Induced Regulatory T cells: Mechanisms of Conversion and Suppressive Potential

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Abstract

Thymus-derived, naturally-occurring CD4^{+} FoxP3^{+} regulatory T cells (nTreg) have suppressive activity that is important for the establishment and maintenance of immune homeostasis in the healthy state. Abundant reports have shown that they can suppress pathogenic processes in autoimmune diseases and inhibit transplant rejection and graft-versus-host disease. Far less is known about induced regulatory T cells (iTreg) that are generated from naïve T cells in the periphery or *in vitro*, by directing naïve T cells to acquire suppressive function under the influence of transforming growth factor-β (TGF-β) and other factors. In this review, we describe mechanisms by which naïve T cells are thought to be converted into iTreg. We also discuss the suppressive potential of iTreg, particularly in comparison to their naturally-occurring counterparts, focusing on those reports in which direct comparisons have been made. Based on current knowledge, we consider the rationale for using iTreg versus nTreg in clinical trials.

Keywords

Naturally-occurring regulatory T cells; induced regulatory T cells; immune suppression; tolerance; transplantation

1. INTRODUCTION

Numerous studies have demonstrated the importance of regulatory T cells in maintaining tolerance to auto- and allo-antigens (Ags). Naturally-occurring Treg (nTreg), that are generated in the thymus, were first described in mice by Sakaguchi et al in 1995 [1] and their suppressive function was later associated with expression of the winged helix transcription factor Forkhead Box P3 (FoxP3) [2]. Additionally, human nTreg can be identified by expression of CD4^{+}, in combination with cell surface CD25(IL-2Rα)[hi], and the absence of CD127(IL-7Rα) [3]. Adoptive transfer of nTreg has been successful in animal

models of autoimmune diseases and transplantation (Tx) [4, 5]. However, as the frequency of nTreg in healthy humans is only ~5% of CD4^{+} T cells, it is difficult to obtain sufficient numbers of these cells for clinical testing [6, 7]. Many groups have reported the marked expansion of nTreg from a CD4^{+} CD25^{hi} (in combination with CD127^{−} or CD45RA^{+}) starting population by T cell Ag receptor (TCR) stimulation in the presence of exogenous IL-2 [8, 9]. However, during prolonged expansion for 3–4 weeks, T effector cells (Teff) outgrow nTreg, resulting in reduction of the suppressive capacity of the cultured cells [3, 7]. A shorter expansion period would avoid this difficulty, but the limited nTreg number that can be generated under these conditions would constitute a problem when Treg are to be used in a clinical setting.

To address this issue, various groups have sought to generate Treg from a naïve T cell starting population. Beginning in 2002, the first reports were published on adaptive or induced Treg (iTreg) that are CD4^{+}FoxP3^{+} cells which are generated in the periphery, and also that have suppressive capacity [10–12]. These iTreg are generated *in vivo* from naïve T cells in peripheral lymphoid organs during immunological responses to Ag stimulation, a phenomenon that is replicated *in vitro* through stimulation of mouse or human T cells under specific conditions. Various groups have observed that iTreg can be generated easily by conversion of much more abundant naïve CD4^{+} T cells *in vitro*, allowing much higher numbers of Treg to be obtained [10–12]. In the in vitro setting, iTreg can be generated in either a polyclonal or an Ag-specific fashion. The percentage of iTreg in the total population of circulating Treg in healthy mice and humans is thought to be in the range <3 to 30% [13, 14]. Here, we review current knowledge on the generation of iTreg and discuss their suppressive function in comparison to nTreg.

2. THE INDUCTION OF TREG FROM NAÏVE T CELLS

2.1 In vivo pathways of iTreg generation

Several pathways through which Treg can be induced in the periphery *in vivo* have been described. In general, all Ag-presenting cells (APC) have the potential to induce Treg. Thus, dendritic cells (DC) in the gut-associated lymphoid tissue (GALT) can promote iTreg conversion [15–18]. However, B cells [19]; mesenchymal stem cells [20, 21] and myeloid-derived suppressor cells [22] are also able to promote conversion.

