Recent Advances in Regulatory T cell therapy of autoimmunity, graft rejection and cancer

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Abstract

Since their initial discover in the 1970s and their subsequent resurgence in the mid 1990s, regulatory T (Treg) cells have become one of the most studied cell subsets. Treg cells prevent autoimmunity and limit aggressive immune responses directed against either pathogen or foreign antigen that might serve to damage host tissue. In contrast, tumour cells have been shown to recruit and/or induce Treg cells, which can impair tumour immunity. The immunoregulatory function of these cells makes them ideal therapeutic candidates for the treatment of autoimmune disease and in the prevention of transplant rejection. Likewise, depletion of Treg cells remains an additional option in the treatment of cancer. Despite significant advances in the treatment of murine models of disease with Treg cells, it has been difficult to transfer this success into the clinic. In this review we will discuss relevant patents and the most recent advances in the use of Treg cells to treat autoimmunity, prevent graft rejection as well as the use of anti-bodies to deplete these cells in cancer.

Keywords: Regulatory T cells (Treg), nTreg, Tr1, Th3, TGF-β, IL-10, adoptive cell therapy, autoimmunity, transplantation, cancer, antibody therapy, *ex-vivo* expansion.

Introduction to Regulatory T cells

Natural regulatory T cells

Regulatory T (Treg) cells include both natural (nTreg) and inducible (also termed adaptive) Treg cells (Figure 1). The term Treg cell will be used throughout this manuscript to include both nTreg and iTreg unless otherwise indicated. $CD4^+CD25^+$ nTreg cells were originally identified through their function in controlling autoimmunity by mediating immunological tolerance to self antigens ([1]). Subsequently nTreg cells were shown to also express the transcription factor FoxP3 which is essential for directing Treg function (2-3). Lack of FoxP3 expression in humans with IPEX syndrome or in scurfy mice results in uncontrollable autoimmune disease due to lack of regulatory function (4). Thus there is no doubt that nTreg cells play a key role in maintaining self tolerance.

Fully differentiated natural Treg (nTreg) cells are derived from the thymus, when CD4 T cells with higher than normal affinity for self antigens are positively selected (5). However, nTreg cells with indistinguishable phenotypic and functional characteristics can also be generated in the periphery from non-Treg cells (6-8). In mice, naïve $CD4^+$ T cells stimulated by antigen in the presence of TGF-β and IL-2 acquire FoxP3 expression and suppressive capacity (9). In humans, however, there is still some controversy as to whether TGF-β induced Treg cells are suppressive (10-11). Other mechanisms have been suggested for the extrathymic generation of nTreg cells including antigen exposure (12-13) and anergy induction (14-15).

There are a number of limitations associated with the markers available to identify and isolate nTreg cells. CD25, the IL-2Rα chain, is an activation marker and therefore can be expressed on activated effector cells as well as Treg cells. Since FoxP3 is expressed intracellularly it cannot be used to sort viable cells. Furthermore in humans, FoxP3 is also upregulated upon activation of non-Treg cells limiting its usefulness as a Treg marker, particularly in inflammatory disease settings (16). FoxP3 expression has also been correlated with cell surface expression of CD39 (17-19) and inversely correlated with CD127 (20). In combination with CD25, these surface markers can facilitate sorting of Treg cells. Recently it has emerged that the nTreg cell population can exhibit some plasticity of function. Particular subsets within the $FoxP3^+$ nTreg cell population have been shown to secrete the pro-inflammatory cytokine IL-17, whilst maintaining their suppressive function (19, 21-22). This would be cause for concern in cases where nTreg cells were used to treat either autoimmunity or transplant rejection. Furthermore, under inflammatory conditions nTreg cells can lose their suppressive function and differentiate into Th17 cells (22). Other common cell surface markers of nTreg cells include glucocorticoid induced tumor necrosis factor receptor family related gene (GITR) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) (23).

nTreg function is usually measured by means of a standard suppression assay, where effector cells are stimulated in the presence of various ratios of nTreg and the amount of suppression measured by proliferation. The suppressive function of nTreg cells *in vitro* requires cell contact with responder cells, although no single mechanism described to date can account for all cases of Treg suppression. Mechanisms described thus far include either modulation of antigen-presenting cells (APC) function via CTLA-4, CD39, LAG-3 or Nrp-1 or direct suppression of the T cell by cytolysis, IL-2 consumption and binding of Galectin-1 (24-25). The ectonuclease CD39 is of interest since it is the first cell surface Treg marker described that has an obvious suppressive function. Extracellular ATP, which has multiple pro-inflammatory effects, is cleaved by CD39 to form adenosine (17-18). CD39 is expressed on all $FoxP3^+$ T cells in mice, however the situation in humans is less clear with only a subset of $FoxP3^+$ cells co-expressing CD39 (18). While no role for soluble mediators can be demonstrated in *in vitro* suppression experiments, IL-10 and to a lesser extent TGF-β have been shown to contribute to Treg cell function *in vivo* (25).

Inducible Treg cells

Inducible Treg cells, including Tr1, Th3 and various subsets of $CD8^+$ regulatory cells are derived in the periphery from uncommitted naïve T cells stimulated by antigen under specific conditions. Tr1 cells exert their suppressive function primarily via IL-10 secretion, and can be generated *in vitro* by stimulating naïve $CD4^+$ T cells in the presence of IL-10 (26). *In vivo*, conditions that favour IL-10 secretion by antigen presenting cells are likely to contribute to Tr1 generation (27). Th3 cells secrete primarily TGF-β and have been associated with the induction of oral tolerance. The induction of Th3 cells from naive $CD4^+$ T cells is favoured by the presence of TGF-β *in vitro* and also *in vivo*, where administration of low dose oral antigen leads to antigen presentation within the TGF-β-rich gut milieu (28).

