Low}*FoxP3*$^{+}$ and CD4^{+}CD25^{-}*FoxP3*$^{+}$ T cells in active SLE patients in comparison with the control group [76–78]. Consistent with these findings, CD4^{+}CD25^{High} T cells from patients with active SLE have diminished suppressive activity *in vitro* [79, 80]. Importantly, the suppressive activity of CD4^{+}CD25^{High} T cells from active SLE patients could be restored after *in vitro* activation with anti-CD3 in the presence of IL-2 [80]. This suggests that *in vivo* (in the diseased situation) other factors may be involved in altering numbers and function of T_{Regs}. As mentioned, pro-inflammatory cytokines, such as IL-1β, IL-23 and possibly IL-15, may mediate the gradual conversion of *FoxP3*$^{+}$ T_{Regs} gradually into IL-17-producing cells. Indeed, increased levels of serum IL-23, IL-15 and IL-17 were also observed in SLE patients reflecting an enhanced Th-17 response [81–85]. Furthermore, increased frequency of circulating Th-17 cells in correlation with disease activity was recently demonstrated in SLE patients [86]. In view of these findings, we propose that, due to increased levels of inflammatory cytokines, T_{Regs} are converted into effector Th-17 cells that may contribute to flares of disease activity in SLE patients. Further investigations directed at the plasticity of T_{Regs} in SLE patients are warranted.

RA

T_{Regs} in patients with RA appear to be present in normal numbers and to exhibit all of the features of T_{Regs}, not only in phenotype but also in their suppression of T-cell proliferation *in vitro* [87, 88]. However, circulating T_{Regs} isolated from patients with active RA are unable to suppress the release of pro-inflammatory cytokines by activated T cells and monocytes [88]. T_{Regs} from RA patients were shown to receive increased co-stimulatory signals from activated monocytes, leading to a decrease in their suppressive capacity [89]. It has recently been demonstrated that monocytes can induce a gradual conversion of T_{Regs}

into Th-17 cells, which may underlie the dysfunction of T_{Regs} *in vivo* [62]. Interestingly, elevated levels of IL-15, produced by monocytes and DCs in response to inflammatory stimuli, were found in serum and SF of RA patients [90–92]. Also, increased numbers of Th-17 cells were observed in the peripheral blood and the SF of RA patients [93, 94]. Moreover, IL-15 was found to trigger IL-17 production in T cells from human RA peripheral blood or synovial mononuclear cells [92]. It is tempting to speculate that IL-15 contributes to a milieu favouring the differentiation of *FoxP3*$^+$ T_{Regs} into IL-17-producing cells. These data strengthen the hypothesis that *FoxP3*$^+$ T_{Rregs} in RA patients display an effector differentiation programme that result in pathogenic IL-17-producing cells. It should be appreciated, however, that IL-17-producing FoxP3$^+$ T_{Regs} in RA patients await to be identified.

WG

WG is a systemic vasculitis associated with ANCAs mainly directed against proteinase 3. This disorder is characterized by granulomatous inflammation, particularly of the airways and pauci-immune vasculitis and GN [95, 96]. Several lines of evidence suggest involvement of T cells in this disease [97–99]. In a recent study [100], we analysed the distribution and function of circulating T_{Regs} in WG. We found that *FoxP3*$^+$ T_{Regs} were significantly increased in WG patients as compared with healthy controls. However, we observed a defective suppressor function of T_{Regs} in this group of patients [100]. It is possible that T_{Regs} from WG patients convert into IL-17-secreting cells in the context of an inflammatory cytokine milieu. A recent study has reported increased levels of serum IL-23 and IL-17 in WG patients [101]. In addition, expression of IL-15 has been demonstrated in areas with granuloma formation of WG patients, which may contribute to a shift of *FoxP3*$^+$ T_{Regs} into IL-17-producing cells [102]. More importantly, we demonstrated an increase in the percentage of Th-17 cells in WG patients as compared with healthy controls [103]. These data favour the conversion of T_{Regs} towards IL-17-producing T cells in WG patients. Therefore, *FoxP3*$^+$CD4$^+$ T cells in WG patients are possibly effector cells rather than T_{Regs}. This may also explain the inconsistency between increased levels of *FoxP3*$^+$ T_{Regs} in WG patients, on the one hand, and a defective suppressor function of these cells, on the other. Future studies in WG patients should analyse the expression of IL-17 and RORγt in *FoxP3*$^+$ T cells.

