The Role of Regulatory T Cell Defects in Type I Diabetes and the Potential of these Cells for Therapy

David Thomas, Paola Zaccane and Anne Cooke

Department of Pathology, Immunology Division, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, United Kingdom. Address correspondence to: Anne Cooke, e-mail: ac@mole.bio.cam.ac.uk.

■ Abstract

Type I diabetes is increasing in incidence in developed countries [1]. Diabetes arises from a breakdown of tolerance to islet antigens, resulting in T cell-driven destruction of the islet cells and concomitant hyperglycemia. In this review, we explore whether this loss of tolerance results in part from a defect in the action of regulatory T cells. We draw on both human data and that obtained from NOD mice, the murine model of autoimmune diabetes. Although insulin-based therapies have been highly successful in treating diabetes, the complications of long-term hyperglycemia are still major

causes of morbidity and mortality. Accordingly, we also discuss whether treatment with regulatory T cells is a viable method for restoring long-term tolerance to self-antigens in recently diagnosed or pre-diabetic individuals. Regulatory T cell therapy offers many potential advantages, including a specific and lasting dampening of inflammation. However, some significant hurdles would have to be overcome before it could become an established treatment.

Keywords: type 1 diabetes · T cell · Treg cell · Foxp3 · dendritic cells

T cell regulation – a crucial part of peripheral tolerance

It is well-established that thymic deletion of autoreactive cells is not a completely stringent process. Consequently, the peripheral T cell repertoire in both mouse and man contains cells that are capable of being activated by self-peptides. It has been shown that in some cases, autoreactive cells do not become activated because they remain “ignorant” of their antigen [2]. Furthermore, it is also clear that autoreactive cells can be deleted or anergized upon encounter with self-antigen in the periphery, particularly if there is no inflammatory context associated with the presentation of that antigen. A further active mechanism for maintain-

ing self-tolerance involves dominant regulation by so-called “suppressor” or regulatory T cells (Treg).

The idea that a subset of T cells is dedicated to preventing over-zealous T cell responses from causing tissue damage has been around for over thirty years. It is an attractive hypothesis; the immune system must mount a fast and effective response to pathogens and consequently there is scope for the positive feedback loops that drive this to become uncontrolled. Many strands of negative feedback exist to calm responses when they are no longer necessary. Regulatory T cells are one of the specific arms of this process. Although a significant literature focused on regulatory T cells built up in the 1970’s, they became rather unfashionable. Since the mid-nineties, however, they have enjoyed a renaissance that has seen them rise once again to the

forefront of studies on self-tolerance and autoimmunity [3].

Regulation by CD4⁺CD25⁺ T cells

In 1995, Sakaguchi and colleagues showed that inoculation of T cell-deficient (nude) BALB/c mice with CD4⁺ T cells that had been depleted of CD25⁺ cells caused a variety of autoimmune diseases in the recipients, including thyroiditis, gastritis, insulinitis, polyarthritis and glomerulonephritis [4]. Furthermore, mice that had been given CD4⁺CD25⁻ cells made heightened responses when immunized with foreign proteins or when given an allogeneic skin graft. The mice could be rescued from the autoimmune manifestations by infusion of CD4⁺CD25⁺ cells within a limited period following transfer of the CD4⁺CD25⁻ cells. This notion of a protective CD25⁺ subset was consistent with the published observation that mice exhibiting defects in IL-2 signaling suffered from autoimmune disease. Sakaguchi's group also linked old data showing that thymectomy of three-day-old mice caused widespread autoimmunity with their work on CD4⁺CD25⁺ cells. They found that CD4⁺CD25⁺ cells begin to appear in the periphery three days after birth and that neonatal thymectomy eliminates CD4⁺CD25⁺ cells from the periphery for several days. Inoculation of mice with CD4⁺CD25⁺ cells following three-day thymectomy prevents the development of autoimmune disease that is normally induced following the procedure [5].

Later, Shevach's group demonstrated that CD4⁺CD25⁺ cells could suppress the proliferation of their CD25⁻ counterparts during *in vitro* stimulation with anti-CD3 or specific antigen [6]. In addition, data has accumulated demonstrating that Treg could suppress B cell responses [7] and influence the manner in which DC and macrophages presented antigen to other T cells. Nevertheless, CD25⁺ cells still struggled to obtain true acceptance as dedicated suppressor cells because of legitimate criticisms that no unique surface marker of Treg had been found. CD25 is also expressed on activated T cells and it has been argued that depletion of activated T cell subsets might simply cause a generalized defect in immune homeostasis, allowing autoreactive clones to proliferate to far beyond their normal precursor frequency. In addition, it has been shown that CD25⁻ T cells specific for an unrelated antigen can suppress autoimmunity if they have a strong capacity for homeostatic proliferation [8, 9].