Since thymically derived nTreg cells are specific for self antigens, they are thought to play a role mostly in preventing autoimmunity, where as inducible Treg cells are more likely to be generated in response to foreign antigens during infection. However the lines between natural and inducible Treg cells are somewhat blurred by the fact that peripheral conversion to nTreg cells can also occur, and it is not clear how these induced cells differ in terms of phenotype, function or antigen specificity from 'classical' thymically derived nTreg cells. Furthermore, although suppression involving IL-10 and TGF-β are the hallmark of inducible Treg cells, the suppressive function of nTreg cells *in vivo* is often cytokine dependent. Therefore the lack of markers, apart from IL-10 or TGF-β secretion, to identify Tr1 or Th3 cells respectively can hamper the interpretation of experiments. It is vital that those who work on Treg cells come to a consensus as to the correct nomenclature for the ever expanding numbers of Treg cell subsets. It remains difficult to differentiate nTreg from iTreg in many cases.

There is no doubt that the various types of Treg cells (both nTreg and iTreg) play a crucial role in maintaining a healthy balance in the immune system. Treg cells have been shown to have a protective role in autoimmunity, allergy and transplantation, while in cancer Treg cells may prevent appropriate immune response to the tumour. During infection by viruses, bacteria or parasites, Treg cells can act to the advantage of either the host or the pathogen or sometimes both (29). Certain pathogens have specific mechanisms to induce Treg cells, thereby subverting the immune response

to favour the infection. Alternatively, Treg induction may favour the host by limiting an excessive immune response to the infection, thereby avoiding destructive immunopathology. The crucial role played by Treg cells in many diseases has generated great interest in manipulating them therapeutically.

Laboratory methods used to expand Treg cells *ex-vivo*

Adoptive Treg cell therapy is fast becoming a viable alternative strategy to combating autoimmunity and transplant rejection. The process involves isolating peripheral blood mononuclear cells (PBMC) from the patient, the subsequent expansion of either polyclonal or antigen-specific Treg cell subsets, culminating with re-infusion (Figure 2, Table 1). The advantage of this process is that the cells can be thoroughly characterised phenotypically and the dose meticulously controlled. Initial studies using mouse models and those in humans concentrated on the expansion of polyclonal Treg cells and their subsequent re-infusion to treat autoimmunity or prevent transplant rejection. The use of polyclonal Treg cells, while successful in treating murine models of disease has come with the concern that under certain circumstances they might induce systemic suppression and lead to the development of tumours and enhanced susceptibility to infection. More recently it would seem that there has been a drive to develop antigen-specific Treg cells that can suppress autoimmunity and prevent graft rejection in a more discriminate manner. It must be pointed out that the authors of many of the protocols described below, do not state categorically whether the expanded Treg cells in their systems reside in either nTreg or iTreg subsets. For this reason, it is impossible for the authors of this review to conclude categorically either way, unless otherwise stated.

***Ex-vivo* generation of polyclonal Treg cells**

Ex-vivo conversion of Treg cells using anti-CD3, anti-CD28 and Rapamycin

One of the first *ex-vivo* Treg expansion protocols involved activating isolated mouse $CD4^+CD25^-$ T cells with immobilised anti-CD3 and soluble anti-CD28 or anti-CD3/anti-CD28-coated beads plus rapamycin and exogenous IL-2 (30-33) Table **1**. Treg cells generated in this way were shown to potently suppress $CD4^+$ effector T cell activation *in vitro* (34-35). Recent work has shed light on the mechanism behind *in vitro* and *in vivo* expansion of Treg cells by Rapamycin. It has been shown that Pim 2 (a transciptionally regulated serine/threonine kinase) is expressed by Treg cells and allows a growth advantage in the presence of rapamycin (36).

This method was subsequently modified by Golovina *et al.* who used good manufacturing process (GMP) to expand human Treg cells *ex-vivo* (37-38). Artificial antigen-presenting cells (APC) cells (K562) were engineered to express the co-stimulatory molecule CD86 as well as the Fc receptor CD32 (K32/86). These APC were subsequently incubated with anti-CD3 and anti-CD28 for 10 minutes prior to co-incubation with $CD4^+$ T cells along with rapamycin and a high dose of IL-2 (300 units/ml) in serum-free medium. This method was very successful in generating human Treg cells from peripheral blood mononuclear cells (PMBC), which could be expanded 1000 fold after just 3 weeks of culture. These cells were shown to have potent suppressor capability *in vitro*. Finally, Tang *et al.* expanded polyclonal Treg cells using anti-CD3/anti-CD28 coated paramagnetic beads plus a very high dose of IL-2 (2000 IU/ml). The Treg cells generated using this method were potent suppressors of autoimmune diabetes in non-obese diabetic mice (39). It is not clear from these studies whether the Treg cells expanded can be classified as nTreg or iTreg.