The presentation of self- or foreign Ag is important for conversion to the Treg phenotype. In vivo generation not only occurs as a homeostatic phenomenon, but also during allo immune responses. iTreg reactive to foreign Ag are generated in response to microbes and food Ags in the intestinal mucosa [17], during the induction of tolerance to chicken ovalbumin (OVA) in the mesenteric lymph nodes [23], and by OVA-presenting DC [24]. They are also found in response to self Ag in chronically inflamed tissues [25], in response to self Ag in a mouse autoimmune diabetes model [26], and during homeostatic repopulation in lymphopenic hosts reconstituted with Treg-depleted cells [27, 28]. Moreover, generation of iTreg in response to donor tissue in transplanted organs has been studied extensively. Plasmacytoid DC play an important role in their generation under alloAg stimulation in the transplant setting [29]. Immunosuppressive therapy is also of major influence; different studies show preferential generation of iTreg with use of non-depleting anti-CD4 mAb [30], anti-CD154 mAb +rapamycin [31], or rapamycin alone [32]. By contrast, cyclosporine is detrimental for iTreg generation *in vitro* as well as *in vivo* [32].

2.2 Infectious tolerance: nTreg can generate iTreg from conventional CD4^{+} T cells

Although the concept of infectious tolerance has long been recognized as a phenomenon in which the T cells of a tolerant mouse or rat can transfer their suppressive activity to conventional CD4^{+} T cells in a naïve host [33–35], a possible mechanism underlying this

phenomenon has been described much more recently. Two groups have reported the induction of Treg from $CD4^+CD25^-$ T cells by nTreg [36, 37]. Both studies showed that human nTreg could induce anergic suppressor cells from a $CD4^+ CD25^-$ population. Conversion occurred in a population that did not contain $FoxP3^+$ cells; during conventional immune responses in vivo, this process is tightly regulated. Homeostatic regulation guarantees the maintenance of an appropriate balance between Treg and conventional T cells. Cell-cell contact between nTreg and naïve $CD4^+$ T cells was necessary for the generation of iTreg, but these iTreg could, in turn, suppress proliferation of Teff in a cell contact-independent fashion. Key cytokines that have been associated with the suppressive activity of iTreg are transforming-growth factor-β (TGF-β) [37] and IL-10 [36]. The mechanisms of infectious tolerance have been further elucidated recently by Kendal et al [38], who have shown that the presence of Treg is crucial for continuous suppression of Teff cells. Peripherally-induced $FoxP3^+$ Treg can sustain tolerance by converting naïve T cells to the next generation of $FoxP3^+$ cells.

3. KEY COMPONENTS OF *IN VITRO* GENERATION OF iTREG: IL-2, TGF-β AND COSTIMULATION

IL-2 is required for the generation and expansion of nTreg, together with stimulation of the TCR (CD3) and costimulation (via CD28) [5, 9]. By contrast, the requirements for iTreg generation and expansion are still under investigation. The main factors that have been identified as crucial for induction of FoxP3 expression in $CD4^+CD25^-$ cells are IL-2 and TGF-β [10, 12]. Zheng et al [12] first showed that $CD4^+$ suppressor cells could be generated *in vitro* from human $CD4^+CD25^-$ cells with TGF-β and stimulation by irradiated superAg-presenting B cells. The iTreg generated had a $CD4^+CD25^{hi}$ cytotoxic T lymphocyte Ag 4 $(CTLA4)^+$ phenotype, exhibited reduced production of interferon (IFN)-γ and IL-10, and suppressed autologous antibody (Ab) production through cell contact as well as TGF-β production. Chen et al [10] reported that TGF-β, together with anti-CD3 and APC stimulation could potently convert mouse $CD4^+CD25^-$ Teff into Treg $(CD4^+CD25^+CD45RB^-)$ that suppressed allergic responses in a mouse asthma model. Subsequently, several groups have demonstrated that strong costimulation provided by B7 through CD28 during iTreg generation prevents FoxP3 upregulation and renders cells with poor suppressive function [11, 19, 39]. By contrast, upregulation of CTLA4, which is the negative counterpart of CD28 in B7-mediated regulation [39] is required for the suppressor function of iTreg [28, 40–43]. Fantini et al [44] observed that exogenous IL-2 is required for *in vivo* generation and expansion of iTreg in a murine colitis model, and that TGF-β-induced FoxP3 expression is associated with downregulation of the inhibitory signaling protein and transcription factor mothers against decapentaplegic (Smad)7, which is a TGF-β type 1 receptor antagonist. This makes iTreg susceptible to TGF-β-mediated regulatory effects. These observations were confirmed by *in vitro* experiments with neutralizing anti-IL-2 Abs in which IL-2 was shown to be crucial for the TGF-β-mediated conversion of naive $CD4^+$ T cells [45]. A recent development is the use of IL-2/anti-IL-2 mAb complexes that deliver a consistent IL-2 level to the cultured cells which is not dependent on the cells' own IL-2 secretion, and is a potent method to generate iTreg with stable FoxP3 expression [46]. Altogether, these observations suggest key roles for IL-2, TGF-β, and negative costimulation through CTLA4 in the generation of iTreg, which are summarized in Table 1.