The search for a marker that could reliably distinguish Treg from other CD4⁺ T cell subsets was ended by three publications [10-12] identifying the gene

Foxp3 as a master controller of regulatory T cell function. It had been previously established that a single gene defect of the Foxp3 gene (FOXP3 in humans) caused widespread autoimmunity in both humans and mice [13-15]. The clinical syndromes observed in both scurfy mice and humans suffering from IPEX are similar to those observed in experimental models in which Treg are selectively depleted. In 2003, three groups demonstrated that these diseases were indeed the result of a regulatory cell deficiency. These publications demonstrated that Foxp3 expression in the mouse was largely confined to CD4⁺CD25⁺ thymocytes and splenocytes. Retroviral transduction of CD25⁻ cells with Foxp3 caused them to acquire a regulatory phenotype that could suppress responses both *in vitro* and *in vivo* [11, 12] while importantly mere activation or Th1/Th2 differentiation of CD25⁻ cells did not drive the expression of Foxp3. Furthermore, mice that transgenically over-expressed Foxp3 had increased numbers of Treg in the periphery [10].

While the role of Foxp3 as the master-switch of regulatory activity in the murine system is conclusive, its role in human T cell physiology is not quite so clear cut. Although it is true that Foxp3 is over-expressed in the CD4⁺CD25^{hi} population in human T cells and that mutations in the human FOXP3 gene give rise to the human disease IPEX (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked), which is extremely similar to the phenotype seen in the scurfy mouse, there seem to be important differences in the stimuli that can induce FOXP3 in human T cells. For instance, a recent publication by Ziegler's group [16] has demonstrated that in human cells, activation with anti-CD3 and anti-CD28 can induce expression of the FOXP3 gene. This was not the case in the original experiments on the mouse. It is, however, shown that these activated, FOXP3-expressing T cells have suppressive capabilities *in vitro*. Nevertheless, there is some evidence to suggest that the expression of FOXP3 need not always be correlated with a regulatory phenotype. For instance, T cell clones specific for *Mycobacterium leprae* and adenovirus express FOXP3 mRNA [17]. Although in both these clones, FOXP3 expression was confined to the CD25^{hi} population, these CD25^{hi} cells were able to proliferate avidly and produce large amounts of IFN- γ in response to antigen. This is in stark contrast to the normal behavior of T regulatory cells *in vitro*. It remains possible that while these cells are positive for FOXP3 by RT-PCR, only a small percentage of them actually express FOXP3 and therefore no regulatory phenotype is seen when they are stimulated *in vitro*. In addition, it could be true that, though

they express FOXP3 mRNA, they do not express the protein itself.

T regulatory cell populations other than CD4⁺CD25⁺ cells

One of the most intriguing features of suppressor T cell biology is that these cells seem to occur in many different forms. In addition to those that become committed to the regulatory pathway during thymic development, there are T cells that acquire suppressor function as a result of encounter with their antigen in specific contexts in the periphery. These include the administration of antigens intranasally or orally [18, 19] and the culture of T cells with allogeneic immature dendritic cells [20]. Interestingly, populations of IL-10-secreting regulatory cells can also be induced upon culture with conventional CD4⁺CD25⁺ Treg [21] and this induction of IL-10 may be dependent on TGF- β . Induced T regulatory cells seem, like conventional ones, to be a feature of both human and murine immune physiology.

The phenotype of peripherally-induced regulatory cells varies depending on the circumstances under which they were formed. Those formed following oral tolerization secrete high levels of TGF- β after *in vitro* stimulation in both mice and humans [22-24]. They have been termed Th3 cells and their purpose may be to drive IgA production by B cells as well as suppression of potentially autoreactive responses [18]. An additional subset of induced regulators predominantly secrete IL-10 and have been termed “Tr1” cells (reviewed in [25]). They can be induced *in vivo*, for example following repeated intranasal administration of antigen or superantigen administration. Intriguingly, it appears that in the mouse a subset of dendritic cells exist that specifically induce their differentiation [26]. Such a population of GAD-specific Tr1 cells has recently been identified in young NOD mice [27]. A role for pathogen-specific Tr1 cells has also been posited as a possible mechanism by which mammals limit potentially harmful immunopathology [28, 29]. Viewed another way, the induction of pathogen-specific Tr1 cells might also represent a strategy by which microbes that cause chronic infections prevent their total elimination.

Tr1 cells can also be formed *in vitro* following the prolonged culture of T cells with immunomodulatory compounds such as IL-10 or combinatorial treatment with vitamin D3 and dexamethasone [30, 31]. These protocols will be discussed later when we explore the *in vitro* generation of therapeutic T regulatory cells.

One could argue that “induced” T regulatory cells merely represent expansion of the CD25⁺ thymic Treg.

There is substantial evidence, however, that suppressor T cells can arise *de novo* in the periphery. Mice that have undergone day three thymectomy (therefore lacking CD4⁺CD25⁺ T cells) and have no prostate gland are capable of developing prostate-specific T regulatory cells when prostate formation is induced by testosterone administration later in life. Studies of Foxp3 expression have helped delineate different species of induced regulators. Firstly, it has been shown that Treg induced by peptide administration or through *in vitro* culture do not have to express Foxp3 at all to be suppressive [32] – this is highly suggestive that they are an entirely different species of suppressor cells. However, it has also been demonstrated that Foxp3 can be induced in peripheral CD25⁺ T cells both *in vitro* and *in vivo* and that these T cells acquire suppressive function [33-37].