Ex-vivo expansion of $CD4^+CD25^+FoxP3^+$ Treg cells based on the synergy between IL-2 and 4-1BB signalling

Anti-CD28 co-stimulation has proved very powerful in the *ex-vivo* expansion of Treg cells. Nevertheless, the fact that almost all naive and effector memory $CD4^+$ and $CD8^+$ T cells express this molecule means that this method has the potential to activate rather that suppress the immune system. This dangerous potential was highlighted recently when life threatening cytokine storms were induced in participants of anti-CD28 antibody trials (40). Despite this, targeting co-stimulatory molecules to expand Treg cells still remains a possibility. Elpek and co-workers chose to target the co-stimulatory molecule 4-1BB since its expression was limited to a subset of $CD4^+$ Treg cells (41-43). Since 4-1BB ligand (4-1BBL) doesn't have co-stimulatory functions as a soluble protein, this group made a stable construct (SA-4-1BBL) by fusing extracellular domains of 4-1BBL to a modified

streptavidin core, which form oligomers capable of cross-linking 4-1BB on T cells (41). $CD4^+CD25^+$ Treg cells, sorted from the spleens of naive mice were co-cultured with irradiated splenocytes in conjunction with anti-CD3, anti-CD28, exogenous IL-2 and the construct SA-4-1BBL. These cells were fed every 4 days and reactivated every 12 days. The result was an up to 110 fold expansion of Treg cells *in vitro*. These cells expressed the canonical Treg cell markers, including FoxP3, GITR, TGF-β_1, CD25 and CD62L as well potently suppressing $CD4^+$ effector T cells in a 1:10 ratio *in vitro* (41). SA-4-1BBL-expanded Treg cells were transferred into chemically-induced diabetic mice, 24 hours prior to transplantation of an allogeneic islet graft. Those animals that received SA-4-1BBL Treg cells showed enhanced survival and reduced graft rejection up to 86 days post transplant (41).

Studies carried out on human umbilical cord blood (UCB) cells have revealed that 4-1BB co-stimulation can be used as part of a protocol to expand Treg cells *in vitro* (44). Cord blood itself is a prominent source to generate Treg cells because it is devoid of memory T cells. As previously described (37), K562 artificial APC cells can be engineered to express co-stimulatory molecules that activate Treg proliferation and expansion. In this case, K562 cells were engineered to stably express CD32 (Fc receptor) and 4-1BB. These cells are subsequently incubated with anti-CD3 and CD28 and are then co-cultured with UCB to expand Treg cells. These expanded Treg cells have been shown to have stable suppressive function *in vitro* (44).

Exogenous IFN-γ/TGF-β and bone marrow dendritic cells (BM DC) can induce and expand murine polyclonal Treg cells in vitro

IFN-γ has been long regarded as the canonical Th1 pro-inflammatory cytokine; however a number of recent studies have revealed that this cytokine can play a regulatory role under certain conditions (45). Evidence from collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) models in mice have shown that IFN-γ-deficient mice are more susceptible to disease (46-47). Furthermore, IFN-γ-deficient recipients of skin allografts more rapidly reject the graft than their wild type counterparts (48). Based on these reports it seems likely that IFN-γ might have a role in the induction or expansion of Treg cells *in vivo* as well as altering the balance of more pro-inflammatory cytokines such as IL-17. Feng *et al.* have devised a protocol whereby bone marrow (BM) DC were incubated with freshly isolated $CD4^+CD25^-Foxp3^-$ T cells and cocktail containing GM-CSF, TGF-β_1 and IFN-γ for 7 days (49-50). On day 7 fresh BM DC and IFN-γ were added to the co-culture and incubated for a further 7 days. The resulting T cells were $CD4^+CD25^+Foxp3^+$ and had potent suppressive function *in vitro*. In addition, co-transfer of these cells with $CD4^+$ effector T cells prevented the rejection of a skin allograft. The authors consider this approach to be ideal in transplantation where donor APC could be used to drive Treg expansion. It is conjectured that Treg cells generated in this fashion could be used alongside a short course of immunosuppressive drugs to improve the chances of graft survival.

***Polyclonal IL-2*/ TGF-β_1-induced Treg (iTreg) cells**

It is now well established that *in vitro* activation of naive $CD4^+$ T cells by DC, in conjunction with TGF-β_1 and IL-6 leads to the differentiation into pro-inflammatory Th17 cells (51). Zheng *et al.* have shown that iTreg and not nTreg cells are resistant to Th17 conversion induced by IL-6. This makes iTreg cells ideal therapeutic candidates in Th17-mediated autoimmunity such as psoriasis and systemic lupus erythematosus (SLE) (52). Briefly, $CD4^+CD25^-$ naive T cells are isolated from the spleens of naive mice and cultured with anti-CD3/CD28-coated beads, TGF-β_1 and IL-2 in serum-free medium for 4 days. The result is the generation of $CD4^+CD25^+FoxP3^+$ iTreg with potent *in vitro* and *in vivo* suppressive function (52-53). To date iTreg generated in this way have been used to treat Myasthenia Gravis in rats (54), Graft Versus Host disease in mice (55), autoimmune Gastritis (56), EAE (57) and Lupus-like Syndrome in mice (58).

