Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY REVIEW ARTICLE

# Extra-thymically induced T regulatory cell subsets: the optimal target for antigen-specific immunotherapy

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doi:10.1111/imm.12458
Received 22 January 2015; revised 20 February 2015; accepted 21 February 2015.
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## Summary

**Antigen-specific immunotherapy aims to selectively restore tolerance to innocuous antigens in cases of autoimmune or allergic disease, without the need for general immune suppression. Although the principle of antigen-specific immunotherapy was discovered more than a century ago, its clinical application to date is limited, particularly in the control of autoimmunity. This has resulted mainly from a lack of in-depth understanding of the underlying mechanism. More recently, the differentiation of extra-thymically induced T regulatory (Treg) cell subsets has been shown to be instrumental in peripheral tolerance induction. Two main types of inducible Treg cells, interleukin-10-secreting or Foxp3$^+$, have now been described, each with distinct characteristics and methods of therapeutic induction. It is crucial, therefore, to identify the suitability of either subset in the control of specific immune disorders. This review explores their natural function, the known mechanisms of therapeutic differentiation of either subset as well as their *in vivo* functionality and discusses new developments that may aid their use in antigen-specific immunotherapy, with a focus on autoimmune disease.**

**Keywords:** antigen specificity; Foxp3; interleukin-10; immunotherapy; regulatory T cell.

## Introduction

Aberrant activation of the immune system can lead to autoimmune disease or allergy. Commonly, these conditions are treated with general immune-modulating substances which, although often highly effective at treating the primary symptoms, frequently lead to adverse effects. By now more than a century has passed since Leonard Noon first discovered that therapeutic administration of the causative antigen of an immune disturbance can educate the immune system and restore a healthy response to the antigen, without affecting general immune function. More recent advances have elucidated that therapeutically induced tolerance involves a range of immunological changes, including the *de novo* differentiation of extra-thymically inducible CD4$^+$ T-cell receptor-$\alpha\beta$ (TCR-$\alpha\beta$) T regulatory (Treg) cell subsets.

## Identification and classification of inducible Treg cells

So far, two broad subsets of inducible Treg cells have been identified; interleukin-10 (IL-10) -secreting, Foxp3$^-$ Treg cells [hereafter referred to as $_{IL\text{-}10}$Treg cells but sometimes also called type 1 regulatory (Tr1) cells], and peripherally induced Foxp3$^+$ Treg cells. Inducible Treg cells are widely recognized as being important for homoeostatic or therapeutically induced T-cell tolerance, yet the lack of specific markers for either subset has complicated the study of their *in vivo* differentiation and function. For example, co-expression of CD49b and LAG-3 has been reported to specifically identify a population of $_{IL\text{-}10}$Treg cells both in man and mouse.[1] However, more recent findings from a study of antigen-specific immunotherapy in a murine model of autoimmune disease

Abbreviations: ASIT, antigen-specific immunotherapy; CNS, central nervous system; IL-10, interleukin-10; $_{IL\text{-}10}$Treg cell, interleukin-10-secreting T regulatory cell; PD-1, programmed cell death protein 1; $_i$Treg cell, *in vitro*-induced T regulatory cell; $_p$Treg cell, peripherally-induced T regulatory cell; TCR, T-cell receptor; Th1, T helper type 1; $_t$Treg, thymic T regulatory cell

 

suggest that these markers are not specific identifiers of all $_{IL\text{-}10}$Treg cells, with co-expression found on a fraction of $_{IL\text{-}10}$Treg cells but also on other T cells that do not express IL-10.[2] Moreover, to distinguish peripherally differentiated Foxp3$^+$ Treg ($_p$Treg) cells from resident thymus-derived Foxp3$^+$ Treg ($_t$Treg) cells, two main differentiating markers, Helios (present on murine and human $_t$Treg but not $_p$Treg cells) and Neuropilin-1 (present only on murine $_t$Treg cells), have been reported, but again neither are undisputed.[3–7] This lack of exclusive markers has limited the ability to track and study inducible Treg cells *in vivo*. In the case of Foxp3$^+$ Treg cells in particular, much of our current understanding results from studies using TCR-transgenic, Rag-deficient mice that lack endogenous Foxp3 expression or from *in vitro* differentiated Treg ($_i$Treg) cells, which are similar but not necessarily identical to *in vivo* differentiated $_p$Treg cells, phenotypically and functionally.[8,9] Of course the latter is likely to be the case when comparing *in vitro* or *in vivo* differentiated $_{IL\text{-}10}$Treg cells as well, although here no formal distinction in nomenclature is made. Despite these shortcomings, we will endeavour to review here the known pros and cons of both subtypes of inducible Treg cell, how to generate them, and their suitability as targets in antigen-specific immunotherapy (ASIT).