4. AUGMENTATION OF iTREG GENERATION

4.1 The role of retinoic acid in iTreg conversion

Vitamin A is an important metabolite of the body that influences immune regulation. Its deficiency has been associated with infectious diseases in children [47]. DC in the GALT

can produce the vitamin A derivative retinoic acid (RA), which is associated with gut-homing of T cells, as well as enhanced FoxP3 expression of CD4+ T cells in mice [19, 48] and humans [49]. RA functions synergistically with TGF-β in the mouse intestine to induce FoxP3 expression in naïve T cells upon Ag activation [15, 17]. When the active derivative of RA, all-trans retinoic acid (ATRA), is added to cultures of CD4+FoxP3− T cells, together with IL-2, anti-CD3/28 and TGF-β, a dose-dependent significant increase in FoxP3 expression is found when compared to culture without ATRA [19]. The cells express high levels of the CC chemokine receptor CCR9 and α4β7, which are homing receptors associated with the intestinal lamina propria [17, 50, 51]. The mechanism by which RA enhances FoxP3 expression and suppressive capacity in iTreg remains under investigation. Different studies demonstrate that it functions either through a cytokine-dependent mechanism, by blocking the secretion of pro-inflammatory cytokines by memory T cells [52], or through cytokine-independent mechanisms, as RA retains its inhibitory effect in the absence of cytokines [53].

The influence of ATRA on iTreg generation has been confirmed in studies showing the generation of human iTreg with stable FoxP3 expression *in vitro* using ATRA+TGF-β. Combination of ATRA+TGF-β significantly enhances FoxP3 expression of iTreg, as shown by our own studies (Figure 1). Moreover, iTreg generated with ATRA+TGF-β have enhanced suppressive capacity compared to either ATRA or TGF-β alone [54].

By contrast, when human nTreg are cultured with ATRA, this is not associated with increased expansion or suppressor function, although culture of nTreg in ATRA in combination with rapamycin enhances their suppressive capacity [55]. These findings suggest an important role for ATRA in the induction, but not in the maintenance, of regulatory function in nTreg.

4.2 Rapamycin functions synergistically with TGF-β

The immunosuppressive pro-drug rapamycin is known to selectively inhibit the expansion and function of Teff by blocking the serine/threonine kinase activity of the mammalian target of rapamycin (mTOR), but still allows nTreg to proliferate [56] (for further details see Thomson et al [57]). Several groups have studied the effects of rapamycin in the context of iTreg generation. Rapamycin can enhance the *de novo* generation of iTreg in bulk CD4+ as well as CD4+CD25− cell populations [58]. Mechanistic studies by Haxhinasto et al [59] have revealed that the active form of the kinase AKT (AKT*) selectively suppresses the *de novo* expression of FoxP3 induced by TGF-β in murine CD4+CD25− cells, without affecting the already established FoxP3 levels of nTreg. This effect of AKT* is reduced by rapamycin, suggesting a protective effect of rapamycin on the induction of Treg. These findings are supported by the study of Delgoffe et al [60], who showed that mTOR is required for the development of effector T cells from bulk CD4+ T cells. In mTOR-deficient mice, T cells differentiate into iTreg even under strong cytokine-induced skewing conditions for Th1, Th2 or Th17 generation. This is associated with decreased signal transducer and activator of transcription (STAT) activation and hyperactive Smad3 phosphorylation, resulting in higher sensitivity of cells to TGF-β.