Induction and expansion of polyclonal Treg cells in vitro with Retinoic Acid and TGF-β_1

DC isolated from the mesenteric lymph nodes (MLN) that drain the gut have been shown to be tolerogenic when compared with those from the spleen. Co-culture of MLN DC with naive T cells supplemented with IL-6 and TGF-β_1 did not result in Th17 differentiation as expected (59). The active metabolite of vitamin A, all trans-retinoic acid (RA), which is produced by DC in the gut and draining lymph nodes was found to be essential to the induction of Treg cells with the help of TGF-β_1 (60). It was subsequently shown that RA had a direct effect on naive $CD4^+$ T cells *in vitro*, since these cells

express the RA receptor. $CD4^+CD25^-$ naive T cells were co-cultured with either MLN DC or irradiated splenocytes in the presence of anti-CD3, IL-2, RA and TGF-β_1. The resultant $CD4^+FoxP3^+$ Treg cells were subsequently co-transferred with $CD4^+CD45RB^{hi}$ naive T cells in a murine model of colitis where they significantly inhibited inflammation (60-61). More recently it has been demonstrated that intravenous administration of RA and TGF-β_1 ameliorated acute heart allograft transplantation and enhanced survival in mice (62).

Ex-vivo generation of antigen-specific Treg cells

Anti-CD3/CD28-expanded versus BM DC-expanded antigen-specific $CD4^+CD25^+FoxP3^+$ Treg cells
In a recent study, Zeng and co-workers compared the function of Treg cells expanded with anti-CD3 and anti-CD28 versus those expanded with mature BM DC. $CD4^+CD25^+$ Treg cells were isolated from the spleen and lymph nodes of ova-transgenic mice. These cells were co-cultured *in vitro* with anti-CD3 and anti-CD28 plus IL-2 or with prostaglandin E2 (PGE2)-matured BM DC (pulsed with ovalbumin) plus exogenous IL-2 (63). The results showed that DC are more effective than anti-CD3/CD28 anti-bodies in expanding and more importantly maintaining the viability of $FoxP3^+$ Treg cells *in vivo*. DC-expanded Treg cells prevented the differentiation of allo-reactive T cells into IFN-γ^+ effectors. This study also highlights the potential advantage that antigen-specific expansion might have over polyclonal Treg cells in treating transplant rejection.

Ex-vivo expansion of IL-10 producing Tr1 cells for chronic disease therapy

IL-10 producing Tr1 cells were first described by Groux *et al.* in 1997 (64). Since then, these cells have been described in both mice and humans, in health and disease (65-66). More recently, Brun and colleagues have used *ex-vivo* expanded antigen-specific Tr1 cells to treat severe refractory Crohn's disease. Most healthy humans have already developed clones to the ubiquitously expressed food antigen, ovalbumin, therefore this group has used a protocol in which Tr1 ovalbumin-specific clones can be expanded from the PBMC of patients with Crohn's disease *ex-vivo* (67) Table **2.** Schneider *Drosophila* feeder cells, transfected with transmembrane murine anti-human CD3 as well as with human CD80, CD58, IL-2 and IL-4 were used to expand Tr1 cells from PBMC in the presence of ovalbumin. Clones were then detected by limiting dilution and cultured for 12 weeks or until they reached 5 billion in number (68). The use of Drosophila feeder cells allows these cells to be expanded in a human and animal sera free manner which is important for good manufacturing practice. Once the optimal number of Tr1 clones were produced these cells were checked for antigen-specific suppressive activity *in vitro*. These cells have an activated phenotype that resembles nTreg cells ($CD25^+FoxP3^+GITR^+$) all of which are down regulated during quiescence. Unlike nTreg cells, Tr1 cells are accepted as an iTreg, which can produce significant amounts of IL-10 and express neither CD25 nor intracellular FoxP3 in the quiescent state (66). Furthermore these cells were checked for genetic aberrations (karyotype check) and also that they possessed the cellular machinery required to reach senescence. A proportion of Tr1 clones were expanded *in vitro* for up to 30 population doublings and checked for normal telomerase activity as well as progressive telomere erosion. It is essential that such characterisations are made prior to re-infusion into the patient. The authors suggest that because these Tr1 clones are antigen-specific there is less likelihood of systemic suppression. However, it has been mooted that although these cells are specific for a single antigen they can suppress in a non-antigen-specific manner via bystander suppression *in vivo* (66). Tr1 clones expanded from the PBMC of Crohn's patients, using the protocol described here went to trial in 2008 for the treatment of severe refractory Crohn's disease (67). The results of this trial have yet to be released.

Expansion of TCR or TCR/FoxP3-Transduced Treg cells

Wright *et al.* have made use of two TCR (αβ) gene transfer strategies to generate antigen-specific Treg cells (69). The first involves the retroviral transduction of a *TCR* gene, specific for ovalbumin peptide (Ova) into nTreg ($CD4^+CD25^+FoxP3^+$) cells. In the second protocol, $CD4^+CD25^-$ T cells were transduced with the ova-specific *TCR* gene as well as the *FoxP3* gene (69). Since gene transfer occurs successfully in cycling cells, the two enriched subsets were activated with anti-CD3/anti-CD28-coated beads plus IL-2 and transduced 2 days later. The resulting cells were $CD25^+Foxp3^+CTLA\text{-}4^+GITR^+$, ova-specific and secreted little IL-2 (characteristic of anergic cells). Furthermore, both the *TCR*-transduced nTreg and the *TCR/FoxP3*-transduced T cells once activated with specific peptide