## The natural role of inducible Treg cells in immune regulation

To understand the therapeutic potential of inducible Treg cell subsets, it is important to first understand the natural development and function of these cells in the prevention of disease. The first *in vivo* demonstration of the regulatory role of $_{IL\text{-}10}$Treg cells was in patients with severe combined immunodeficiency who received HLA-mismatched haematopoietic stem cell transplants, where donor T cells expressed high quantities of IL-10 and were responsible for tolerance to host antigens.[10] A role for $_{IL\text{-}10}$Treg cells in maintaining immune homoeostasis to gut flora in mice was suggested after the discovery of their presence in the intestinal lamina propria.[11] In 2004, Akdis *et al.*[12] first clearly demonstrated a natural role for $_{IL\text{-}10}$Treg cells in maintaining a healthy immune balance in humans by revealing that, in comparison to allergy sufferers; healthy individuals harbour a greater frequency of IL-10-secreting rather than interferon-$\gamma$-secreting or IL-4-secreting CD4$^+$ T cells specific for common environmental antigens. This study was later followed up by demonstrating that, in healthy individuals, the frequency of allergen-specific $_{IL\text{-}10}$Treg cells among CD4$^+$ T cells increases with higher exposure to the antigen.[13] Akin to the allergy study, the Peakman group demonstrated that in healthy individuals the T-cell response to islet antigens shows a bias towards IL-10, in contrast to diabetes patients who exhibited polarization towards a T helper type 1 (Th1) response.[14,15] In multiple sclerosis, $_{IL\text{-}10}$Treg cells from patients demonstrated a reduction in IL-10 secretion, associated with a reduced suppressive ability.[16,17] Finally, $_{IL\text{-}10}$Treg cells were shown to curtail collateral damage caused by enduring immune responses to chronic infection.[18] Differentiation of $_{IL\text{-}10}$Treg cells from chronically activated effector T cells therefore seems a generally conserved negative feedback mechanism.

Experimental animal models suggest that, similar to $_{IL\text{-}10}$Treg cells, Foxp3$^+$ $_p$Treg cells are important for the induction and maintenance of mucosal tolerance. Three independent groups reported simultaneously that $_p$Treg cells are generated in the intestine under the influence of the vitamin A metabolite all-*trans* retinoic acid, secreted by mucosal dendritic cells.[19–21] In addition, short-chain fatty acids produced by commensal microorganisms in mice were shown to promote extra-thymic Foxp3 induction in CD4$^+$ T cells that mediate an anti-inflammatory response.[22,23] In addition to mucosal sites, $_p$Treg cells can develop within other peripheral tissues. In a murine model of uveoretinitis, tissue-resident and locally differentiated $_p$Treg cells protected from retinal damage.[24] The $_p$Treg cells have also been reported to develop in response to chronic inflammation resulting from asthma, autoimmune disease or infection and therefore appear to play a role in limiting the tissue damage that inevitably results from long-lasting inflammation, although these findings are not universally supported (as reviewed thoroughly by Bilate and Lafaille[25]). Interestingly, comparison of various animal models of autoimmune disease, each carrying the same modified version of Foxp3 protein that affects the development of $_p$Treg cells but not $_t$Treg cells, suggests that $_p$Treg cells play a pivotal role in preventing the onset of type 1 diabetes but not arthritis or autoimmune encephalomyelitis.[26–28] Disease-specific conditions therefore seem to play an important role in the functionality of $_p$Treg cells.

Clearly, both subsets of inducible Treg cells fulfil a diverse natural role in immune homoeostasis and both seem potent, albeit not universal, inhibitors of undesirable immune responses. This supports the notion that the therapeutic differentiation of these T cells should be a prime aim for immunotherapy of hyperimmune conditions.

## Immune regulation by inducible Treg cells; a pivotal role for IL-10

Several mechanisms have been reported for the suppressive function of $_{IL\text{-}10}$Treg cells and inducible Foxp3$^+$ Treg cells. These include cell contact-dependent negative co-stimulatory molecules including cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed death-1 (PD-1), lymphocyte activation gene-3 (LAG-3) and inducible T-cell costimulator (ICOS) and surface


 

molecules that mediate metabolic disruption such as CD39 and CD73.[29] It is becoming increasingly clear, however, that the immunosuppressive cytokine IL-10 plays a vital role in mediating the function of not only $_{IL\text{-}10}$Treg cells but also Foxp3$^+$ Treg cells. Interleukin-10 has recently been shown to be important not only for Foxp3$^+$ Treg cell-mediated *in vitro* suppression[30] but also for *in vivo* regulation in models of colitis[31] and central nervous system (CNS) autoimmune disease[32]. Interleukin-10 is produced by a wide variety of other immune cells, yet CD4$^+$ T-cell-derived IL-10 has a dampening effect on the immune response either by directly affecting other T cells or through the regulation of dendritic cell function.[33] It not only suppresses T cells that may otherwise exert undesirable immune responses, but appears to actively promote further differentiation and stability of $_{IL\text{-}10}$Treg cells and Foxp3$^+$ Treg cells,[31,34,35] although IL-10 is not required for initial *in vitro* differentiation of Foxp3$^+$ $_i$Treg cells.[32] Despite being a common mediator of suppression for both types of inducible Treg cells, IL-10 on its own has not proven a suitable candidate for immunotherapy as clinical trials with the administration of exogenous IL-10 have shown limited benefit and considerable adverse effects.[36] Clearly, a cellular source of this pleiotropic cytokine is required to direct adequate delivery for immune regulation.

## *In vitro* differentiation of inducible Treg cells for immunotherapy

To harness their therapeutic potential, many laboratories have developed methods for the *in vitro* differentiation of inducible Treg cells, with the ultimate aim of achieving immune regulation by adoptive transfer.