In a study of non-human primate (baboon) cells, rapamycin increased the induction of FoxP3+ cells from purified CD4+ cells by ~2-fold (11.2% to 20.6%). CD4+ CD25+ cells isolated from the expanded population potently suppressed a xenogeneic T cell proliferative response [61]. In a recent study of human cells [55]. nTreg expanded in the presence of rapamycin showed improved capacity to induce infectious tolerance. Thus, CD4+ FoxP3− cells cocultured with nTreg for 3 days showed 8% FoxP3+ expression and significant IL-2 production; however, if the nTreg were expanded in the presence of rapamycin (or rapamycin+ATRA), there was a 2-fold increase in FoxP3 induction in Teff, and IL-2

production was abrogated. Another recent study describes the potent ability of a combination of rapamycin and TGF-β to induce FoxP3 expression in human naïve T cells after expansion in culture for up to 2 weeks [62]. Moreover, the iTreg that were generated by this method suppressed GVHD in a xenogeneic model.

An important role in the conversion and function of iTreg in relation to mTOR activity has been found for the coregulatory B7 family member programmed death-ligand1 (PD-L1, = B7-H1), which is expressed on hematopoetic and parenchymal cells. Expression of PD-L1 is associated with the inhibition of autoreactive CD4^{+} T cell responses and peripheral T cell tolerance [63]. PD-L1 synergizes with TGF-β to inhibit the AKT-mTOR axis, thereby promoting iTreg development and function via the same pathway as rapamycin [64]. Cobbold et al [65] have linked the effect of mTOR inhibition to infectious tolerance by showing that enzymes produced by nTreg consume certain amino acids that are essential for T cell proliferation, resulting in inhibition of the mTOR pathway and induction of FoxP3 expression. These studies suggest that upregulation of PD-L1 may be used as a strategy to further optimize iTreg conversion.

Taken together, these studies show that mTOR inhibition with rapamycin can enhance induction of FoxP3^{+} Treg from a naïve T cell population, and function synergistically with ATRA (Table 1). This supports the concept that use of rapamycin as maintenance therapy may constitute tolerogenic immunosuppression [66, 67].

5. ALTERNATIVE APPROACH: GENETIC ENGINEERING TO INDUCE FOXP3 EXPRESSION

An alternative strategy to induce suppressor function in conventional T cells is *ex vivo* FoxP3 gene transfer using a viral vector. This approach was studied by Hori et al [68], who demonstrated the conversion of 30–60% of mouse CD4^{+}CD25^{−} cells to the Treg phenotype after forced expression of FoxP3 was induced by retroviral transduction. The iTreg generated had reduced cytokine production and a potent inhibitory effect on autologous Teff proliferation. Moreover, the FoxP3^{+} cells generated were capable of abrogating autoimmune disease in a mouse inflammatory bowel disease model. Lentivirus-based transduction of the FoxP3 transcription factor was explored by Allen et al [6], after it was found that overexpression of FoxP3 by regular retroviral transduction did not lead consistently to generation of suppressive Treg [69]. In contrast to conventional retroviral vectors, lentiviral vectors have the ability to replicate in non-cycling cells, rendering them very efficient in gene transduction. A lentiviral vector was used to induce FoxP3 in human FoxP3^{−}cells, and a maximum transduction efficiency of 76–97% was achieved. The FoxP3^{+} cells had an nTreg phenotype and potent suppressor function. Additionally, it was shown that lentivirus-driven FoxP3 expression could be maintained for several weeks, whereas expanded nTreg lost FoxP3 expression upon prolonged culture.

These studies indicate that there are several ways to obtain high numbers of FoxP3^{+} suppressor cells from a naïve CD4^{+} T cell population, and promising results provide a strong rationale for the use of viral transduction in further studies.

6. *IN VIVO* PREVALENCE OF iTREG

The incidence of iTreg *in vivo* has been determined in different settings. When their frequency was assessed by enumeration of FoxP3^{+} cells after homeostatic repopulation in a FoxP3^{−} mouse model, it was found to be 8% [28] and 4–7% [27] of total CD4^{+} cells, respectively. However, in a study without lymphodepletion, little to no conversion was found upon stimulation with self Ag [14]. A solution to the issue of distinguishing nTreg

from iTreg would preferably be through a marker that is expressed specifically on one, but not the other cell type. The development of the T lymphocyte lineage is linked to one of the members of the Ikaros transcription factor family, Helios [70]. Expression of Helios is upregulated in nTreg by binding to the FoxP3 promoter; upon Helios elimination, FoxP3 expression and the suppressive function of nTreg is inhibited [71]. A recent study [13] used Helios expression to assess nTreg/iTreg proportions in healthy mice and humans. Helios was expressed by 100% of thymic $CD4^+FoxP3^+$ nTreg, but was not expressed when mouse or human $FoxP3^+$ iTreg were generated *in vitro*. Both mouse and human $CD4^+FoxP3^+$ cells from healthy subjects are 70% $Helios^+$[13], suggesting that 30% of circulating $FoxP3^+$ Treg are peripherally-induced iTreg. Based on these studies, it can be concluded that there is a significant percentage of circulating iTreg in healthy individuals, although the exact proportion of iTreg within the total $FoxP3^+$ population needs to be better defined.