suppressed $CD4^+$ effector T cells of unrelated specificity *in vitro*. Despite the fact that both subsets could suppress *in vitro*, the *TCR*-transduced nTreg cells were more potent. Importantly, both subsets retained their phenotype and function up to 6 weeks post *in vivo* transfer. These subsets were subsequently used to treat murine antigen-induced arthritis (AIA), where mice were immunised with methylated bovine serum antigen (mBSA) and ovalbumin followed by intra-articular knee re-challenge. The resultant Treg cells were specific for Ova, which is included in BSA. Transduced Treg cells were transferred prior to re-challenge with mBSA in the knee and the resulting swelling and cellular infiltration was measured at fixed time points thereafter. Mirroring *in vitro* results, antigen-specific *TCR*-transduced nTreg and the *TCR/FoxP3*-transduced T cells reduced swelling and the number of infiltrating $IL\text{-}17^+$ proinflammatory cells (69). The benefits of this protocol include the fact these Treg cells are antigen-specific and that they have potential as a monotherapy without the need for lymphodepletion or immunosuppressive drugs such as cyclosporine. These transduced Treg cells, particularly TCR transduced nTreg cells; prevent tissue damage by $CD4^+$ effector T cells with unrelated specificity. Therefore they represent a promising therapeutic option for the treatment of autoimmunity without any knowledge of the initiating auto-antigens.

Use of immature DC (iDC) versus CD40-activated B cells (CD40-B) to generate Treg cells

iDC have been used in the past to generate antigen-specific $CD4^+$ Treg as well $CD8^+$ Treg cells from human PBMC, *in vitro* (70). The process involves expansion of iDC using granulocyte/monocyte-colony stimulating factor (GM-CSF) and exogenous IL-4 followed by co-culture with enriched $CD4^+$ or $CD8^+$ T cells in the presence of antigen and high dose IL-2 in serum free medium (70-72)]. These Treg cells were capable of suppressing in an antigen-specific manner both *in vitro* and *in vivo*.

CD40-activated B cells have also been used as APC to generate Treg cells *in vitro* (73-75). B cells from human PBMC were stimulated via CD40 using NIH3T3 cells, transfected with the human CD40 ligand (t-CD40-L cells) plus IL-4 and cyclosporine. Cyclosporine was used at a concentration that inhibited T cell proliferation but not that of B cells. After 14 days of co-culture approximately 95% of the cells in the culture were positive for the immature B cell marker CD19. These B cells were subsequently activated in the presence of freshly isolated allogeneic $CD4^+CD25^-$ T cells plus IL-2 and re-activated every 7 days. The resulting alloantigen-specific Treg cells expressed and bore the standard phenotypic markers, including FoxP3 and suppressed effector cell proliferation *in vitro* (73-74).

Zheng and co-workers compared the induction of Treg cells using either iDC or CD40-B, which were generated using the protocols described above (76). $CD4^+CD25^-$ T cells were isolated from PBMC and co-cultured with either iDC or CD40-B in a 10:1 ratio. While both APC induced antigen-specific $CD25^+$ Treg cells that suppressed via cell contact, CD40-B-induced Treg cells were more suppressive and expressed more FoxP3 and CTLA-4 (76).

Anti-CD52-induced Treg cells

CD52 is a small glycopeptide that is expressed on most T cells (77). Anti-CD52 provides a co-stimulatory signal to T cells, when cross-linked, which can inhibit proliferation and has been used to treat T and B cell lymphomas (78) and is currently undergoing clinical trial for the treatment of ovarian cancer (79). An unexpected finding of anti-CD52 treatment was its co-stimulatory impact on Treg cells, which has piqued the interest of those interested in treating autoimmunity and graft rejection. Recently, Watanabe and colleagues have used the representative humanised anti-CD52 monoclonal antibody (mAb), Campath-1H to expand polyclonal and antigen-specific Treg cells *in vitro* (77, 80). Their protocol involved isolating $CD4^+CD25^-$ T cells and stimulating them with anti-CD3 and anti-CD52 or allogeneic T cells, DC and anti-CD52. Exogenous IL-2 (100 IU/ml) was used in subsequent re-stimulations in order to enhance proliferation. However, the authors note that the final round of stimulation must include anti-CD52 only and not IL-2, since the latter has been shown to inhibit suppressive capacity when used at this stage of expansion. The Treg cells induced and expanded in this fashion have slightly enhanced FoxP3 expression when compared with naive T cells, must be activated with specific antigen and were found to suppress xenogeneic GvHD in SCID mice that received human PBMC. While anti-CD52-induced Treg cells are less suppressive that nTreg cells, the authors postulate that they have a number of advantages. First, it is easy to obtain a large number of $CD4^+CD25^-$ T cells for Treg induction and second, the purity of the enriched $CD4^+CD25^-$ T cells is not

as crucial when compared with isolating $CD4^+CD25^+$ nTreg cells. It remains to seen whether this protocol can be applied to the clinic in the treatment of autoimmunity or transplantation.

α-melanocyte-stimulating hormone (α-MSH) and TGF-β_2 induce antigen-specific Treg cells in mice

α-MSH is a neuropeptide with anti-inflammatory properties including the inhibition of macrophages and neutrophils induced by IL-1, TNF-α and IFN-γ (81-82). Namba *et al.* generated α-MSH-induced antigen-specific Treg cells which were used to treat a murine model of human posterior uveitis (experimental autoimmune uveoretinitis; EAU) (83-84). Briefly, APC were derived from murine splenocytes and co-cultured with $CD3^+$ T cells isolated from immunised mice in the presence of α-MSH and TGF-β_2. In this case, mice were immunised with a number of ocular proteins that included purified retinal antigen. Transfer of Treg cells generated using this protocol inhibited the symptoms of EAU in an antigen-specific manner. It was shown in a subsequent study that *α*-MSH/TGF-β_2-induced ocular antigen-specific Treg cells could also enhance intravitreous allogeneic retinal graft survival in mice (85). Despite the fact that Treg cells generated in this way demonstrate potential in treating autoimmunity and transplant rejection, no work has yet been carried out on human disease.