*In vitro*, inducible Treg cells can be differentiated and expanded efficiently. The differentiation of $_{IL\text{-}10}$Treg cells was first described after chronic activation of naive CD4$^+$ T cells in the presence of IL-10.[37] A similar subset of $_{IL\text{-}10}$Treg cells can be produced by stimulating human T cells with antibodies against CD3 and the co-stimulatory molecule CD46 in the presence of IL-2.[38] Furthermore, both human and murine $_{IL\text{-}10}$Treg cells can be produced by activation of CD4$^+$ T cells in the presence of vitamin D3 and dexamethasone.[39] 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), which binds the ligand-activated transcription factor aryl hydrocarbon receptor, also promotes the induction of IL-10 expression in human naive CD4$^+$ T cells upon activation.[40] It is important to note, though, that none of these methods generate a pure population of IL-10-secreting T cells. Generally, not more than 50% of the differentiated cells express IL-10, whereas both IL-10$^+$ and IL-10$^-$ retain the ability to produce effector cytokines. The addition of neutralizing antibodies to IL-12, interferon-$\gamma$ and IL-4 during differentiation does, however, abrogate this.[39]

*In vitro* differentiation of Foxp3$^+$ $_i$Treg cells at very high purity (> 90%) can be achieved by activating naive CD4$^+$ T cells with anti-CD3 and anti-CD28 in the presence of transforming growth factor-$\beta$ and high doses of IL-2.[3] Differentiation of $_i$Treg cells using antigen and antigen-presenting cells, however, typically does not give rise to a population more than 75% Foxp3$^+$, even if using naive TCR-transgenic CD4$^+$ T cells.[28] Several agents have now been demonstrated to augment Foxp3 expression and improve *in vivo* functionality upon adoptive transfer of $_i$Treg cells. As expected from its natural role in the gut, all-*trans* retinoic acid was shown to promote iTreg cell differentiation. It is debated, however, whether this results from a direct effect on signalling downstream of cytokine receptors[41,42] or is an indirect result of relieving the inhibitory effect of pre-activated/memory T cells in the culture.[43] According to a recent study, retinoic acid promotes the suppressive capacity and stability of $_i$Treg cells upon *in vivo* transfer in a skin allograft model.[44] Similar to $_{IL\text{-}10}$Treg cells, the aryl hydrocarbon receptor plays a role in the differentiation of Foxp3$^+$ $_i$Treg cells. Whether the aryl hydrocarbon receptor promotes the development of $_{IL\text{-}10}$Treg cells, Foxp3$^+$ $_i$Treg cells or indeed potentially pathogenic Th17 cells depends on a combination of the cytokines in the culture and the nature of the specific ligand.[40,45,46] PD-L1, the ligand for the co-inhibitory receptor programmed death 1, has been shown to promote the induction of Foxp3 expression in human and murine CD4$^+$ T cells.[47–50] This suggests that modulation of the PD-1–PD-L1 axis could be used to amplify conversion of naive T cells into $_i$Treg cells, although a recent study using PD-1 knock-out mice found that PD-1 was non-essential for Foxp3 induction.[51] Other, less well-defined methods of promoting Foxp3 induction that have been reported recently include the use of rapamycin,[52] blocking antibody to the adhesion molecule leukocyte function-associated antigen-1,[53] the growth factor progranulin,[54] the glucocorticoid-induced leucine zipper protein,[55] the Notch ligand Delta-like 1 (DL1 or DLL1),[56] depletion of essential amino acids,[57] or drugs that prevent the proteolysis of the transcription factor Krüppel-like factor 2[58] (see also Table 1). Finally, changing common cell culture conditions to a hypoxic environment has been suggested to improve Foxp3 induction *in vitro*.[59]

## Efficacy of immunotherapy based on the transfer of *ex vivo* differentiated inducible Treg cells

Pre-clinical studies have revealed that adoptive transfer of CD4$^+$ Treg cells can provide effective immune suppression. For example, transferring *ex vivo* expanded Foxp3$^+$ $_t$Treg cells can suppress inhibitory antibody formation in haemophilia,[60] delay allograft rejection[61] and protect against autoimmune cholangitis[62] and rheumatoid

**Table 1.** Factors that promote inducible regulatory T (Treg) cell differentiation upon activation, *in vitro*

| IL-10 Treg cells | | $Foxp3^+$ $_i$Treg cells | |
|---|---|---|---|
| Exogenous IL-10 | Groux *et al.*[37] | Retinoic acid | Xiao *et al.*[41], Mucida *et al.*[42], Hill *et al.*[43] |
| CD46 ligation | Kemper *et al.*[38] | Aryl hydrocarbon receptor ligation | Gandhi *et al.*[40], Quintana *et al.*[45], Mezrich *et al.*[46] |
| Vitamin D3 and dexamethosone | Barrat *et al.*[39] | PD-1–PD-L1 interaction | Wang *et al.*[47], Francisco *et al.*[48], Amarnath *et al.*[49], Chen *et al.*[50] |
| Aryl hydrocarbon receptor ligation | Gandhi *et al.*[40] | Rapamycin | Hippen *et al.*[52] |
| | | Blockade of leukocyte function-associated antigen-1 | Verhagen *et al.*[53] |
| | | Progranulin | Wei *et al.*[54] |
| | | Glucocorticoid-induced leucine zipper (GILZ) | Bereshchenko *et al.*[55] |
| | | Delta-like 1 mediated Notch signalling | Mota *et al.*[56] |
| | | Inhibition of Krüppel-like factor 2 (KLF2) | Pabbisetty *et al.*[58] |
| | | Depletion of essential amino acids | Cobbold *et al.*[57] |

