REVIEWS

Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans

Maria-Grazia Roncarolo[*‡] *and Manuela Battaglia*[*§]

Abstract | Substantial progress in understanding the biology of regulatory T cells and their roles in health and disease has been achieved in the past 10 years. This has led to an increasing interest in the possibility of using regulatory T cells as a biological therapy to preserve and restore tolerance to self antigens and alloantigens. Immunotherapy by the adoptive transfer of regulatory T cells may have several advantages over conventional treatments. However, several hurdles have to be overcome before such a therapy can enter clinical practice. This Review summarizes our current knowledge of regulatory T cells, illustrates the ongoing regulatory T-cell-based clinical trials, analyses the strengths and pitfalls of this new therapeutic approach, and highlights the future perspectives.

Regulatory T cells are fundamental in controlling various immune responses. Compelling data generated in preclinical animal models indicate that adoptive transfer of regulatory T cells can prevent or cure several T-cell-mediated diseases, including autoimmune diseases and allograft rejection, by restoring immune tolerance to self antigens or alloantigens. Absence or defective function of regulatory T cells has been correlated with autoimmunity in humans, whereas their presence has been associated with tolerance. Several different regulatory T-cell subsets have been described (reviewed in REF. 1). The $CD4^+$ regulatory T cells have been categorized into two major subgroups based on their ontogeny: the naturally occurring forkhead box P3 ($FOXP3^+CD4^+CD25^+$ regulatory T cells (referred to here as T_{Reg} cells), which develop in the thymus and are present in normal naive mice and healthy individuals from birth (BOX 1), and the inducible regulatory T cells, which are generated in the periphery under various tolerogenic conditions. Many different subsets of inducible regulatory T cells have been reported. Among them, the T-cell subset (known as T regulatory type 1 (T_R1) cells) that produces high levels of interleukin-10 (IL-10) was originally described by our group[2] (BOX 2). In addition, inducible $FOXP3^+CD4^+CD25^+$ human regulatory T cells can be generated *in vitro* from $CD4^+CD25^-$ T cells in the presence of transforming growth factor-β (TGFβ)[3]. A detailed description of $CD4^+$ regulatory T-cell subsets has been the subject of other reviews[1,4] and is beyond the scope of this Review. Here, we focus on T_{Reg} cells, being the only naturally occurring regulatory T-cell subset described so far, and on T_R1 cells, being the only inducible T-cell subset that is currently being used in the clinic. This does not exclude the possibility that other regulatory T-cell subsets may be effective in providing protection from immunopathology and be suitable for immunotherapy in the near future.

There are several key differences between mouse and human regulatory T cells (TABLE 1) and this should be kept in mind, as these differences may represent obstacles in transferring knowledge gained from mouse models to human diseases.

The possibility that regulatory T cells might be used for the treatment of T-cell-mediated diseases has recently gained increasing momentum. The advantages of the adoptive transfer of regulatory T cells over conventional treatments are numerous. Some of these benefits include: the potential for antigen specificity with the lack of general immunosuppression, the possibility of inducing 'physiological' long-lasting regulation *in vivo*, and the fact that regulatory T-cell-based immunotherapy could be a custom-made product, designed *ad hoc* for each patient, with very limited or absent side effects. However, it is still unclear to what extent observations made in preclinical animal models will be applicable to humans; which human regulatory T-cell subset will be most appropriate for adoptive transfer; which method should be used to safely and efficiently

*San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, 20132 Milan, Italy.
‡Vita-Salute San Raffaele University, 20132 Milan, Italy.
§San Raffaele Scientific Institute, Immunology of Diabetes Unit, 20132 Milan, Italy.
Correspondence to M.-G.R.
e-mail: m.roncarolo@hsr.it
doi:10.1038/nri2138

Box 1 | **FOXP3$^+$CD4$^+$CD25$^+$ naturally occurring regulatory T (T_{Reg}) cells**

Mouse and human T_{Reg} cells are selected in the thymus and represent about 5–10% of total CD4$^+$ T cells in the periphery. They are crucial for maintaining tolerance by downregulating undesired immune responses to self and non-self antigens. T_{Reg} cells are defined on the basis of constitutive expression of high levels of CD25, the transcription factor FOXP3 (forkhead box P3)[72], low or absent expression of CD127 (REFS 49,50) and the inability to produce interleukin-2 (IL-2) and to proliferate *in vitro*[72]. Human CD4$^+$CD25$^-$ effector T cells lack detectable levels of FOXP3, but *FOXP3* mRNA and FOXP3 protein are upregulated after polyclonal stimulation[73].