7. TREG PLASTICITY: Th17 DIFFERENTIATION

Conversion of naïve T cells carries the risk that not all cells will be converted to Treg, and that remaining $CD4^+$ T effector cells will contaminate the cultures. However, during the activation of murine naïve $CD4^+$ T cells in the presence of TGF-β, there is a crucial role for IL-6, a common pro-inflammatory cytokine produced by Teff and macrophages. When combined with TGF-β, this acute-phase protein is associated directly with differentiation of $CD4^+$ T cells into pro-inflammatory Th17 cells, rather than into the $FoxP3^+$ iTreg phenotype in the mouse model, suggesting that an inflammatory milieu can drive naïve cells to convert into effector rather than suppressor cells [72, 73]. Moreover, IL-6 can convert already established $FoxP3^+$ nTreg into Th17 cells [74, 75]. The capacity of IL-6 (or an inflammatory milieu in general) to direct T cells to an immune effector rather than regulatory phenotype is a problematic issue for expansion of a pure Treg population *in vitro*, and could also be of crucial importance when Treg are used in a clinical setting of autoimmune disease or transplantation, where, despite immunosuppressive therapy, some degree of inflammatory response can be expected.

In addition to the influence of IL-6, high levels of TCR/CD28 costimulation are associated with induction of nuclear factor-κB (NF-κB) activation, which potently inhibits iTreg differentiation in naïve $CD4^+$ T cells. Indeed, when naïve $CD4^+$ T cells are stimulated with anti-CD28 and high doses of anti-CD3, expression of the proinflammatory cytokines IL-17 and IL-9 is enhanced significantly, and the frequency of $FoxP3^+$ cells diminished [39]. As the main goal of iTreg induction is to generate potent suppressor cells, contamination by outgrowth of effector cells is likely to have a detrimental effect on their potential application. These recent observations raise concerns with regard to the preparation of Treg for therapeutic application, and indicate that a reliable method to assess suppressive capacity might become a necessary goal.

A promising and straightforward solution to overcome the detrimental outgrowth of Th17 cells in the Treg population is the addition of ATRA or rapamycin during the expansion of naïve T cells. ATRA potently suppresses Th17 differentiation of naïve T cells and drives them to iTreg conversion with stable $FoxP3^+$ expression, despite an IL-6-induced inflammatory environment [23, 76]. Recently, it has been shown that ATRA has similar effects on nTreg stability as on iTreg, since it prevents their conversion to Th17 cells [77]. Similar to the inhibitory effect of ATRA on TGF-β-mediated iTreg/Th17 differentiation, rapamycin inhibits the development of Th17 cells from bulk $CD4^+$ cells, as well as $CD4^+CD25^-$ populations under Th17-skewing conditions [58]. Therefore, it is likely that iTreg generated *in vitro* under the influence of ATRA or rapamycin will have a stable phenotype and function. It is not clear if this same effect can be obtained by *in vivo* administration of ATRA or rapamycin.

Importantly, in experiments involving human cells, it has been found that naïve T cells do not differentiate into Th17 cells when stimulated with TGF-β and IL-6 alone, but that this requires additional stimulation by IL-1β ([78], IL-23 [79], or DC [80]. Unfortunately, reports on human iTreg have not determined the Treg-skewing effects of ATRA or rapamycin on iTreg/Th17 differentiation. Although this knowledge is perhaps not essential for *in vitro* generation of these cells (since human naïve T cells need additional stimulation to differentiate into Th17 cells, and cells and cytokine levels can be strictly controlled during laboratory experiments), it will certainly be an important issue for future clinical application of iTreg.