Do defects in T regulatory cells contribute to autoimmune diabetes?

Defects in CD4⁺CD25⁺ cells

Whether a deficiency in the number or function of Treg can contribute to the onset of diabetes mellitus has been addressed by several studies of the NOD mouse. A study by McDevitt's group found that NOD mice have significantly lower numbers of Treg in the spleen and thymus when compared with BALB/c mice. This difference was found to be present at 3, 8 and 15 weeks of age. NOD mice also have lower numbers of Treg in the thymus when compared with other mouse strains that do not develop spontaneous autoimmunity. However, they have similar numbers of Treg when compared with strains that exhibit spontaneous autoimmune manifestations. This “gradation of regulation” through the strains was also evident to a certain extent when splenic Treg were examined. NOD mice had lower numbers of Treg than BALB/c, C57Bl/6, DBA/2 but similar numbers to (C57Bl/6 x NOD)F1 mice which have little or no spontaneous autoimmunity. Further, this study showed that the total number of Treg is likely to be regulated to some extent by the pro-inflammatory cytokine TNF- α . It is known that injection of TNF- α to neonatal NOD mice accelerates the onset of diabetes whereas anti-TNF- α therapy at the same time abrogates the disease. Interestingly, neonatal TNF- α blockade increases the number of CD4⁺CD25⁺ T cells in spleen and thymus, whereas neonatal injection with TNF- α itself significantly decreases Treg numbers in both of these organs. By contrast, a study by Berzins *et al.* [38] examined the percentage of CD25 in the CD4 compartment in six-

week-old pre-diabetic mice rather than the absolute number of Treg in the lymphoid organs. In an extensive analysis, these authors detailed the percentage of CD4⁺CD25⁺ cells amongst thymocytes, recent thymic emigrants, splenocytes and pancreatic lymph node lymphocytes. Using this method, they found no significant differences in the percentage of Treg between NOD mice and other strains of mice – some of which were the same as those discussed in the study by Wu *et al.* [39].

While it remains unclear whether NOD mice are deficient in the absolute number of Treg, there is a growing consensus that they do have a deficit in the function of these cells, particularly as they age. One publication showed that CD4⁺CD25⁺ cells from eight-week-old NOD mice are able to suppress the ability of diabetogenic splenocytes to transfer diabetes to NOD.*scid* mice. The CD4⁺CD25⁺ cells from 16-week-old NOD mice, however, cannot prevent disease transfer mediated by diabetogenic splenocytes [40]. These findings were confirmed by *in vitro* assays of suppressor cell activity and it was additionally noted that the CD4⁺CD25⁺ cells from older mice are also more difficult to suppress. A second publication (while broadly confirming these findings) pointed out that even diabetic NOD mice do have some functional Treg that can mediate *in vitro* suppression if only the CD25^{hi} cells are selected [41]. Therefore, the results observed by Gregori and colleagues may represent the fact that in mice with significant autoimmune inflammation, more of the CD4⁺CD25⁺ cells are activated cells rather than regulatory cells and the absolute number of Treg or the function of individual Treg may not necessarily have decreased. Nevertheless, it is likely that some decline in Treg function occurs in NOD mice, as demonstrated in a recent paper by Gregg [37] and colleagues in which they show that T regulatory cell function declines with age and that this is associated with a loss of membrane-bound TGF- β from the surface of CD4⁺CD25⁺ T cells.

Defects of regulation in human diabetics

A recent study has demonstrated that there does indeed appear to be a defect in T cell regulation in diabetic individuals [42]. CD4⁺CD25⁺ T cells from the peripheral blood of patients who had been recently diagnosed with type 1 diabetes were compared with those from healthy, age and HLA-matched controls with respect to surface phenotype and *in vitro* suppressive function. These authors found that while there appeared to be no difference in the frequency of CD4⁺CD25⁺ cells between patients and controls, there

was a significant impairment in the ability of diabetic patients' Treg to suppress proliferation of CD4⁺CD25⁺ cells *in vitro*. In co-cultures with CD4⁺CD25⁺ T cells, the Treg from diabetic patients were approximately two-fold less effective in suppressing proliferation when effector T cells and Treg were mixed at a 1:1 ratio. In addition, analysis of the cytokines produced in such co-cultures showed that there were higher levels of the pathogenic cytokine IFN- γ and lower levels of the more immunosuppressive cytokine IL-10. This data is consistent with an earlier study by the same group which showed that diabetic patients made a pro-inflammatory response to islet antigens whereas HLA-matched controls made one that was dominated by the secretion of anti-inflammatory cytokines such as IL-10 [43].