Treg cell therapy and Transplantation

Transplantation is now the preferred method of treating organ failure. However, successful transplantation is mitigated by direct and in direct allo-recognition by donor and recipient DC. It is becoming more evident that the balance between effector T cell and Treg cell subsets is crucial to long-term graft survival. There are many examples in the literature where Treg cell therapy has been used to treat graft rejection in animals and humans. We have focused on 3 recent studies which highlight the pitfalls and the way forward in using adoptive transfer of Treg cells to prevent graft rejection.

Recently, Park and colleagues used a promising combination therapy to promote the survival of skin allograft in mice, whereby $CD4^+$ and $CD8^+$ T cells are depleted in the host followed by Treg cell transfer (86). The rationale behind the T cell depletion is to remove the source of aggressive graft-specific effector T cells which might combat the benefit afforded by the Treg cell transfer. $CD4^+CD25^+CD62L^+$ Treg cells were enriched from spleen and lymph node cells and co-cultured with allogeneic, irradiated allogeneic splenocytes plus 300 IU/ml of IL-2. The second round of stimulation included the addition of recombinant TGF-β_1. Mice were injected with anti-CD4 and anti-CD8 depleting antibodies 4 hours prior to skin transplant. *In vitro* expanded $CD4^+CD25^+CD62L^+$ Treg cells were transferred into recipient mice 3 days later (86). The results showed that combination therapy prolonged graft survival when compared with Treg cell transfer alone (86). Nevertheless, the skin grafts were ultimately rejected in those mice that received combination therapy, implying that more work is needed to optimise this protocol, especially if it is to be transferred to the clinic.

As previously described, a number of protocols have now been developed whereby retinoic acid in conjunction with TGF-β_1 has been employed to induce iTreg from $CD4^+FoxP3^-CD25^-$ cells *in vitro* (59-60, 62). One of these strategies in particular has been successful in preventing acute heart rejection in mice (62). Interestingly, a recent study by Koenecke *et al.* has issued a caution over the use of iTreg to treat GvHD, which might have implications in other models of transplantation (87). This group have demonstrated that iTreg cells generated from $CD4^+$ effectors, using a combination of matured BM DC, TGF-β_1, retinoic acid and 333 IU/ml of IL-2, lost FoxP3 expression and suppressive function within 8 days of adoptive transfer. On the other hand, $CD4^+FoxP3^+CD25^+$ nTreg cells retained FoxP3 expression and protected the recipient mice from experimental GvHD. These results should be taken on board in devising future strategies to treat transplant rejection with iTreg cells. nTreg cells are very effective in preventing graft rejection and are now widely accepted as being crucial for preventing autoimmunity. Despite this, their low frequency and the associated difficulties in expanding antigen-specific nTreg clones, makes this Treg cell subset an unlikely candidate for treating graft rejection in humans.

On the contrary, large numbers of antigen-specific Tr1 inducible Treg cells can be generated *in vitro* to treat graft rejection in mice and humans (64, 66-67, 88). Using a mouse model of pancreatic islet cell transplantation in chemically-induced diabetes, Gagliani *et al.* showed that donor-specific Tr1 cells could promote long-term survival (89). In a continuation of the debate between the use of

polyclonal versus antigen-specific Treg cells, these researchers demonstrated that polyclonal Tr1 cells could only inhibit transplant rejection in less stringent murine models. *Polyclonal Tr1 cells* were induced *in vitro* from enriched $CD4^+CD62L^+$ naive T cells and stimulated with anti-CD3/CD28 plus 1000 IU/ml of human IL-10. On the other hand, *antigen-specific Tr1 cells* were generated *in vivo* by injecting mice (that received an islet cell transplant under the renal capsule) with a cocktail containing rapamycin, human IL-10 and anti-CD45RB depleting antibody. Anti-CD45RB antibody applied in this method served to deplete naive $CD4^+CD45RB^+$ naive cells with the potential to become potent effector anti-donor cells. Furthermore, rapamycin predominantly inhibits effector $CD4^+$ T cell growth. 30 days post transplant, $CD4^+CD25^-$ cells were isolated from the spleens of these animals and co-transferred into mice that received an islet cell graft in a stringent transplant model. All mice that received this graft in conjunction with antigen-specific Tr1 cells survived in comparison with those mice which received polyclonal Tr1 cells, where none survived. In short, inducible antigen-specific Tr1 Treg cells were more potent than their polyclonal inducible Tr1 counterparts. A clinical trial is now underway using a similar approach, where *ex-vivo* generated host-specific Tr1 cells were transferred into patients with leukaemia to prevent the rejection of haploidentical haematopoietic stem cells (89-90). Hopefully the results of this trial will be positive and open up a new option for the treatment of transplant rejection in the future.