8. iTREG VS nTREG: DIRECT COMPARISON

Induced Treg have significant suppressive function when compared to baseline levels of T cell proliferation (baseline = responder cells + stimulation only; no Treg or control T cells added), but a wide range of suppressive efficacy has been reported depending on methods of induction [10, 12, 54, 58]. To adequately compare the function of iTreg with that of nTreg, it is necessary to test both cell types in the same system and under identical conditions. Interestingly, only a few groups that have generated iTreg have undertaken this direct comparison of the function of iTreg versus nTreg. The few studies on iTreg in the mouse model that have done so all show the level of suppression of <u>fresh</u> nTreg compared to <u>expanded</u> iTreg.

Conversion of $CD4^+CD25^-$ T cells by 5 days stimulation with anti-CD3, irradiated APC and IL-2 +/− TGF-β showed, when compared to fresh $CD4^+CD25^-$ T cells, a significant increase in normalized FoxP3 mRNA levels which was about 50% of the nTreg level. These 5-day expanded iTreg potently suppressed baseline $CD4^+CD25^-$ Teff proliferation to levels comparable to those when nTreg were assessed [11]. In another study using a similar method of induction, murine $CD4^+CD25^-$ T cells were cultured for 5 days with plate-bound anti-CD3 and soluble anti-CD28 +/− IL-2 +/− TGF-β. The frequency of $CD25^+FoxP3^+$ iTreg increased significantly from 3% (no TGF-β) to 53% when IL-2+TGF-β was added to the cultures. In a suppression assay, the addition of TGF-β-expanded iTreg or fresh nTreg (1:1 to responders) resulted in similar suppression of Teff proliferation to 5–10% of baseline under both conditions. These findings were then translated to an *in vivo* colitis model, where iTreg infusion resulted in a marked decrease in colonic inflammation, although these results were not compared to nTreg [81].

Very promising results have also been reported by Benson et al [19], who showed almost 100% conversion to $CD4^+FoxP3^+$ iTreg by 4 days expansion of $CD4^+FoxP3^-$ Tcells with anti-CD3, anti-CD28, IL-2, TGF-β and ATRA. Expanded iTreg exhibited similar suppressive activity as fresh nTreg when added to responder $CD4^+$ cells in a 1:1 ratio. At a ratio of 1:8 (Treg:responder), iTreg retained some suppressive effect, whereas fresh nTreg had lost their suppressive capacity. These studies in the mouse model provide evidence that iTreg can exhibit suppressive function at a similar strength as nTreg.

In a study in which baboon bulk $CD4^+$ T cells were expanded in the presence of anti-CD3/28 beads, IL-2 and rapamycin for 4 weeks, increased expression of $FoxP3^+$ of 20% (11% when cultured without rapamycin) was found. The $CD25^{hi}$ cells were purified after expansion, and added to an MLR of autologous baboon $CD4^+CD25^-$ responders stimulated with xenogeneic (porcine) irradiated PBMC. Compared to freshly-isolated nTreg, iTreg cultured with rapamycin showed similar, potent inhibition of the xenogeneic T cell response [61].

Interestingly, a recent study describes the induction of human Treg by bacterial superAgs, which provide potent polyclonal activation [82]. Stimulation of human $CD4^+CD25^-$ T cells

with Streptococcal pyrogenic exotoxin and autologous APC alone (without the addition of IL-2 or TGF-β) resulted in the induction of 20% $CD25^+FoxP3^+$ T cells after 3 days stimulation (5% without bacterial Ag stimulation). Subsequent addition of Treg in an autologous $CD4^+CD25^-$ proliferation assay showed that, at 1:1 ratio, fresh nTreg could reduce proliferation to 20% of baseline, compared to 40% for iTreg; however, at 1:10, nTreg were still suppressive (55%), whereas iTreg had lost their suppressive effect (80%).

Overall, these studies provide promising evidence that, similar to the findings in the mouse model, in humans and primates iTreg generated from naïve T cells can acquire suppressive function of similar strength as their nTreg counterparts.

9. FUTURE PROSPECTS

Many questions remain unresolved regarding pathways and optimal conditions for the induction of Treg from a naïve T cell population and how the resulting population compares to nTreg. Although recent reports are promising, there are very few studies that provide a direct comparison of nTreg and iTreg. This will be of great importance when such studies are translated to clinical trials in the near future, especially in regard to the quantity of cells needed. Notably, a recent study shows that human nTreg can be expanded on a much greater scale than had been reported previously and used for the first time in clinical trials [83, 84], thus making the cell number that can be obtained of less importance when proposing the use of iTreg. To make iTreg true competitors of their naturally-occurring counterpart, the suppressive function of iTreg should be at least equal to that of nTreg.