Most *in vitro* studies indicate that T_{Reg} cells mediate suppression by an undefined cell-contact-dependent mechanism. However, *in vivo*, suppression may be mediated by suppressive cytokines. T_{Reg} cells may facilitate the production of IL-10 and transforming growth factor-β (TGFβ) by other T cells through 'infectious tolerance'[72]. Alternatively, under certain conditions, T_{Reg} cells might acquire the ability to produce TGFβ and/or IL-10 (REF. 74).

After activation through their T-cell receptor, T_{Reg} cells suppress proliferation of CD4$^+$ and CD8$^+$ T cells by inhibiting *IL2* transcription[75]. T_{Reg} cells can also suppress other cell types, such as antigen-presenting cells.

IL-2 is crucial for the generation, expansion, survival and effector function of T_{Reg} cells (reviewed in REF. 76). As T_{Reg} cells do not produce IL-2 themselves, their capacity to use IL-2 secreted by target T cells appears to be essential for their suppressive activity[76].

expand and generate regulatory T cells *ex vivo*; which human disease will be likely to benefit the most from regulatory T-cell transfer; and whether a combined therapy with other drugs is necessary. Some of these questions will undoubtedly be addressed by the ongoing clinical trials with adoptive transfer of regulatory T cells, whereas others will need further preclinical investigation and a better understanding of the basic biology of regulatory T cells. This Review provides an update on the most recent and exciting findings that might help to make regulatory T-cell therapy a feasible approach for the cure of numerous human diseases. Important limiting financial and regulatory aspects are also discussed.

Regulatory T cells control tolerance in humans

Several studies have shown that regulatory T cells have a crucial role in controlling tolerance to self antigens and alloantigens in humans. This section describes the correlation between, on the one hand, the presence of regulatory T cells and tolerance and, on the other hand, the absence of regulatory T cells and immunopathology. Furthermore, the human genetic diseases in which regulatory T-cell dysfunction occurs are discussed.

Role of regulatory T cells in responses to self antigens. Negative selection of developing autoreactive thymocytes is not a wholly efficient process and some T cells with high affinity for autoantigens can escape the deletion process in the thymus and migrate to the periphery. Peripheral tolerance is required to suppress those autoreactive T cells that escape thymic selection. Proliferative responses of human peripheral-blood mononuclear cells (PBMCs) from healthy individuals to the self antigens heat-shock protein 60 (REF. 5), myelin oligodendrocyte glycoprotein (MOG)[6], the type-1-diabetes-associated antigen glutamic-acid decarboxylase 65 (GAD65) and the vitiligo-associated antigen tyrosinase[7] are indeed significantly enhanced *in vitro* upon removal of naturally occurring T_{Reg} cells. Similarly, T_R1 cells specific for self MHC molecules[8] and for pancreatic-islet-derived peptides[9] have been found in healthy individuals. These observations indicate that autoreactive T cells are present and circulate in healthy subjects and that regulatory T cells actively suppress their function.

Also consistent with an important role for regulatory T cells in controlling autoreactivity is the demonstration that a wide variety of autoimmune diseases are associated with defects in regulatory T-cell function, raising the interesting possibility that this may be a common basis for the uncontrolled immune responses to self antigens[10]. T_R1 cells have been isolated from the synovium of patients with rheumatoid arthritis, but they seem to be present in significantly lower numbers compared with control individuals with non-autoimmune-mediated joint inflammation[11]. Similarly, T_{Reg}-cell defects in peripheral blood from patients with multiple sclerosis, type 1 diabetes, psoriasis, myasthenia gravis, rheumatoid and juvenile idiopathic arthritis have been described (reviewed in REF. 10). However, it should be noted that all these studies tracked the percentages of circulating CD4$^+$CD25$^+$ T cells, which might also include non-regulatory activated T cells.