arthritis.[63] Focusing on Treg cells differentiated *in vitro*, adoptive transfer of $_{IL\text{-}10}$Treg cells has been shown to protect against colitis,[11,37] rheumatoid arthritis[64] and CNS autoimmune disease,[39] whereas $Foxp3^+$ $_i$Treg cells have been shown to suppress colitis,[31] graft rejection,[65] spontaneous abortion,[66] graft-versus-host disease[52] and CNS autoimmune disease (Table 2).[32,67,68] This success has led to the translation of Treg cell therapy to the clinic, with several trials using adoptive transfer of *ex vivo* expanded thymic Treg cells or *in vitro* differentiated extra-thymic Treg cells to treat autoimmune disease, transplant rejection or graft-versus-host disease recently completed, currently underway or recruiting patients (see clinicaltrials.gov and Table 2). The first results have been promising and appear to show some efficacy and indicate that the principle of Treg cell therapy is safe.[69–73] Crucially, however, an increasing number of studies have shown that antigen-specificity improves Treg cell functionality, regardless of the subset of interest, and reduces the risk of off-target immunosuppressive effects.[30,32,39,74–76] It is important to note, though, that simply inducing the expression of a regulatory factor like Foxp3 in antigen-specific T cells does not necessarily produce a suppressive phenotype.[77] Although it is possible to differentiate antigen-specific extra-thymic Treg cells *in vitro* before adoptive transfer, it is challenging to obtain significant cell numbers at high purity and also costly to provide a bespoke treatment for every patient. In addition, concerns have been raised about the stability of the phenotype of *in vitro* differentiated $Foxp3^+$ $_i$Treg cells in particular, following reports that these may revert to a pathogenic phenotype.[63] These concerns and others suggest that *in vivo* differentiation of inducible Treg cells may be preferable over transfer of *ex vivo* differentiated cells, although both methods have their own advantages and challenges (Fig. 1).

## Antigen-mediated differentiation of inducible Treg cells *in situ*

It has long been known that immune tolerance, associated with the differentiation of inducible Treg cells, can be

**Table 2.** Pre-clinical disease models and early stage clinical trials (Italics) showing efficacy and/or safety of regulatory T (Treg) cell transfer

| | Expanded thymic $Foxp3^+$ Treg cells | $Foxp3^+$ iTreg cells | $_{IL\text{-}10}$Treg cells |
|---|---|---|---|
| Colitis | | ✓ [31] | ✓ [11,37] |
| Rheumatoid arthritis | ✓ [63] | | ✓ [64] |
| Central nervous system autoimmune disease | | ✓ [32,67,68] | ✓ [39] |
| Graft-versus-host disease | ✓ *[69–71]* | ✓ [52] | ✓ *[73]* |
| Graft rejection | ✓ [61,65] | ✓ [65] | |
| Antibody formation in haemophilia | ✓ [60] | | |
| Autoimmune cholangitis | ✓ [62] | | |
| Spontaneous abortion | | ✓ [66] | |
| Crohn's disease | ✓ *[72]* | | |


 