One concern that may arise when translating studies on iTreg to clinical trials, is that not all cells from the starting population would be converted, thus leaving a remainder of contaminating Teff. However, this concern could be overcome by sorting the iTreg after expansion (e.g., based on expression of $CD25^{hi}$, $CD45RA^+$, or CCR9, depending on the method of iTreg generation).

Another critical point is the potential to drive a subset of naïve T cells to the Th17 or other effector phenotype, rather than to iTreg during stimulation - a risk that cannot be underestimated. Although it is well-demonstrated that this can be abrogated by the use of ATRA or rapamycin in *in vitro* culture, the potential of Th17 differentiation *in vivo* may remain. Interestingly, RA has also been associated recently with promotion of Teff responses, suggesting a dual function for proinflammatory as well as suppressive T cell responses [85]. A possible solution to overcome the release of pro-inflammatory under RA stimulation would be by blocking IL-15 during iTreg stimulation [86]. Moreover, most studies on iTreg have been carried out in mice, but Treg in humans have already proven different from those in the mouse. These differences can be found, for example, in the conversion of naïve T cells to Th17 effector cells (in humans this does not occur when stimulation is with TGF-β and IL-6 alone) or in the expression of FoxP3. In mice, FoxP3 expression is strictly related to suppressor function, while human activated T cells can transiently express intermediate levels of FoxP3. This however, is at a lower level than that of human nTreg, and is not associated with suppressor function [87, 88]. Moreover, activated human conventional $CD4^+$ T cells can acquire suppressive capacity while not expressing FoxP3.

Another point of interest is that in certain diseases, e.g., in autoimmune disorders, patients may have defective nTreg, which may be the immunologic basis of their disease. Although studies have shown that e.g., in type-1 diabetes, nTreg can be expanded and exert suppressive function that is equal to that of healthy controls [89], this may not be the case in other diseases. In diseases where the nTreg of the patient are less functional, e.g., the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome,

generation of iTreg may be beneficial and more effective than expansion of autologous nTreg. Moreover, these iTreg can be generated in an Ag-specific fashion, thereby making the cells a disease- (or donor-organ) specific treatment.

Remaining concerns that need to be addressed before clinical trials are initiated, include the stability of the iTreg (and nTreg) phenotype, particularly considering their interaction with inflammatory cells and immunosuppressive therapy. As described by Feuerer et al [90], various subtypes of Treg have different gene expression profiles, suggesting a different function for each type. Furthermore, the importance of appropriate migration of the cells (reviewed by Zhang et al [91]), which determines their fate after *in vivo* injection, and a detailed comparison of the efficacy of nTreg versus iTreg *in vivo* need to be studied. Many factors are likely to affect homeostasis of transferred Treg, including IL-2, TGF-β levels and those of other regulators such as mTOR inhibitors or Vitamin A. Non-human primate models can provide valuable information on the *in vivo* function of Treg (reviewed by Dons et al [92]); in this regard, comparison of iTreg to nTreg might provide further information on the function of both cell types, before use of these cells is translated to clinical trials.

10. CONCLUDING REMARKS

Regulatory T cells have been the subject of intense investigation for the last decade, and as the first clinical trials to ascertain the safety of ex-vivo expanded nTreg infusion in humans are being initiated [83, 84], determination of the necessary numbers of Treg has become increasingly pressing. The induction of a regulatory phenotype from naïve $CD4^+CD25^-$ Teff has become a well-established approach to convert large numbers of T cells into functional Treg. Although various methods for the generation of iTreg have been published, a consensus exists in regard to the basic procedure of induction, using IL-2, TGF-β and low costimulation to generate potently suppressive $FoxP3^+$ iTreg. Rapamycin and ATRA are proven to be valuable, as these reagents not only enhance FoxP3 induction, but also actively prevent the differentiation of naïve T cells into Th17 cells, thus providing a more stable phenotype. iTreg have proven to be stable and potent suppressor cells that seem of similar suppressive strength to nTreg. Perhaps optimal results will be achieved when both cell types are being used in concert, a concept that still needs to be explored.