Box 2 | **T regulatory type 1 (T_R1) cells**

T_R1 cells arise in the periphery after encounter with antigen in the presence of interleukin-10 (IL-10). The unique cytokine production profile (IL-2$^{low/-}$IL-4$^-$IL-5$^+$IL-10$^+$TGFβ$^+$) distinguishes T_R1 cells from T helper 0 (T_H0), T_H1 and T_H2 cells[2]. To date, no specific cell-surface marker for T_R1 cells has been identified. T_R1 cells have a very low proliferative capacity following activation *in vitro* through the T-cell receptor (TCR), in part due to autocrine production of IL-10. However, their proliferative activity *in vivo* is unknown[52].

T_R1 cells regulate immune responses through the secretion of the immunosuppressive cytokines IL-10 and transforming growth factor-β (TGFβ), and they suppress both naive and memory T-cell responses[52] and downregulate the expression of co-stimulatory molecules and pro-inflammatory cytokines by antigen-presenting cells (APCs). Furthermore, T_R1 cells favour the production of IgD, IgA and IgG by B cells[52]. Importantly, T_R1 cells are inducible, antigen specific and need to be activated through their TCR to exert their suppressive functions. However, once activated, they mediate suppression in an antigen non-specific manner[52].

IL-10 is absolutely required for the differentiation and function of mouse T_R1 cells. For human T_R1-cell differentiation, IL-10 is necessary but probably not sufficient[77,78]. Other necessary factors include the presence of APCs, which provide, in addition to IL-10, other soluble and surface molecules that are crucial for T_R1-cell generation. Many different approaches have been explored for the induction of T_R1 cells both *ex vivo* and *in vivo*[52].

Negative selection
The deletion of self-reactive thymocytes in the thymus. Thymocytes expressing T-cell receptors that strongly recognize self peptide bound to self MHC molecules undergo apoptosis in response to the signalling generated by high-affinity binding.

Table 1 | **Differences between mouse and human regulatory T cells**

Feature	Mouse	Human
CD4⁺CD25⁺ naturally occurring regulatory T cells		
Phenotype	$CD25^{hi}CD127^{low/-}FOXP3^{+}$	$CD25^{hi}CD127^{low/-}FOXP3^{+}$
	Anergic *in vitro*	Anergic *in vitro*
	IL-2-dependent *in vivo* proliferation	ND
	Granzyme-B expression on activation	Granzyme-A expression on activation
FOXP3	One isoform[79]	Two isoforms[73]
	Lack of FOXP3 expression by $CD4^{+}CD25^{-}$ T cells activated *in vitro*[79]	FOXP3 expression by $CD4^{+}CD25^{-}$ T cells activated *in vitro*[73]
	Deletion of *Foxp3* in scurfy mice[80]	Mutations in *FOXP3* in patients with IPEX[25]
Suppressive mechanism	Cell–cell contact *in vitro*	Cell–cell contact *in vitro*
	Cytokine mediated in some *in vivo* models[74]	ND
	Downregulation of *Il2* transcription in target T cells[75]	Downregulation of *IL2* transcription in target T cells[75]
	Apoptosis of effector T cells via a granzyme-B-dependent[81] and perforin-independent pathway[82]	Killing of monocytes, DCs, $CD4^{+}$ T cells, $CD8^{+}$ T cells and B cells via a granzyme-A- and perforin-dependent pathway[83]
	Suppression of innate immunity[84]	ND
T_R1 cells		
Phenotype	$IL\text{-}4^{-}IL\text{-}5^{+}IL\text{-}10^{+}IFN\gamma^{-}TGF\beta^{+}$	$IL\text{-}4^{-}IL\text{-}5^{+}IL\text{-}10^{+}IFN\gamma^{low}TGF\beta^{+}$
	$CD4^{+}CD25^{-}CD45RB^{low}$ (REF. 69) ($IFN\gamma R^{+}IL\text{-}10R^{+}$)[85]	$CD4^{+}CD25^{-}$ (REF. 77)
	Anergic *in vitro*[2]	Anergic *in vitro*[2]
FOXP3	Constitutively $FOXP3^{-}$	Constitutively $FOXP3^{-}$ (REF. 77)
	ND	Upregulation on activation *in vitro*
Differentiation *in vitro*	Exogenous IL-10 (REF. 2)	Exogenous IL-10 with IFNα and APCs[78]
Suppressive mechanism	IL-10 and TGFβ mediated[52]	IL-10 and TGFβ mediated[52]

APC, antigen-presenting cell; DC, dendritic cell; FOXP3, forkhead box P3; IFN, interferon; IL, interleukin; IPEX, immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome; ND, not determined; R, receptor; TGFβ, transforming growth factor-β; T_R1 cell, T regulatory type 1 cell.