This result obtained in diabetic patients is similar to that observed in a variety of other autoimmune diseases. It has previously been established that there is a defect in the efficacy of suppression mediated by regulatory cells from patients suffering from multiple sclerosis (MS) compared with Treg from healthy controls. The MS patients' CD4⁺CD25⁺ cells were unable to suppress proliferation or pro-inflammatory cytokine production effectively [44]. Similar results were obtained by Ehrenstein and colleagues who observed that Treg taken from the peripheral blood of patients suffering from rheumatoid arthritis, although able to suppress the proliferation of CD4⁺CD25⁺ cells *in vitro*, were unable to suppress the production of IFN- γ and TNF- α by these cells. T regulatory cells from rheumatoid patients were also defective in their ability to suppress TNF- α production by monocytes [45]. This study also neatly illustrates the point that suppression of inflammatory cytokine production and suppression of T cell proliferation can be uncoupled as shown in other studies. Intriguingly, Treg function was restored in patients who responded to anti-TNF- α therapy. Defects in regulatory T cell function have also been cited as causative factors in autoimmune polyglandular syndrome and myasthenia gravis.

T regulatory cell therapy

As it has been established that defects in T cell regulation may contribute to a number of human autoimmune diseases, a major advance would be the introduction of therapies that specifically re-established tolerance to the autoantigens that were driving the autoimmune process. One way in which this could be accomplished would be to treat individuals with regulatory T cells that were specific for such autoantigens.

These cells could then mediate local immunosuppression by abrogating responses to these antigens and also by inducing bystander suppression. In bystander suppression responses to other autoantigens being primed in the same location are also affected either by direct cell-cell interaction between Treg and T effector cells or via interactions between Treg and antigen-presenting cells (Figure 1).

T regulatory cell treatment may be advantageous for other reasons too. Mouse models inform us that Treg can be long-lived *in vivo* [46, 47] and this may obviate the need for frequent treatment. Most importantly, Treg can condition other cells of the immune system to behave differently. They can lessen the costimulatory abilities of antigen-presenting cells [48] and it has been suggested that they can induce non-regulatory cells to produce the immunosuppressive cytokine IL-10 [49, 50], in some cases conferring on them regulatory activity. In this way, limited treatment with Treg may be able to induce a long-term dominant tolerance to autoantigens rather than just the short-term amelioration of inflammation.

Regulatory T cells – attempts to induce them for therapy

One of the major obstacles to regulatory T cell therapy is the low frequency at which these cells are found in blood. *In vitro* culture methods must therefore be established to facilitate the bulk production of large numbers of regulatory T cells. One of the first successful examples of the large-scale culture of suppressor cells involved generating Tr1-like cells rather than conventional CD4⁺CD25⁺ T cells. In 1997, it was shown that expansion of human or mouse CD4⁺ T cells in the presence of IL-10 led to the generation of T cells that produced very high amounts of IL-10 and some IL-5 but low levels of IL-2 and IL-4. These cells could inhibit the antigen-specific proliferation of naïve

Figure 1. Regulation of islet inflammation. The figure depicts some of the cells which are involved in β -cell destruction in the pancreas and ways in which they might be inhibited by antigen-specific Treg cells. Pancreatic β -cells can be killed by effector cells which have been activated in the pancreatic draining lymph node. The mechanisms by which effector cells can kill β -cells includes an MHC class I restricted cytotoxicity by CD8⁺ T cells, FAS/FASL interaction by both CD4⁺ T cells and CD8⁺ T cells and also cytokine-mediated destruction. Treg cells can inhibit these processes through the production of cytokines such as IL-10 and TGF- β . IL-10 secretion by Treg cells may also influence DCs such that they become tolerogenic.

T cells *in vitro* and the murine cells could suppress inflammatory bowel disease in a *scid* transfer model [31]. However, the finding that these cells did not make immunosuppressive cytokines exclusively but could also make some proteins that could facilitate Th2 responses made them potentially dangerous as therapeutics. Using a combination of vitamin D3 and dexamethasone, though, a T cell population could be differentiated that secreted only IL-10 in significant quantities. These cells were suppressive in an *in vivo* EAE model [30]. Further investigation showed that the cells could also mediate *in vitro* suppression but surprisingly did so in an IL-10-independent manner, suggesting that there was more to their regulatory phenotype than the mere ability to secrete large amounts of IL-10. Interestingly, they were not Foxp3⁺, lending further weight to the argument that Tr1 cells are a separate lineage of suppressors [32].

Culture in the presence of TGF- β has also been used to induce regulatory function in naïve human T cells as a putative therapy for autoimmune diseases [51]. In this study, the induced regulatory cells exhib-

ited a phenotype that was similar to that of conventional T regulatory cells in that they expressed CD25 and CTLA-4. This is consistent with later work showing that TGF- β can induce Foxp3 [33, 34]. However, it is also possible that conventional Treg in the naïve cell population were being expanded as depletion of CD25 prior to culture with TGF- β significantly impaired the generation of a suppressor cell population.