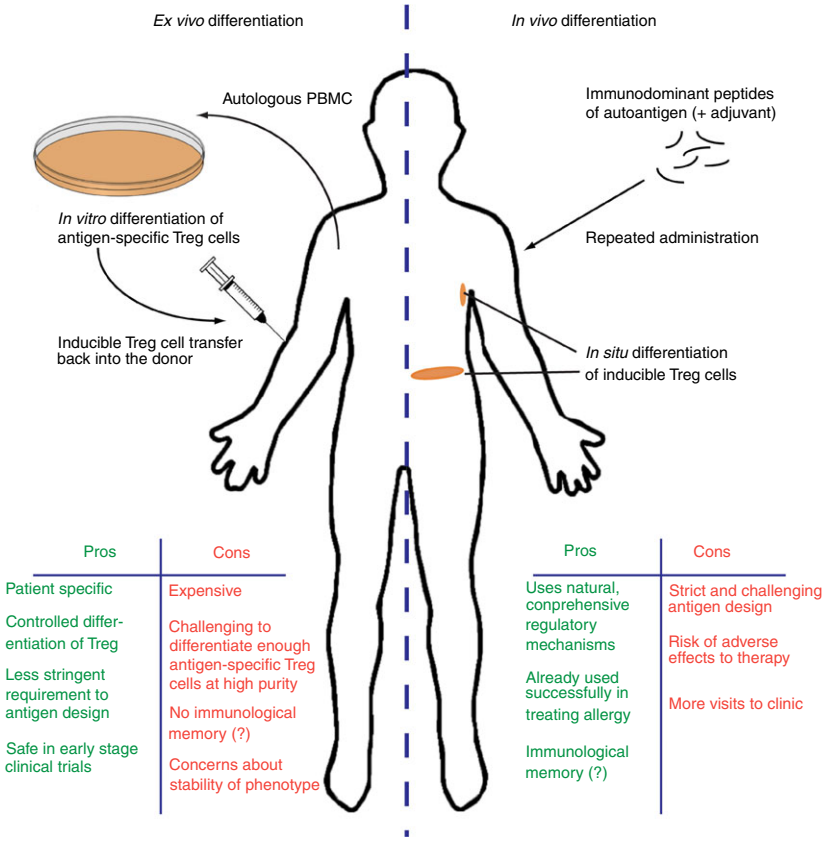


**Figure 1.** Transfer versus *in situ* differentiation of inducible regulatory T (Treg) cells in antigen-specific immunotherapy of autoimmune disease. Two different antigen-mediated treatment strategies aimed at using the therapeutic potential of inducible Treg cells are considered for immunotherapy; *ex vivo* differentiation of autologous $CD4^+$ T cells followed by transfer back into the donor or *in situ* differentiation by administration of tolerogenic peptide, either alone or in combination with a tolerogenic adjuvant.

achieved by therapeutic administration of relevant antigen.[75] Importantly, it is here that we find a fundamental difference between the differentiation of $_{IL\text{-}10}$Treg cells and $Foxp3^+$ $_p$Treg cells.

The therapeutic differentiation of $_{IL\text{-}10}$Treg cells *in vivo* is the best-established method of ASIT and generally mimics the natural differentiation of such cells, as described above for allergen-specific cells.[13] As shown in our laboratory and by others, the induction of $_{IL\text{-}10}$Treg cells by ASIT requires the repeated exposure to high doses of specific antigen (reviewed by Ng *et al.* and Sabatos-Peyton *et al.*[33,75]). Expression of IL-10 is up-regulated in pre-differentiated Th1, Th2 as well as Th17 cells upon repeated activation, which demonstrates that this negative feedback mechanism is applicable to dampening the immune response in a wide range of conditions. The requirement for high doses of antigen carries an inherent risk of adverse effects, particularly in patients who already demonstrate an undesirable immune response to the relevant antigen. Therefore, before applying ASIT to target the induction of $_{IL\text{-}10}$Treg cells, careful considerations ought to be made regarding the route of administration and dosing strategy. We have shown previously, in a murine model, that intranasal administration of myelin basic protein-derived peptide provides tolerance and protection from CNS autoimmune disease without severe adverse effects.[78] In the clinic, intranasal administration is less practical because the dose of antigen administered is relatively difficult to control. Recently, we demonstrated that the same peptide can be used safely for subcutaneous tolerization, provided that a dose escalation protocol is followed.[2] Analysis of the $CD4^+$ T-cell transcriptome during the dose escalation protocol revealed progressive suppression of pro-inflammatory mediators and repression of the cell cycle pathway, coinciding with up-regulation of IL-10 and co-inhibitory receptors. With this knowledge, it may now be easier to find suitable adjuvants that, in combination with specific antigen, can be used to obtain the desired phenotype for $_{IL\text{-}10}$Treg cells more efficiently, while also further reducing the risk of undesirable immune activation and adverse effects. It has been shown already that targeting antigen uptake by dendritic cells via a scavenger receptor favours specifically the differentiation of $_{IL\text{-}10}$Treg cells.[79] In a similar vein, antigen coupled to either autologous apoptotic cells or, perhaps more elegantly, synthetic biodegradable microparticles, which promotes antigen uptake via scavenger receptors, has been shown to promote immune tolerance, although a clear role for $_{IL\text{-}10}$Treg cells in this system has yet to be defined.[80–82] It further remains to be elucidated if other factors that promote the differentiation of $_{IL\text{-}10}$Treg cells *in vitro*, such as ligation of aryl hydrocarbon receptor[40] or rapamycin and anti-CD45RB[83], may be used in combination with antigen-specific therapy to augment IL-10 production *in vivo*.

Both $_{IL\text{-}10}$Treg cells and Foxp3$^+$ $_p$Treg cells develop in response to chronic antigen encounter, but whereas the development of $_{IL\text{-}10}$Treg cells requires high antigen doses, Foxp3$^+$ $_p$Treg cells develop in response to very low levels of antigen,[84,85] whereas strong TCR signals actively prevent Foxp3 expression.[86] Several methods of antigen administration have been demonstrated to give rise to $_p$Treg cell differentiation, including subcutaneous infusion,[84] targeting of the antigen to dendritic cells using DEC-205,[85] oral administration[87] and ectopic expression of antigen in the liver.[88,89] In an interesting novel approach, mice received systemic sublethal irradiation to induce apoptosis of immune cells before antigen administration.[90] This approach was shown to improve Foxp3$^+$ $_p$Treg cell differentiation and antigen-specific tolerance in models of multiple sclerosis and diabetes but, importantly, without affecting the antibacterial response. Many of these methods take advantage of either naturally high or therapeutically enhanced levels of transforming growth factor-$\beta$ at the site of treatment, which promotes $_p$Treg cell development.