Role of regulatory T cells in responses to alloantigens. The role of human T_{Reg} cells in the suppression of graft-versus-host disease (GVHD) after haematopoietic stem-cell transplantation (HSCT) has been investigated in several studies by correlating the presence of T_{Reg} cells with the incidence of GVHD, but these studies have yielded conflicting results. A positive correlation between high numbers of circulating $FOXP3^{+}CD4^{+}CD25^{hi}$ T_{Reg} cells and reduced GVHD has been found in some studies[12–14], whereas in other studies the outcome was the opposite[15,16]. A straightforward comparison between all these studies is not feasible as patients were not homogeneously selected, different immunosuppressive therapies were administered, different sources of HSCs were used and analyses were carried out at different time points after transplantation. Furthermore, an important limitation of these studies is the analysis of peripheral-blood T cells, which might not mirror the situation in tissues.

An elegant study by Rieger and colleagues showed that T_{Reg} cells are numerically deficient in the intestinal mucosa, which is a preferential site of GVH reactivity, in patients with acute and chronic GVHD[17]. Overall, although studies in preclinical models have clearly shown protection of GVHD after T_{Reg}-cell transfer (see later), definitive clinical evidence that cell therapy with T_{Reg} cells is effective in the cure or prevention of this disease is still lacking. Conversely, in solid-organ transplantation, the scenario is clearer. In patients transplanted with lung[18], liver[19] or kidney grafts[20], a positive correlation between graft survival and the number of circulating T_{Reg} cells has been shown.

T_R1 cells were originally described as an important cellular component in maintaining peripheral tolerance *in vivo* in severe combined immunodeficient (SCID) patients after allogeneic HLA-mismatched HSCT. $CD4^{+}$ host-reactive T_R1-cell clones generated from transplant recipients produced very high levels of IL-10 and IL-5 but not IL-4 and IL-2 after alloantigen-specific stimulation *in vitro*. The presence of these cells *in vivo* correlated with the absence of GVHD and with long-term graft tolerance without the need for drug-induced immunosuppression[21]. In addition, high levels of IL-10 produced spontaneously by PBMCs from an immunocompetent host before bone-marrow transplantation (BMT) have been associated with a subsequent low incidence of GVHD

Graft-versus-host disease (GVHD). A frequent complication after allogeneic stem-cell transplantation caused by the expansion of donor lymphocytes with helper and cytotoxic reactivity against host histocompatibility antigens. It can occur as two distinct syndromes: acute GVHD (occurring within 100 days) and chronic GVHD (occurring after 100 days). Complete depletion of T cells from the transplant largely eliminates GVHD but significantly increases the risk of graft failure and infections.

and transplant-related mortality[22]. A high frequency of donor cells producing IL-10 in response to host alloantigens was found to correlate with the absence of acute GVHD after BMT, whereas a low frequency of these cells was strongly associated with severe GVHD[23]. We have also recently found increased frequencies of T_R1-cell clones in patients with thalassaemia with a persistent state of mixed chimerism after successful HSCT from HLA identical or matched unrelated donors (G. Serafini and M.-G.R., unpublished observations). $CD4^+$ T_R1 cells are also present in patients who spontaneously developed tolerance to a kidney or liver allograft[24]. Taken together, these data indicate that T_R1 cells can naturally regulate immune responses and induce tolerance to alloantigens *in vivo* in both BMT and solid-organ transplantation. These findings provide a strong rationale for the clinical application of cell therapy with antigen-specific T_R1 cells generated *ex vivo*.