Recent advances

Previous attempts to induce suppressor T cells *in vitro* involved culturing naïve peripheral T cells in the presence of compounds that would skew their differentiation towards a suppressive phenotype. While T cells with regulatory properties have been generated *in vitro*, they have been shown to be distinct from the CD4⁺CD25⁺ subset that exists in the intact animal. The last three years, however, have witnessed major advances in the *in vitro* manipulation of conventional CD4⁺CD25⁺ T cells. The development of two major techniques underlie these steps forward. Firstly, it is now possible to expand CD4⁺CD25⁺ cells *in vitro* through continuous rounds of cell division. Secondly, CD4⁺CD25⁺ Treg can be generated *de novo* from CD25⁻ cells via retroviral transduction with the Foxp3 gene.

Regulatory T cell expansion

The *in vitro* expansion of CD4⁺CD25⁺ T cells always posed a difficult task; investigators may have been deterred by the legendary proliferative unresponsiveness of these cells in culture. It has become clear, however, that T regulatory cells are capable of vigorous proliferation in response to antigenic stimulation *in vivo*. The question then arose of how one could recreate the conditions that facilitate Treg proliferation in the whole animal in the *in vitro* setting. Moreover, could this be achieved without the outgrowth of small numbers of contaminating CD4⁺CD25⁻ cells that will always vigorously proliferate in cell culture? Several publications had demonstrated that Treg could multiply in response to some stimuli *in vitro*, such as IL-2 [6, 52] and IL-4 [53]. It was subsequently shown that human Treg could be expanded with allogeneic feeder cells and high doses of IL-2 without losing their suppressive capacity [54]. However, to determine whether such expanded T regulatory cells are still capable of mediating *in vivo* suppression, one must look to animal models.

Expanded Treg are capable of suppressing disease in a graft-versus-host model of disease [55]. In this

study, murine T regulatory cells were expanded using combinatorial therapy with anti-CD3, IL-2 and TGF- β . Approaches involving the expansion of T cells specific for a known antigen are now being used to provide insight into therapeutic Treg action.

Recently, a systematic protocol for large-scale Treg expansion *in vitro* using dendritic cells has been published [56]. This group expanded OVA-specific T regulatory cells using CD86^{hi} dendritic cells as antigen-presenting cells and showed that these were more efficient stimulators than whole splenocytes. It has also been shown that LPS-matured bone-marrow derived dendritic cells can facilitate Treg expansion more efficiently than immature dendritic cells and, while this is dependent on costimulation, neither IL-2 nor IL-15 production is required from the dendritic cells [57]. Such studies give important insights into the role that dendritic cells play in facilitating Treg expansion *in vivo* and give us some sense of the conditions that would best drive such proliferation physiologically.

Importantly, Steinman's group then expanded their findings from BALB/c mice to the field of autoimmunity in a paper by Tarbell and colleagues [58]. In this study, they expanded the T regulatory cells of the BDC2.5NOD mouse. This mouse is a TCR transgenic in which the cDNA for the TCR is derived from an islet-reactive clone, BDC2.5. *In vitro*, T regulatory cell function was strikingly enhanced by the expansion. Crucially, the expanded Treg cells also passed with flying colors when put through their suppressive paces *in vivo*. When co-transferred with diabetogenic splenocytes from NOD mice to NOD.*scid* recipients, they were able to efficiently suppress diabetes onset at remarkably low numbers.

Another excellent publication details a protocol for Treg expansion based on stimulation with anti-CD3 and anti-CD28 monoclonal antibodies bound to beads [59]. This group had already demonstrated that Treg cells from NOD mice are highly sensitive to CD28 costimulation by demonstrating the deficiency of these cells and an accelerated progression to diabetes in CD28 knockout mice [60]. As in the protocol described by Steinman's group, very high doses of IL-2 were also used. The necessity for IL-2 for Treg expansion in these protocols is interesting in light of the fact that both the IL-2R α knockout mouse and the IL-2 knockout mouse develop systemic autoimmunity and that this autoimmunity can be ameliorated by the presence of Treg [61].

The bead-bound antibody protocol yielded extremely high numbers of expanded Treg (up to 100-fold expansion over five days' culture) and, in common

with the findings of Tarbell *et al.*, these authors also demonstrated augmented Treg function in the expanded Treg population. This was evident *in vitro* and *in vivo* and the antibody-stimulated T cells were able to suppress diabetes onset upon co-transfer with BDC2.5 CD4⁺CD25⁻ cells to NOD.*scid* mice. Impressively, expanded Treg were able to suppress disease in CD28^{-/-} NOD mice – demonstrating that the cells can function in a non-lymphopenic animal. Even more strikingly, these CD4⁺CD25⁺ cells were able to prevent the rejection of a syngeneic islet graft that had restored normoglycemia to previously overtly diabetic NOD mice and also to restore normoglycemia to newly diabetic mice. Taken together these findings hold great promise for future human immunotherapy. Figure 1 shows some of the mechanisms that Treg cells might employ in prevention of type 1 diabetes.