Whereas $_{IL\text{-}10}$Treg cells may develop from differentiated effector T cells, Foxp3$^+$ $_p$Treg cells are generally considered to develop from naive T cells only. This requirement for a naive T-cell phenotype has discouraged some researchers from attempting *in vivo* differentiation of Foxp3$^+$ $_p$Treg cells considering that, in the clinic, ASIT will inevitably follow the onset of disease, meaning that effector/memory CD4$^+$ T cells will be present. However, even during inflammation, not all T cells in the body that are specific for relevant antigens will be activated. From unpublished personal observations we would conclude that although pre-activated T cells impair Foxp3 induction in naive T cells, the conversion is not fully abrogated. It remains to be elucidated whether the remaining conversion suffices for immune suppression *in vivo*. Excitingly, several of the adjuvants described to promote the (*in vitro*) differentiation of inducible Foxp3$^+$ Treg cells have been reported to either relieve the suppressive effect of memory T cells[43] and inflammatory conditions[54] on the induction of Foxp3 expression in naive CD4$^+$ T cells or even augment the conversion of pre-activated/memory CD4$^+$ T cells[56]. *In situ* differentiation of either subset of inducible Treg cells seems a feasible approach, provided that an optimized protocol is applied.

## Functionality and phenotypic stability of inducible Treg cells *in vivo*

In dose escalation immunotherapy, $_{IL\text{-}10}$Treg cell-mediated tolerance could be induced after antigen priming and provided long-lasting homoeostatic protection.[2] The allergy beekeeper model has demonstrated that the frequency of $_{IL\text{-}10}$Treg cells correlates with the level of antigenic exposure and that a high level of antigen is required for an enduring response dominated by IL-10.[13] Although the number of $_{IL\text{-}10}$Treg cells diminishes in the absence of specific antigen, this is rapidly restored upon subsequent encounter with high levels of antigen.

The question of whether Foxp3$^+$ $_p$Treg cells can be differentiated and are functional as well as stable in an inflammatory setting is highly contentious. Probably, this is due to a range of factors, including the general inability to distinguish $_p$Treg cells from $_t$Treg cells, the fact that most of the data available are achieved using $_i$Treg cells rather than $_p$Treg cells, and the broad heterogeneity in the specificity and phenotype of Treg cell populations used. First, although some groups have reported the differentiation of $_p$Treg cells in response to chronic inflammation, so suggesting that they provide a negative feedback mechanism similar to that offered by $_{IL\text{-}10}$Treg cells, this was not observed by others.[25] Moreover, several groups have reported that chronic activation of inducible Foxp3$^+$ Treg cells with specific antigen and the presence of pro-inflammatory cytokines such as IL-6 and tumour necrosis factor-$\alpha$ can impair the stability of the regulatory phenotype and lead to the conversion of Treg cells to pathogenic Th1 or Th17 cells.[91–94] In direct contrast, tumour necrosis factor receptor 2 was shown to be critical for Treg cell stability in a colitis model,[95] whereas in a model of CNS autoimmune disease IL-6 was not only found to be ineffective in converting Treg cells into Th17 cells,[96] it also abrogated granulocyte–macrophage colony-stimulating factor production in $_i$Treg cells specifically and thereby suppressed pathogenic conversion.[97] Similarly, murine $_i$Treg cells have been reported to retain their suppressive ability under Th1-polarizing conditions,[98] whereas a study on Foxp3$^+$ Treg cells from patients with relapsing–remitting multiple sclerosis demonstrated that IL-12 promoted interferon-$\gamma$ secretion and reduced suppressive function.[99] These conflicting results indicate that the differentiation, functionality and phenotypic stability of $_p$Treg cells, under inflammatory conditions, may vary greatly depending on the origin of cells that gave rise to them, the method used for their differentiation and the disease-specific conditions. It seems clear that there is a degree of plasticity in the phenotypic stability of Foxp3$^+$ $_p$Treg cells, but it remains to be elucidated if this plasticity has a negative impact on their suitability as a target for immunotherapy or if, just like $_{IL\text{-}10}$Treg cells, $_p$Treg cells can retain a memory of suppressive function, as proposed as part of the recently coined 'revised heterogeneity model'.[100]

## Requirements of antigen suitable for Treg cell differentiation *in vivo*

As mentioned earlier, Foxp3$^+$ $_p$Treg cell differentiation can be achieved through the administration of sub-immunogenic levels of antigen. Despite this, a strong binding of the peptide to MHC II seems to be required. The Von Boehmer group reported that only a mimotope


 