It has been demonstrated in another report by the same group, that monotherapy of type 1 diabetic patients with rapamycin, prior to islet graft transplantation, can enhance nTreg suppressive function and promote the long term survival of the graft (91). As described previously, rapamycin has the ability to selectively inhibit the proliferative capacity of $CD4^+$ effector T cells whilst having a limited effect on Treg cells. It was shown here that while monotherapy with rapamycin didn't alter the number, phenotype or proliferative capacity of $CD25^+$ nTreg cells, it did enhance their suppressive function (91). The authors also advise against co-treatment of diabetic patients with anti-CD25 depleting antibody (daclzumab) and rapamycin, since the former will also deplete nTreg cells and abrogate the tolerogenic effects of rapamycin (91).

Antibody therapy and Treg cell induction *in vivo*

The use of monoclonal antibodies to generate Treg cells directly in the patient represents a cheaper and less cumbersome method of treating autoimmunity and graft rejection. However, the worry still remains that this type of therapy might induce a wave of non-specific suppression that could lead to opportunistic infection or malignancy. Moreover, these therapies, the majority of which are developed in animal models, might not transfer well into humans. This was none more evident than in a clinical trial involving anti-CD28, which showed promising results in primates but lead to devastating consequences in man (40).

In vivo Treg cell expansion using the anti-CD28 mAb TGN1412

Initial studies revealed that the mAb drug TGN1412, a recombinantly expressed humanised superagonist anti-CD28, could stimulate T cells independently of TCR signalling (92). It was shown in a subsequent report that this drug could be used to treat EAE in mice without detectible toxic or pro-inflammatory effects (93). TGN1412 selectively enhanced Treg cell numbers and function *in vivo* (93). All the reports indicated that this drug showed promise as a therapeutic candidate in the treatment of autoimmunity. Therefore in a phase 1 trial conducted in 2006, six volunteers received TGN1412 and the remaining two received placebos, with disastrous consequences (40). While all six survived, intravenous infusion of TGN1412 caused a sudden and rapid release of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-6) leading to a cytokine storm. The physiological impact of the drug, measured over 6 days included acute renal impairment, haemodynamic instability, respiratory distress syndrome, disseminated intravascular coagulation, nausea, vomiting, myalgia, raised liver enzymes, partial amnesia and severe headaches (40). While the motive was undoubtedly good and the preliminary results in animal models promising, the results of this trial should highlight the significant peril in trying to manipulate T cells with a single agent, especially when that agent is known to target co-stimulatory molecules on the majority of T cells.

IL-2:anti-IL-2 mAb-induced Treg cells

Recombinant IL-2 therapy has been used to treat cancer with variable success. In fact it has been suggested that IL-2 therapy is detrimental in cancer considering its observed potential to expand Treg

cells *in vivo* (94). This however, led to the idea that while IL-2 therapy might be damaging in cancer, it might have a role to play in treating autoimmunity, by the very fact that it can increase Treg cell numbers. Chen and co-workers demonstrated that infusion of daily doses of recombinant human (rh) IL-2 (25000 IU/mouse) alleviated the symptoms of EAE in mice (95). However, it was shown in a subsequent study that IL-2 therapy alone could not control airway hypersensitivity in mice (96). These variable results made it increasingly difficult to consider IL-2 as a viable therapy for treating autoimmunity in humans. Serendipity led to the discovery of an alternative approach, whereby rhIL-2 complexed to a specific monoclonal anti-IL-2 anti-body (JES6-1) was found to amplify Treg cell responses in mice (97). Wilson *et al.* used this protein complex to treat a murine model of allergen-induced airway inflammation with considerable success (96). The amplified Treg cells in this model suppressed in an IL-10-dependent fashion, however the precise mechanism of action behind IL-2/anti-IL-2 Treg expansion remains unknown (98). While this monotherapy has potential in treating allergy in humans, the paradoxical action of IL-2 rings similar to that observed with using anti-CD28 therapy and any future progress into the clinic must be made with caution.

Anti-CTLA-4-induced Treg cells

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is a close relative of CD28, which is up regulated on T cells following activation. CTLA-4 on T lymphocytes binds to both CD80 and CD86 on antigen-presenting cells with 20-fold greater affinity than CD28 and serves to inhibit T cell activation. It has a critical role in maintaining T cell homeostasis and is constitutively expressed on nTreg cells. Anti-CTLA-4 therapy, with a number of different monoclonal anti-bodies has been used in the treatment of cancer in the guise of Ipilimumab and Tremelimumab (99). Coquerelle *et al.* started with the premise that anti-CTLA-4 (UC10-4F10) in their murine model would enhance T cell helper function but found that this particular mAb had the opposite effect (100). This group showed in subsequent experiments that anti-CTLA-4 (UC10-4F10) ameliorated chemical-induced colitis (trinitrobenzene sulfonic acid; TNB) in mice. Treatment enhanced the number of IL-10-secreting Treg cells that also expressed FoxP3 and inducible co-stimulator (ICOS), which was dependent on the production of indoleamine 2,3 dioxygenase (IDO) by DC (100). The pitfalls here are similar to those attributed to the use of anti-CD28 and IL-2/anti-IL-2 therapy. What is certain is that while antibody therapy might be cheaper than Treg cell therapy, the potential hazards preclude the application of these therapies in the clinic, for the time being at least.