Transduction of CD25⁻ cells with Foxp3

In contrast to expansion by cell division, an alternative approach to the generation of sufficient numbers of T regulatory cells for immunotherapy would be to transduce the more numerous CD25⁻ T cells with Foxp3 in order to convey regulatory activity upon them. This prospect was made a reality by some of the original publications that described Foxp3 as a T regulatory cell-specific gene in the mouse.

Both publications by Hori *et al.* and Fontenot *et al.* demonstrated that cloning of the Foxp3 gene into a retroviral vector and then transducing CD25⁻ T cells undergoing activation with the retrovirus could turn these cells into *bona fide* CD25⁺ regulatory T cells *in vitro* and in an *in vivo* colitis model. The implications of these developments for T cell immunotherapy of autoimmune disease were exciting but the experiments were carried out in a non-autoimmune prone strain. It remained to be seen whether the same approach could be as successful in mice with documented defects in T suppressor cell function [40, 41, 62]. Is Foxp3 as powerful a dictator of Treg cell development and function in the NOD mouse as it appears to be in the BALB/c strain? Would the transduced cells be able to stably maintain their regulatory phenotype?

These questions were answered in an impressive study by Jaeckel *et al.* [63]. They successfully transduced CD25⁻ cells from BDC2.5 NOD mice with the transcription factor; despite noting a lower transduction efficiency than that seen in other strains, they found that the T cells were fully suppressive *in vitro* and could reverse disease in newly diabetic female NOD mice – the toughest test of any neo-Treg. Despite the low transduction efficiency, it is conceivable that the

newly formed Treg could be expanded as described previously to optimise regulatory T cell production.

Future challenges

Despite the successes of the past few years in the induction and expansion of T regulatory cells, several major hurdles still need to be overcome before they could make it as far as the clinic. There are potential problems relating both to the safety and efficacy of these cells as human therapeutics.

One significant obstacle in terms of efficacy is that we still do not know enough about the antigen specificities of pathogenic islet-specific T cells in humans. The work done in murine systems has the great advantage that it has been able to utilize a TCR transgenic system in which it is known that the T cells concerned are (1) specific for an islet cell autoantigen, (2) will home to this site and (3) will proliferate in response to that antigen – a process which many studies have shown is crucial for Treg function *in vivo*. It is also clear from the above studies that expanded Treg cells that are islet-specific are considerably more potent than polyclonal expanded Treg cells at suppressing disease *in vivo*. Therefore, for human therapy, the challenge will be to identify islet-specific Treg and selectively expand them. The difficulty of finding appropriate T cell specificities is illustrated by results from the murine work demonstrating that even GAD-65-specific regulatory T cells from a GAD-65 TCR transgenic mouse are not capable of suppressing disease as are BDC2.5 T cells [59]. Even though they are islet-specific and will respond to injected GAD-65 protein, it seems either that the antigen is not expressed at high enough levels in the draining lymph node or that the T cells do not have a high enough affinity for the peptide-MHC complexes.

Despite the perceived difficulty of identifying and expanding islet-specific T reg in humans, there are a few approaches that could be used, including the identification of antigen-specific cells with MHC class II tetramers. The identification of new epitopes is also being furthered by peptide elution studies [43].

The *in vivo* life-span of human T regulatory cells is also something that requires investigation; while T reg can be long-lived following injection to mice, this may not be true in humans. Experiences with CD8⁺ T cells in tumor immunotherapy have shown that most of the transferred T cells are lost reasonably soon after inoculation. In some treatment protocols, persistence of the transferred population has been improved by depleting the patient's lymphocytes prior to transfer [64]. This might be an inappropriate treatment for patients who

are predisposed to autoimmune disease as it is known that transient lymphopenia itself can induce autoimmunity. Similarly, the survival of transferred CD8⁺ T cells can be enhanced by treating recipients with IL-2 at the same time. Again, treatment of patients who have ongoing autoimmunity with such a potent T cell mitogen might also precipitate an exacerbation of their existing disease.

There are also potential concerns regarding the safety of T cell immunotherapy. As a concept it has been trialed in cancer immunotherapy. Generally, there is little data on the long-term safety of such treatment as it is often given as a last-ditch treatment to patients with a very low chance of long-term survival. In the setting of administration to a juvenile diabetic, the patient might expect 70-80 more years of life and it must be proven that treatment with T cells is as safe as the existing treatment of daily insulin injections. One of the major problems with regulatory T cell therapy is that the Treg may suppress responses to pathogens. From the point of view of autoimmunity, it is advantageous that Treg can suppress responses to antigens other than the ones they are specific for (bystander suppression.) However, one would not want this bystander suppression to spill over into preventing antimicrobial defense. This might not be a problem as the presence of pro-inflammatory stimuli can sometimes break suppression [65] but it is an important issue to consider.