Defective regulatory T cells in genetic diseases. IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome), WAS (Wiskott–Aldrich syndrome), APS type 2 (autoimmune polyglandular syndrome type 2) and ALPS (autoimmune lymphoproliferative syndrome) are the few autoimmune diseases caused by known genetic defects. Interestingly, patients with IPEX[25], with WAS[26] or with APS type 2 (REF. 27) have a clear defect in T_{Reg}-cell suppressive function. Patients with ALPS type Ia are characterized by an expansion of T cells, but a reduction in the $CD4^+CD25^+$ T-cell subset, and this defect may be indicative of disturbed lymphocyte immunoregulation in ALPS[28]. Although the molecular mechanisms underlying these defects in T_{Reg} cells are still under scrutiny, these data suggest that regulatory T-cell-based therapy might also represent a good immunomodulatory strategy for the treatment of these genetic diseases.

Therapy with regulatory T cells in mice

Adoptive transfer of regulatory T cells in animal models of autoimmune diseases. Several preclinical animal studies have established that the adoptive transfer of regulatory T cells can prevent various autoimmune diseases (TABLE 2). As the development of autoimmunity in humans is rarely a foreseeable phenomenon, to be of use as therapeutic agents regulatory T cells must inhibit ongoing T-cell responses and reverse established pathology. However, among the numerous published studies only three studies showed that T_{Reg}-cell transfer is efficacious in reverting active disease[29–31]. Interestingly, of these three studies, only one used polyclonal wild-type T_{Reg} cells to show that reversal of disease can be mediated by these cells[29], whereas the other two studies used transgenic T_{Reg} cells expressing a T-cell receptor (TCR) specific for the pathogenic antigen[30,31]. Importantly, the positive results obtained with polyclonal wild-type T_{Reg} cells[29] were generated in lymphopenic hosts in which activation or expansion of T_{Reg}-cell subsets can be influenced by homeostatic proliferation. Overall these data indicate that the suppression of an ongoing autoimmune disease by the adoptive transfer of T_{Reg} cells might only be feasible when the T_{Reg} cells are specific for the pathogenic antigen. Alternatively, transfer of polyclonal T_{Reg} cells may cure ongoing disease only in lymphopenic hosts, in which the massive expansion of T_{Reg} cells may lead to the generation of a sufficient number of antigen-specific T_{Reg} cells. If this is the case also in humans, it may represent a significant limitation for the clinical application of T_{Reg} cells in autoimmune diseases, as, to date, human self-antigen-specific T_{Reg} cells have not been successfully expanded *ex vivo*. By contrast, T_R1 cells can be generated in an antigen-specific manner *ex vivo* and/or directly *in vivo* and therefore may represent an ideal therapeutic alternative. However, additional preclinical studies need to be carried out to show that antigen-specific T_R1 cells can revert ongoing autoimmunity.

Adoptive transfer of regulatory T cells in animal models of transplantation. The transfer of freshly isolated T_{Reg} cells together with the bone-marrow allograft has been shown to ameliorate GVHD and facilitate engraftment in mouse models of BMT[32–34] (TABLE 2). GVHD was also the first model in which it was shown that the adoptive transfer of donor T_{Reg} cells that were polyclonally expanded *ex vivo* is as efficient as the transfer of freshly isolated T_{Reg} cells in curing disease that results from transplantation in mice[35,36]. Interestingly, contrary to what was observed in the experimental models of autoimmunity, the transfer of T_{Reg} cells enriched for alloantigen specificity showed only moderately improved efficacy compared with the transfer of a polyclonal T_{Reg}-cell population[37]. These different results might be ascribed to the higher frequency of alloantigen-specific T cells compared with cells specific for self antigens within the T_{Reg}-cell subset. However, this hypothesis contrasts with the concept that T_{Reg} cells are enriched for self-antigen specificity because they develop in the thymus. An alternative explanation for the different outcome in the experimental models of autoimmunity versus BMT might be the presence of a lymphopenic environment that supports the expansion of transferred effector T cells and regulatory T cells in BMT recipients and that it may be different to that in normal mice. Thanks to these promising results generated in the animal models and to the lack of antigen-specific requirements for the transferred T_{Reg} cells, BMT is the setting for the first human clinical trial with T_{Reg} cells generated *ex vivo*, which will be carried out to test the ability of T_{Reg} cells to suppress GVHD (see later).