Treg cell depletion to treat cancer

While Treg cells are crucial to maintaining central and peripheral tolerance in animals and humans, malignancies have co-opted these cells to suppress tumour-mediated immunity, which permits uncontrolled growth. Currently, one of the goals in cancer treatment remains the eradication of Treg cells using anti-CD25-depleting antibodies in combination with standard treatment such as cyclophosphamide (101). The anti-CD25 depleting antibody (PC61) has been used extensively in murine studies of cancer to eliminate $CD25^+$ Treg cells in an attempt to augment anti-tumour immunity (101-102). Another strategy has been to deplete $CD25^+$ cells in conjunction with DNA vaccination in an attempt to simultaneously deplete Treg cells while enhancing anti-tumour memory responses (103). However, to date few of these studies have been translated into human clinical trials and there have been some concerns regarding the development of autoimmunity following the immunotherapy of tumours (104). Nevertheless, a recent study on the use of a diphtheria toxin-IL-2 fusion protein to treat melanoma metastases in humans has shown promise (105).

Treg-depletion and melanoma metastases

Cognate immune reactions against tumour cells depend on a balance between activated tumour antigen-specific $CD4^+$ and $CD8^+$ T cells and Treg cells. While Treg depletion has proved successful in mouse models, many have failed when examined in human clinical trials (106-108).

Denileukin difitox (DAB/IL2) is a recombinant DNA-derived cytotoxic protein composed of diphtheria toxin fragments A and B and IL-2. DAB/IL2 binds to CD25 and following internalisation, inhibits protein synthesis and causes cell death within hours (109). This drug is licensed for the treatment of cutaneous T cell lymphoma (CTCL), which is believed to be a malignant proliferation of $CD25^+$ Treg cells (110). Based on the fact that DAB/IL2 can successfully treat CTCL, Rasku *et al.* decided to use this drug to treat patients with stage IV melanoma by depleting $CD25^+$ Treg in (105). DAB/IL2 was

administered over a 4 day period at 21 day cycles in 18 patients. This transient depletion of $CD25^+$ cells proved effective in causing a regression of metastases. It is believed that temporary depletion is followed by a repopulation with tumour-specific CD8+ T cells which effectively halt tumour progression (105). This phase II trial has now been expanded to garner further evidence and it is hoped that DAB/IL2 treatment can be applied to other cancer types (105).

CURRENT & FUTURE DEVELOPMENTS

The treatment of autoimmunity and graft rejection by the transfer of *ex-vivo* expanded Treg cells or directly by using antibodies or other immunomodulatory agents remains fraught with difficulties and danger. As things stand, the *ex-vivo* expansion of antigen-specific IL-10 producing Tr1 cells using modified Drosophila feeder cells, represents the most advanced protocol for the treatment of autoimmunity; colitis in this instance. This protocol is very stringent in terms of karyotypic examination, functional and phenotypic assessments and makes use of good manufacturing process prior to transfer. In addition, induced Treg cells have an advantage over nTreg cells since in theory, $CD4^+$ T cells of any specificity can induced to become a Treg cell *in vitro*. It is more difficult to expand self-antigen-specific nTreg cells in sufficient numbers to treat patients.

It is becoming clearer that antigen-specific Treg cells trump their polyclonal counterparts with regard to treating autoimmunity or graft rejection. It is thought that the use of polyclonal Treg cells could contribute to systemic suppression resulting in opportunistic infection or malignancy, while antigen-specific Treg cells are confined to the target organ. Despite this, it should be noted that while many of the antigen-specific Treg cells described here require activation with their cognate antigen, they can then engage in bystander suppression.

Significant progress is being made in the development of effective and efficient protocols for treating patients with expanded Treg cells. While the use of antibodies and immunomodulatory agents as montherapies for autoimmunity have shown some promise, the dangers far outweigh the benefits to the patient. Further work needs to be carried out to completely elucidate the mechanisms of action as a result of these antibodies binding to their ligands. Perhaps combination therapies involving *ex-vivo* expanded antigen-specific Treg cells and immunomodulatory agents represent the way forward. Despite the technical, safety and fiscal concerns, we are moving ever closer to manipulating Treg cells in order to treat autoimmunity, graft rejection and cancer.

CONFLICT OF INTEREST

No financial contribution to the work has been declared. No patents are reported in any stage of legal litigations.

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Figure 1

Schematic representation of Treg subsets in humans and mice.

Figure 2

Protocols used to generate polyclonal and antigen-specific Treg cells *ex-vivo*.

A, *Ex-vivo* expansion of polyclonal Treg cells. K562 artificial APC cells can be engineered to express co-stimulatory molecules such as 4-1BBL and CD86 as well as CD32 (FC receptor). After a short incubation with anti-CD3 and anti-CD28 (that bind to the K562 cell via CD32) the APC are co-cultured with naive $CD4^+CD25^-$ T cells isolated from either whole blood, umbilical cord blood (UCB) or murine splenocytes. The co-culture serum free medium is either supplemented with high dose exogenous IL-2 or IL-2 plus rapamycin. After a number of rounds of stimulation, the result is the induction and expansion of $CD4^+CD25^+FoxP3^+$ Treg cells with regulatory function. ***B,*** *Ex-vivo expansion of ovalbumin-specific IL-10 producing Tr1 cells*. *Drosophila* feeder cells, transfected with transmembrane murine anti-human CD3, in conjunction with human CD80, CD58, IL-2 and IL-4 are co-cultured with PBMC in the presence of ovalbumin, in serum free medium. Ovalbumin-specific clones are then detected by limiting dilution, isolated and cultured for 12 weeks under similar conditions. The result is the generation of up to 5 $x10^9$ antigen-specific Tr1 clones with potent suppressive capacity.