We also need to be sure that T regulatory cells that have been proliferated through many rounds of *in vitro*

division in the presence of such high doses of IL-2 are no more likely to become T cell tumors than freshly isolated T regulatory cells. In the case of T regulatory cells that have been retrovirally transduced there are two possible causes for concern. There is the possibility of retroviral integration causing transformation of the T cells. Furthermore, it is also possible that the patient may mount an immune response against viral epitopes. This may result in the destruction of the infused T cells if they express viral peptides on their MHC class I molecules, although this does not appear to have been a problem in the murine models used. In any case, retroviral transduction could possibly be circumvented by using TGF- β or anti-CD4 to induce Foxp3 [33-35]. However, in this situation not all of the treated cells would become Foxp3⁺ and some autoreactive CD25⁻ cells might therefore still be transferred.

Conclusions

Defects in T cell regulation play a crucial role in many human and murine autoimmune diseases. Recent advances in our understanding of regulatory T cell biology have made regulatory T cell therapy a potential means by which autoimmunity could be specifically ameliorated. If subsequent work shows that Treg cells represent a safe and efficient source for therapy, they could become an important weapon in the fight against immune-mediated pathology.

References

1. Cooke A, Zacccone P, Raine T, Phillips JM, Dunne DW. Infection and autoimmunity: are we winning the war, only to lose the peace? *Trends Parasitol* 2004. 20:316-321.
2. Ohashi PS, Oehen S, Buerki K, Pircher H, Ohashi CT, Odermatt B, Malissen B, Zinkernagel RM, Hengartner H. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 1991. 65:305-317.
3. Sakaguchi S. Naturally arising CD4⁺ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004. 22:531-562.
4. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995. 155, 1151-1164.
5. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 1996. 184:387-396.
6. Thornton AM, Shevach EM. CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998. 188:287-296.
7. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4⁺CD25⁺ regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001. 194:629-644.
8. Stockinger B, Barthlott T, Kassiotis G. The concept of space and competition in immune regulation. *Immunology* 2004. 111:241-247.
9. Stockinger B, Barthlott T, Kassiotis G. T cell regulation: a special job or everyone's responsibility? *Nat Immunol* 2001. 2:757-758.
10. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat Immunol* 2003. 4:337-342.
11. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003. 299:1057-1061.
12. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat Immunol* 2003. 4:330-336.
13. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. The immune dysregulation, polyen-

- docrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 2001. 27:20-21.
14. **Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L et al.** X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 2001. 27:18-20.
 15. **Brunkow ME, Jeffery EW, Hjerrild KA, Paepers B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F.** Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 2001. 27:68-73.
 16. **Walker MR, Kaspruwicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF.** Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J Clin Invest* 2003. 112:1437-1443.
 17. **Morgan ME, van Bilsen JH, Bakker AM, Heemskerk B, Schilham MW, Hartgers FC, Elferink BG, van der Zanden L, de Vries RR, Huizinga TW.** Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol* 2005. 66:13-20.
 18. **Weiner HL.** Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes Infect* 2001. 3:947-954.
 19. **Hanninen A., Harrison LC.** Mucosal tolerance to prevent type 1 diabetes: can the outcome be improved in humans? *Rev Diabetic Stud* 2004. 1(3):113-121.
 20. **Roncarolo MG, Levings MK, Traversari C.** Differentiation of T regulatory cells by immature dendritic cells. *J Exp Med* 2001. 193:F5-F9.
 21. **Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH.** Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 2000. 192:1213-1222.
 22. **Fukaura H, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Hafler DA.** Antigen-specific TGF-beta1 secretion with bovine myelin oral tolerization in multiple sclerosis. *Ann N Y Acad Sci* 1996. 778:251-257.
 23. **Hafler DA, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Fukaura H.** Oral administration of myelin induces antigen-specific TGF-beta 1 secreting T cells in patients with multiple sclerosis. *Ann N Y Acad Sci* 1997. 835:120-131.
 24. **Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL.** Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 1994. 265:1237-1240.
 25. **O'Garra A, Vieira PL, Vieira P, Goldfeld AE.** IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. *J Clin Invest* 114, 1372-8 (2004).
 26. **Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H.** Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 2003. 18:605-617.
 27. **You S, Chen C, Lee WH, Brusko T, Atkinson M, Liu CP.** Presence of diabetes-inhibiting, glutamic acid decarboxylase-specific, IL-10-dependent, regulatory T cells in naive nonobese diabetic mice. *J Immunol* 2004. 173:6777-6785.
 28. **McGuirk P, McCann C, Mills KH.** Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. *J Exp Med* 2002. 195:221-231.
 29. **McGuirk P, Mills KH.** Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. *Trends Immunol* 2002. 23:450-455.
 30. **Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, de Waal-Malefyt R, Coffman RL, Hawrylowicz CM, O'Garra A.** In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 2002. 195:603-616.
 31. **Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG.** A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997. 389:737-742.
 32. **Vieira PL, Christensen JR, Minaee S, O'Neill EJ, Barrat FJ, Boonstra A, Barthlott T, Stockinger B, Wraith DC, O'Garra A.** IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol* 2004. 172:5986-5993.
 33. **Huber S, Schramm C, Lehr HA, Mann A, Schmitt S, Becker C, Protschka M, Galle PR, Neurath MF, Blessing M.** Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4+CD25+ T cells. *J Immunol* 2004. 173:6526-6531.
 34. **Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM.** Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003. 198:1875-1886.
 35. **Cobbold SP, Castejon R, Adams E, Zelenika D, Graca L, Humm S, Waldmann H.** Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol* 2004. 172:6003-6010.
 36. **Apostolou I, von Boehmer H.** In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 2004. 199:1401-1408.
 37. **Gregg RK, Jain R, Schoenleber SJ, Divekar R, Bell JJ, Lee HH, Yu P, Zaghoulani H.** A sudden decline in active membrane-bound TGF-beta impairs both T regulatory cell function and protection against autoimmune diabetes. *J Immunol* 2004. 173:7308-7316.
 38. **Berzins SP, Venanzi ES, Benoist C, Mathis D.** T-cell compartments of prediabetic NOD mice. *Diabetes* 2003. 52:327-334.
 39. **Wu AJ, Hua H, Munson SH, McDevitt HO.** Tumor necrosis factor-alpha regulation of CD4+CD25+ T cell levels in NOD mice. *Proc Natl Acad Sci U S A* 2002. 99:12287-12292.
 40. **Gregori S, Giarratana N, Smiroldo S, Adorini L.** Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development. *J Immunol* 2003. 171:4040-4047.
 41. **Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L.** TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* 2003. 9:1202-1208.
 42. **Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI.** Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. *Diabetes* 2005. 54:92-99.
 43. **Arif S, Tree TI, Astill TP, Tremble JM, Bishop AJ, Dayan CM, Roep BO, Peakman M.** Autoreactive T cell responses