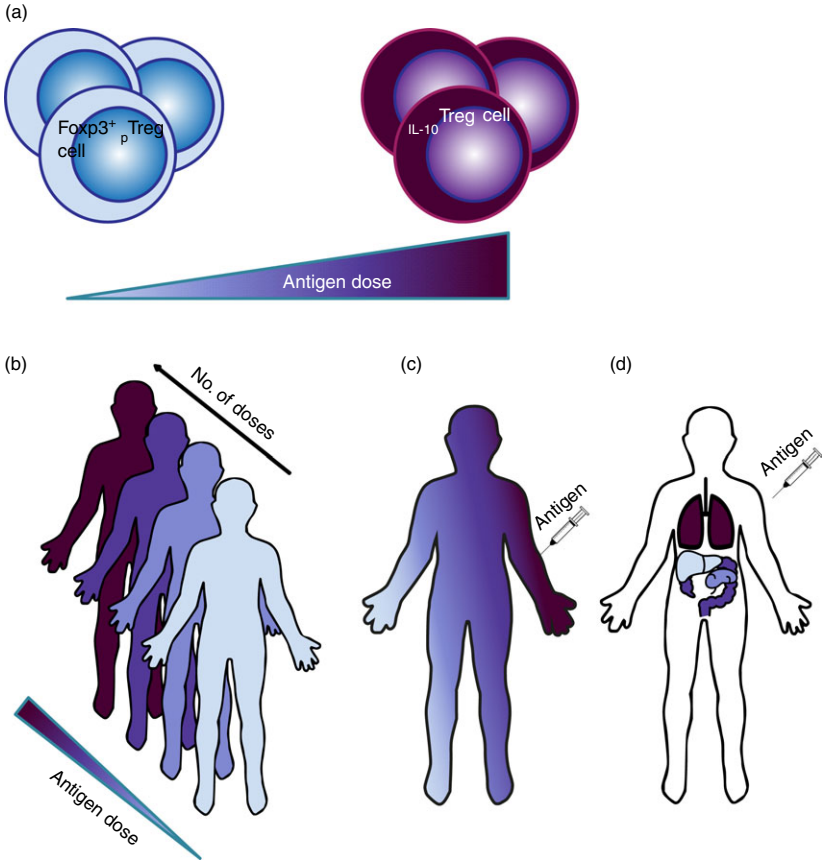


**Figure 2.** Theoretical correlation between Foxp3$^+$ peripherally induced regulatory T ($_p$Treg) cell and interleukin-10-secreting Treg ($_{IL\text{-}10}$Treg) cell formation in response to therapeutic antigen. (a) Quantity of antigen forms a crucial decider in determining whether Foxp3$^+$ $_p$Treg cells or $_{IL\text{-}10}$Treg cells are formed. This may affect the appearance and distribution of inducible Treg cells in several ways. (b) In dose escalation immunotherapy, the early low doses appear to favour Fox3$^+$ $_p$Treg cell formation, whereas the later high doses promote $_{IL\text{-}10}$Treg cell differentiation. It remains to be elucidated whether there is a causal or functional link between the two. (c) Proximity to the site of injection, and therefore the level of antigen exposure, may determine if $_{IL\text{-}10}$Treg cells or Foxp3$^+$ $_p$Treg cells are formed. (d) $_{IL\text{-}10}$Treg and Foxp3$^+$ $_p$Treg cells may develop simultaneously after antigen administration but in distinct physiological sites (chosen at will for this illustration). This would depend on antigen penetrance, but also on the local variety of antigen-presenting cells and/or the local cytokine environment (e.g. presence of transforming growth factor-$\beta$).

for the insulin B:9–23 peptide with increased MHC affinity but not the natural peptide induced the differentiation of $_p$Treg cells and protected from diabetes in NOD mice.[101] This is in line with the finding that T cells of high antigen affinity are more readily converted into Foxp3$^+$ Treg cells compared with T cells that recognize the same antigen with lower affinity.[102] In our own studies of the efficacy of $_{IL\text{-}10}$Treg cell and Foxp3$^+$ $_i$Treg cell differentiation and their suppressive function *in vivo*, we revealed a direct correlation between MHC II affinity of variants of the immunodominant myelin basic protein peptide Ac1-9 and $_{IL\text{-}10}$Treg cell formation,[103] but were able to generate functional Foxp3$^+$ $_i$Treg cells using the lower affinity variant, *in vitro*.[28] However, although subcutaneous administration of the low-affinity peptide variant alone promotes the development of Foxp3$^+$ $_p$Treg cells *in vivo*, we have yet to achieve protection from CNS autoimmune disease with this approach (J. Verhagen, unpublished observation). Although further study of the role of antigen affinity is required, these results emphasize that epitope selection forms a crucial step in the design of ASIT. This is the case particularly in autoimmune disease where CD4$^+$ T cells that recognize auto-antigens of high MHC affinity will mostly have been deleted during thymic selection and as a result immunodominant epitopes responsible for pathology are often of relatively low affinity. The use of altered peptide ligands to treat autoimmune disease is controversial after complications following high-dose administration of peptide antigen with augmented TCR affinity in a phase 2 trial in multiple sclerosis.[104,105] This, however, should not occur when targeting the induction of $_p$Treg cells instead of $_{IL\text{-}10}$Treg cells, as no adverse effects were observed at lower doses. Moreover, this effect is unlikely to occur with peptide of altered MHC affinity rather than altered TCR affinity.[2] Nevertheless, the alteration of peptide affinity may not be required for successful antigen-specific differentiation of Treg cells. As mentioned above, an increasing number of adjuvants have been reported that may aid the development of $_{IL\text{-}10}$Treg cells or Foxp3$^+$ $_p$Treg cells by modifying the activatory signals through the TCR or co-factors such as co-stimulatory molecules, cytokine receptors or adhesion molecules. The adjuvants could allow for tolerance induction with peptides of lower MHC affinity. In addition, it remains critical for the success of ASIT of many autoimmune diseases to further identify suitable immunodominant epitopes. We have already discussed that in several autoimmune settings inducible Treg cells have been reported to be important for a healthy homoeostatic balance. It is currently unclear if autoimmune disease results primarily from a defect in central or peripheral tolerance, but from the importance of inducible Treg cells in homoeostasis we can conclude that natural self-antigens of an affinity sufficient to induce extra-thymic Treg