Altogether, the induction of a regulatory phenotype provides a promising means to generate potently suppressive Treg with stable FoxP3 expression in significantly higher numbers than can be obtained by expansion of nTreg, thereby having the potential for use in large-scale clinical trials.

Acknowledgments

ABBREVIATIONS

Ab	antibody
Ag	antigen
APC	antigen-presenting cells
ATRA	all-trans retinoic acid

CTLA4	cytotoxic lymphocyte antigen 4
DC	dendritic cells
FoxP3	Forkhead box P3
iTreg	induced regulatory T cells
mTOR	mammalian target of rapamycin
nTreg	naturally-occurring regulatory T cell
RA	retinoic acid
Teff	T effector cells
TGF-β	transforming growth factor β
Treg	regulatory T cells
Tx	transplantation

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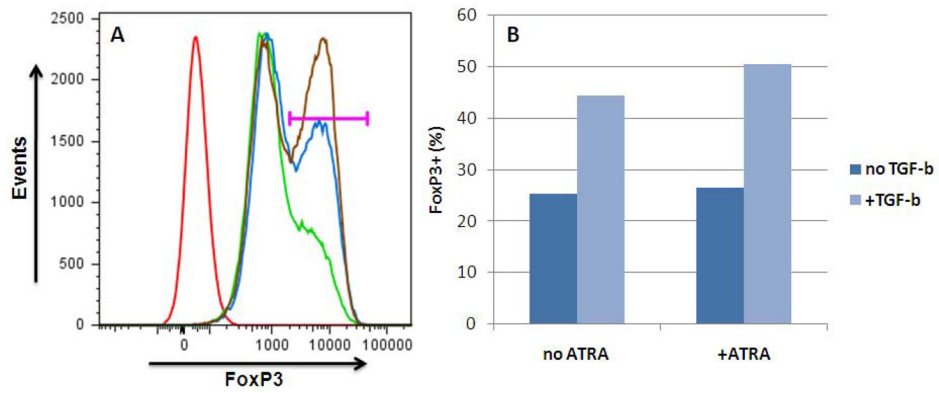
Figure 1. Human iTreg generation with TGF-β and ATRA

Human CD4^{+} T cells were obtained by magnetic bead sorting (CD4^{+} kit, Miltenyi) of PBMC (purity >90%; 5% FoxP3^{+}) and stimulated with high-dose IL-2 +/− TGF-β (20 ng/ ml) +/− ATRA (10 ng/ml). After 6 days, live CD4^{+} cells were assessed for FoxP3 expression by flow cytometry. Cells cultured in the presence of TGF-β + ATRA showed 50% FoxP3^{+} expression while cells cultured with only IL-2 were only 25% FoxP3^{+}; the remaining 75% of the cells showed an intermediate level of FoxP3.

(A) Gating for FoxP3^{+} cells (horizontal bar). Red: isotype; green: no TGF-β, no ATRA; blue: TGF-β only; brown: TGF-β + ATRA.

(B) Quantification of FoxP3^{+} cells: 50% of cells cultured with TGF-β + ATRA acquired FoxP3^{+} phenotype.

Table 1

Properties of nTreg and iTreg

		nTreg (references)	iTreg (references)
Generation	Tissue	Thymus (1, 2)	Secondary lymphoid organs, inflamed tissue, site of transplant (13, 17, 25, 30)
	Cytokines required	IL-2 or IL-15 (6, 9)	IL-2 +TGF-β (10, 12, 45)
	Costimulation required	CD28 (6, 9)	CTLA4 (28, 39, 42, 43)
Improvement of suppressive function by reagent	ATRA	No (54)	Yes (15, 17, 19, 53)
	Rapamycin	Yes (54)/No (58)	Yes (57, 59, 63)
Specificity		Self or alloAg (1, 2)	AlloAgs, food allergens, gut microbes, transplanted tissue, self (inflammation) (15, 17, 25, 26, 30)
Th17 differentiation	IL-6-mediated	Yes (73, 74)	Yes (39, 71, 72)
	Antagonized by ATRA	Yes (76)	Yes (23, 75)
	Antagonized by rapamycin	Has not yet been addressed	Yes (57)
Intracellular marker	FoxP3	Yes (1, 2)	Yes (10, 11)
	Helios	Yes (70)	No (13)