- show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J Clin Invest* 2004. 113:451-463.
44. **Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA.** Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 2004. 199:971-979.
 45. **Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C.** Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF α therapy. *J Exp Med* 2004. 200:277-285.
 46. **Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R, Salomon BL.** Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. *J Exp Med* 2003. 198:737-746.
 47. **Walker LS, Chodos A, Eggena M, Doms H, Abbas AK.** Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med* 2003. 198:249-258.
 48. **Cederbom L, Hall H, Ivars F.** CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* 2000. 30:1538-1543.
 49. **Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA.** Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF- β , and IL-10. *J Immunol* 2004. 172:5213-5221.
 50. **Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W.** Transforming growth factor (TGF)- β 1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF- β 1-mediated fibrosis. *J Exp Med* 2003. 198:1179-1188.
 51. **Yamagiwa S, Gray JD, Hashimoto S, Horwitz DA.** A role for TGF- β in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *J Immunol* 2001. 166:7282-7289.
 52. **Thornton AM, Donovan EE, Piccirillo CA, Shevach EM.** Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. *J Immunol* 2004. 172:6519-6523.
 53. **Xu D, Liu H, Komai-Koma M, Campbell C, McSharry C, Alexander J, Liew FY.** CD4+CD25+ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, Leishmania major infection, and colitis in mice. *J Immunol* 2003. 170:394-399.
 54. **Levings MK, Sangregorio R, Roncarolo MG.** Human CD25+CD4+ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 2001. 193:1295-1302.
 55. **Taylor PA, Lees CJ, Blazar BR.** The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 2002. 99:3493-3499.
 56. **Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, Steinman RM.** Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 2003. 198:235-247.
 57. **Fehervari Z, Sakaguchi S.** Control of Foxp3+ CD25+CD4+ regulatory cell activation and function by dendritic cells. *Int Immunol* 2004. 16:1769-1780.
 58. **Tarbell KV, Yamazaki S, Olson K, Toy P, Steinman RM.** CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J Exp Med* 2004. 199:1467-1477.
 59. **Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, Masteller EL, McDevitt H, Bonyhadi M, Bluestone JA.** In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 2004. 199:1455-1465.
 60. **Boehm BO, Bluestone JA.** Differential roles of costimulatory signaling pathways in type 1 diabetes mellitus. *Rev Diabetic Stud* 2004. 1(4):156-164.
 61. **Almeida AR, Legrand N, Papiernik M, Freitas A.** A. Homeostasis of peripheral CD4+ T cells: IL-2R α and IL-2 shape a population of regulatory cells that controls CD4+ T cell numbers. *J Immunol* 2002. 169:4850-4860.
 62. **Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA.** B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 2000. 12:431-440.
 63. **Jaekel E, von Boehmer H, Manns MP.** Antigen-specific FoxP3-transduced T-cells can control established type 1 diabetes. *Diabetes* 2005. 54:306-310.
 64. **Blattman JN, Greenberg PD.** Cancer immunotherapy: a treatment for the masses. *Science* 2004. 305:200-205.
 65. **Pasare C, Medzhitov R.** Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 2003. 299:1033-1036.