cell differentiation do exist. One might hypothesize that immunotherapy based on Treg cell differentiation would benefit most from promoting tolerance to antigens that are of an affinity that falls within the, as yet undefined, range that makes them naturally susceptible to regulation by inducible Treg cells. To extrapolate this even further, one might argue that, based on their natural role, differentiation of $_{IL\text{-}10}$Treg cells should be more suitable for tolerance induction to abundant antigen (e.g. proteolipid protein or myelin basic protein in the case of myelin sheath antigens involved in CNS autoimmune disease). On the other hand, $_{p}$Treg cell differentiation may better suit rarer antigens (such as myelin oligodendrocyte glycoprotein). An unequivocal method of distinguishing $_{IL\text{-}10}$Treg cells, $_{p}$Treg cells and $_{t}$Treg cells in combination with novel single cell omics analysis may reveal if indeed tolerance to individual antigens within the same tissue is regulated by distinct subtypes of Treg cells. Furthermore, if a clear division of labour as hypothesized exists, it will be important for the efficacy of immunotherapy to demonstrate if both $_{IL\text{-}10}$Treg cells and Foxp3$^{+}$ $_{p}$Treg cells can exert bystander suppression. For example, can $_{IL\text{-}10}$Treg cells specific for protein A suppress immune responses to the related protein B, even if protein B is normally regulated by Foxp3$^{+}$ $_{p}$Treg cells or vice versa?

## Interaction between Foxp3$^{+}$ $_{p}$Treg cells and $_{IL\text{-}10}$Treg cells

So far, we have considered whether it would be advantageous to target the differentiation of either $_{IL\text{-}10}$Treg cells or Foxp3$^{+}$ $_{p}$Treg cells. However, the greatest success for safe and enduring tolerance induction using ASIT may not rely on the choice of the preferred subset, but rather on inducing the differentiation of both. After all, both subsets have been demonstrated to have a wide range of specificity and the ability to suppress Th1-, Th2- and Th17-dominated disease. Furthermore, although the nature of the cells that can convert into either subtype as well as the quantity of antigen required may vary greatly, the nature of the optimal antigens themselves seems comparable for both $_{IL\text{-}10}$Treg cells and Foxp3$^{+}$ $_{p}$Treg cells. This dichotomy in peripheral T-cell regulation does indeed seem to occur. Depending on environmental signals, both $_{IL\text{-}10}$Treg cells and Foxp3$^{+}$ $_{p}$Treg cells were found to be involved in regulating the immune response to the same antigen during fungal infection.[106] Similarly, in a model of transplant tolerance both $_{IL\text{-}10}$Treg cells and Foxp3$^{+}$ Treg cells were found to play diverse and non-redundant roles during long-term immune regulation, each in distinct physiological sites.[107] These authors found that while Foxp3$^{+}$ Treg cells initiated tolerance in their model, $_{IL\text{-}10}$Treg cells provided enduring protection. This is reminiscent of our own findings in dose-escalation immunotherapy, where the initial, low, doses of antigen triggered the accumulation of Foxp3$^{+}$ T cells, whereas the higher doses needed for enduring tolerance induced $_{IL\text{-}10}$Treg cell differentiation.[2] In this model, however, we have yet to confirm if the surge in Foxp3$^{+}$ Treg cells at the early stages of tolerization results from *de novo* differentiation of Foxp3$^{+}$ $_{p}$Treg cells or relative expansion of $_{t}$Treg cells. In any case, it seems reasonable that given the right antigen, both $_{IL\text{-}10}$Treg cells and Foxp3$^{+}$ $_{p}$Treg cells may differentiate simultaneously at distinct sites considering that both develop under different environmental conditions (Fig. 2). A recent comprehensive analysis of human T-cell compartmentalization elegantly demonstrated that the distribution of naive, effector and memory T cells throughout the human body varies greatly from site to site.[108] This, combined with variations in the level of antigen exposure and natural variation in environmental factors (e.g. transforming growth factor-$\beta$) at various physiological sites, strongly supports a devolved mechanism of tolerance.

## Conclusions

Novel insights increasingly support the notion that peripheral regulation plays a crucial role in the maintenance of autoimmune homeostasis. Improved understanding of inducible Treg cell differentiation and function allows for the development of more refined approaches to ASIT by advancing the design of therapeutic peptide and the use of adjuvants to augment inducible Treg cell conversion. Although the first results of immunotherapy trials based on Treg cell transfer have been promising, we feel that *in situ* differentiation of inducible Treg cells remains the optimal strategy for the induction of efficacious and enduring immune regulation in autoimmune disease. With the arrival of the omics era we will be able to improve our understanding of the spatiotemporal contribution of inducible Treg cell subsets in tolerance induction. This should aid the further development of dosing strategies, including the optimal quantity and route of administration. The exciting developments in this field promise to propel the development of immunotherapeutic strategies and will hopefully lead to ASIT of autoimmune disease finally accomplishing its promise of a wide-scale treatment of unprecedented specificity and efficacy.

## Acknowledgements

JV and DCW are supported by Wellcome Trust programme grant 091074. AW receives a grant from the Marie Curie Initial Training Networks (NeuroKine).

## Disclosures

The authors declare no financial or commercial conflict of interest.


 

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