SS

SS is an autoimmune exocrinopathy, characterized by chronic inflammation and destruction of the lacrimal and salivary glands resulting in dryness of the eyes (KCS) and mouth (xerostomia) [104]. Similar to other autoimmune diseases, impairment in number or function of T_{Regs} could be involved in the development and perpetuation of SS. However, studies on T_{Regs} in SS patients have yielded controversial results. Gottenberg *et al.* [105] reported that SS patients have increased CD4$^+$CD25^{+High} T cells in the peripheral blood, and that these cells exert normal suppressive activity. Contrary to this study, other studies have reported decreased proportions of CD4$^+$CD25^{+High} T cells in the peripheral blood and also in the salivary glands of SS patients [106, 107]. Recently, Christodoulou *et al*. [108] studied *FoxP3*$^+$ T_{Regs} in the peripheral blood and in minor salivary glands of SS patients. Levels of *FoxP3*$^+$ T_{Regs} in the peripheral blood of SS patients were comparable with those in healthy controls, but correlated negatively with the frequency of *FoxP3*$^+$ T_{Regs} in the salivary gland lesions. Importantly, numbers of infiltrating *FoxP3*$^+$ T cells in these lesions were positively correlated with the focus scores in the salivary gland biopsy, which suggests an association of infiltrated T_{Regs} with the grade of severity of the autoimmune lesion in SS patients [108]. Moreover, increase in IL-15 expression was observed in biopsies from SS patients with ectopic germinal centre formation [109]. Furthermore, infiltrating CD4$^+$ T cells in the salivary glands of SS patients predominantly expressed IL-17 [110, 111]. In addition, expression levels of IL-17 in the salivary glands progressively increased with higher levels of focus scores [112], as did numbers of *FoxP3*$^+$ T_{Reg} cells [108]. These data suggest that the inflammatory cytokine milieu within the lesions promotes the conversion of infiltrated *FoxP3*$^+$ T_{Regs} into IL-17-producing cells that may underlie the development of lymphocyte infiltrates in SS.

Strategy for expansion and isolation of highly pure *FoxP3*$^+$ T_{Regs} to be used in cellular therapy

T_{Regs} have been suggested to be important for intervention as an alternative to conventional therapy in human autoimmune diseases. In view of the recent findings discussed above, transfer of T_{Regs} may be less beneficial or even harmful in established inflammatory conditions, since a fraction of *FoxP3*$^+$ T_{Regs} may differentiate into Th-17 cells with pathogenic potential. Therefore, depletion of IL-17-producing T_{Regs} from the real T_{Regs} is proposed. Recently, Kleinewietfeld *et al*. [113] introduced a new approach to isolate a highly purified population of *FoxP3*$^+$ T_{Regs} free of contaminating effector T cells by depleting cells double positive for CD49d and CD127 from the CD4$^+$ T-cell population. Thus, isolation of CD49d$^-$CD127$^-$ T_{Regs} by a negative selection procedure provides access to a highly pure population of *FoxP3*$^+$ T_{Regs} that have not been tagged by an antibody, which is expected to be more suited for clinical applications. Indeed, Kleinewietfeld *et al.* [113] have determined the efficacy of CD49d$^-$CD127$^-$ T_{Regs} in an acute graft-*vs*-host disease (GVHD) mouse model. They induced an acute aggressive form of GVHD in mice by transfer of CD25-depleted human peripheral blood mononuclear cells (PBMCs) into Rag2$^{-/-}\gamma c^{-/-}$ mice. They found that addition of CD49d$^-$CD127$^-$ T_{Reg} cells completely prevented GVHD. Taken together, CD49d$^-$CD127$^-$ T_{Regs} seem to be potent suppressor cells capable of controlling pro-inflammatory immune responses *in vivo*.

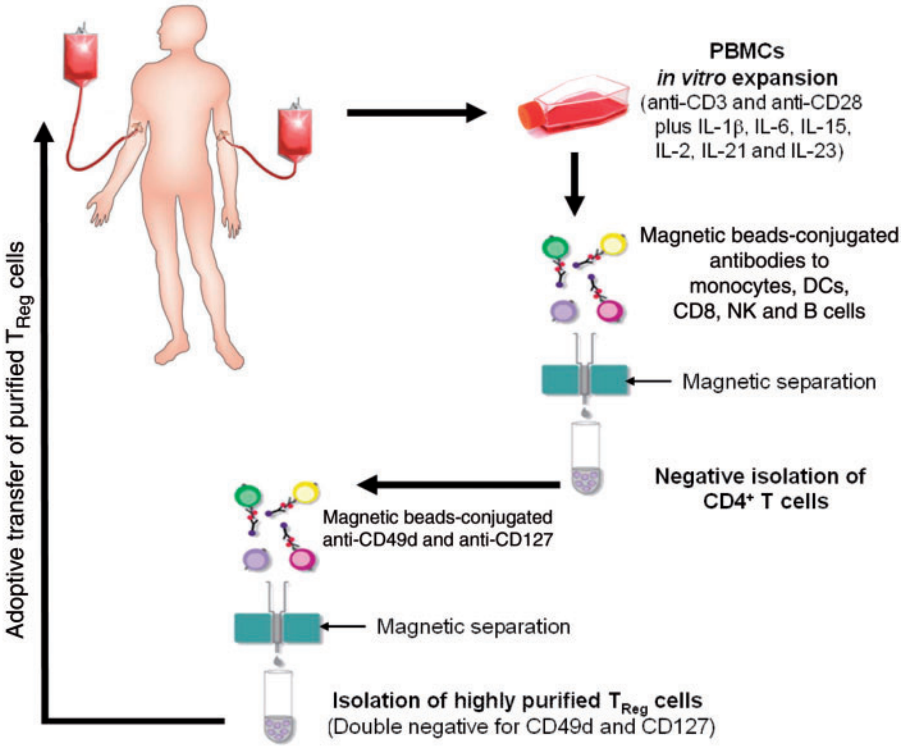
Similar to the aforementioned approach, we propose a two-step, new strategy for adoptive T_{Reg}-based forms of therapy (Fig. 3). In this two-step approach, we first seek to expand the number of T cells (and thus also the nT_{Regs}), and secondly to isolate pure T_{Regs} via negative selection. To this end, peripheral blood mononuclear cells are isolated from a patient, and stimulated polyclonally with anti-CD3 and anti-CD28 monoclonal antibodies for 7 days in the presence of IL-1β, IL-2, IL-6, IL-15 and IL-23. In this culture condition, numbers of T_{Regs} will increase, and a fraction of *FoxP3*$^+$ T cells will differentiate towards IL-17-producing cells. The fraction of *FoxP3*$^+$ T_{Reg} cells that does not produce IL-17 (or does not convert into Th-17 cells) is characterized by lack of surface expression of CD49d and CD127 [113]. The latter fraction of T_{Regs} can be isolated negatively by two steps using immunomagnetic beads technique (Fig. 3). Here, CD8$^+$ T cells, γ/δ T cells, B cells, NK cells, DCs, monocytes, granulocytes and erythroid cells, will be removed by immunomagnetic separation using cell-specific mAbs coupled with magnetic beads. Next, the negatively isolated CD4$^+$ T cells will be labelled with magnetic beads-conjugated anti-CD49d and anti-CD127, to remove the CD49d$^+$CD127$^+$ cells, and the fraction of pure *FoxP3*$^+$ T_{Regs} (double negative for CD49d and CD127) will be collected, and finally re-injected into the patients in order to restore the T_{Reg}/T-effector balance in autoimmune diseases.

It was previously shown that epigenetic modification underlies the phenomenon of T_{Reg} plasticity [62]. Thus, it is feasible that concomitant treatment with histone deacetylase inhibitors may help stabilize the T_{Reg} phenotype and may thus add to the success of cellular T_{Reg} therapy. Clearly, further clinical studies aimed at optimizing this approach for treating human systemic autoimmune diseases are warranted.

Summary

Major focus has been placed on the role of *FoxP3*$^+$ T_{Regs} in autoimmune diseases. Although decrease in number or function of *FoxP3*$^+$ T_{Regs} may favour the autoimmune response in systemic autoimmune diseases, the aetiology of this defect as well as the exact mechanisms of suppression are still elusive and need further studies.

Fig. 3 Highly purified population of *FoxP3*$^+$ T_{Reg} cells for adoptive cell therapy. Peripheral blood mononuclear cells will be isolated from the peripheral blood of a patient, and stimulated *in vitro* with anti-CD3 and anti-CD28 mABs in the presence of IL-1β, IL-6, IL-2, IL-15, IL-21 and IL-23 for 7 days. This step is necessary to increase the limited number of circulating *FoxP3*$^+$ T_{Regs}, and to induce differentiation of a fraction of *FoxP3*$^+$ T cells with potential to become IL-17-producing cells. Next, CD4$^+$ T cells will be isolated by negative selection using an immunomagnetic beads technique. Subsequently, CD4$^+$ T cells will be stained for bead-conjugated anti-CD49d and anti-CD127. In this step, the cells double positive for CD49d and CD127 (which are the IL-17-producing cells) will be removed, and highly purified *FoxP3*$^+$ T_{Regs} will thus be isolated by negative selection (being double negative for CD49d and CD127). Finally, this fraction of functional T_{Reg} cells, which are not positively but negatively selected by antibodies, can be infused into the patient.



Recent studies in humans demonstrate skewing in a fraction of *FoxP3*$^+$ T$_{\text{Regs}}$ towards pathogenic IL-17-secreting cells in the context of a pro-inflammatory cytokine milieu. In this regard, interpretation of impaired function/numbers of *FoxP3*$^+$ T$_{\text{Regs}}$ in systemic autoimmune diseases should be handled with caution and needs to be updated. In addition, the current strategies for T$_{\text{Reg}}$-based forms of therapy in autoimmune diseases should take this issue into account. In view of recent findings, removal of CD49d$^+$CD127$^+$ cells from the CD4$^+$ T cells provides access to highly pure populations of immune-suppressive *FoxP3*$^+$ T$_{\text{Regs}}$ free of IL-17-secreting cells. Thus, future perspectives for cellular therapy in human autoimmune diseases could be built on re-injection of *ex vivo* expanded CD49d$^-$CD127$^-$ T$_{\text{Regs}}$.

Rheumatology key messages

- *FoxP3*$^+$ T$_{\text{Regs}}$ are not terminally differentiated, but may convert into IL-17-producing effector T cells.
- Reduced numbers of *FoxP3*$^+$ T$_{\text{Regs}}$ in autoimmune inflammation may occur due to their conversion into IL-17-producing cells.
- T$_{\text{Reg}}$-based cellular therapy could be built on re-injection of *ex vivo* expanded, highly pure T$_{\text{Regs}}$.

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