T_R1 cells generated *ex vivo* upon stimulation with alloantigens in the presence of IL-10 and TGFβ have been shown to be potent regulators of GVH responses after allogeneic BMT. Infusion of unmanipulated cultured T cells induced lethal GVHD in all transplant recipients, whereas only 25% of mice receiving *ex vivo* generated T_R1 cells died[38]. A clinical trial with *ex vivo* generated alloantigen-specific T_R1 cells in BMT is ongoing at the San Raffaele Scientific Institute, Milan, Italy (see later).

In contrast to what has been reported in GVHD, to our knowledge there are no reports showing that the transfer of freshly isolated T_{Reg} cells can prevent

Mixed chimerism
A state of coexistence of the host and allogeneic donor haematopoietic cells.

IPEX
(Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). A disease caused by mutations in *FOXP3* (forkhead box P3) and characterized by refractory enteritis and, in some patients, autoimmune endocrinopathies, autoimmune diabetes and thyroiditis. Unlike scurfy mice, peripheral-blood mononuclear cells from IPEX patients fail to produce cytokines after *in vitro* stimulation.

WAS
(Wiskott–Aldrich syndrome). A life-threatening X-linked immunodeficiency caused by mutation in the WAS protein. It is characterized by thrombocytopaenia with small platelets, eczema, recurrent infections caused by immunodeficiency, and an increased incidence of autoimmune manifestations and malignancies.

APS
(Autoimmune polyglandular syndrome; also known as APECED). APS type 1 is caused by the loss of central tolerance due to mutations in autoimmune regulator (*AIRE*), whereas APS type 2 is of unknown pathogenesis besides an association with polymorphisms in *CTLA4* and an HLA-extended haplotype. It is characterized by multiple endocrine diseases initiated by an autoimmune process.

ALPS
(Autoimmune lymphoproliferative syndrome). A syndrome associated with diffuse autoimmune manifestations. ALPS type Ia patients have mutations in *TNFRSF6*, which encodes CD95; ALPS type Ib patients have mutations in *TNFSF6*, which encodes CD95 ligand; ALPS type II patients have mutations in *CASP10*, which encodes caspase-10.

Table 2 | **Adoptive transfer of regulatory T cells in experimental mouse models**

Disease	Regulatory T cells	Mouse model	Effect on disease	Refs
Autoimmune disease				
Type 1 diabetes	BDC2.5 TCR-transgenic T_{Reg} cells (from NOD mice)	NOD.*Rag*$^{-/-}$ mice reconstituted with diabetogenic T cells	Prevention	30
		NOD.*Cd28*$^{-/-}$ mice (which lack T_{Reg} cells)	Prevention	30
		Diabetic NOD mice receiving syngeneic islets	Prevention	30
		NOD mice with new onset diabetes	Remission (60%)	30
	BDC2.5 TCR-transgenic T_{Reg} cells expanded by DCs *in vitro*	BDC2.5 TCR-transgenic mice treated with high doses of cyclophosphamide	Prevention	86
		NOD.SCID mice reconstituted with diabetogenic T cells	Prevention	86
		Pre-diabetic NOD mice	Prevention	86
		NOD mice with new onset diabetes	Remission (50%)	81
	Antigen-specific NOD T_{Reg} cells expanded *in vitro*	NOD.*Cd28*$^{-/-}$ mice	Prevention	87
	GAD65-specific T_R1 cells	NOD.SCID mice reconstituted with diabetogenic T cells	Prevention	88,89
Multiple sclerosis (EAE)	TCR-transgenic MBP-specific T_{Reg} cells	*Rag*$^{-/-}$ TCR-transgenic (MBP-specific) mice	Prevention of spontaneous disease	90
	T_{Reg} cells from naive C57BL/6 mice	C57BL/6 mice immunized with $MOG_{35–55}$ peptide	Prevention of induced disease	91
	OVA-specific T_R1 cells	BALB/c mice immunized with mouse spinal-cord homogenate and with heat-killed *Mycobacterium tuberculosis*	Prevention of induced disease	44
	T_R1 cells induced by B7H1–immunoglobulin fusion protein plus immobilized CD3-specific antibody	C57BL/6 mice immunized with $MOG_{35–55}$ peptide	Prevention of induced disease	85
Rheumatoid arthritis	T_{Reg} cells	Collagen-induced arthritis	Inhibited progression of early stage disease	92
Inflammatory bowel disease	T_{Reg} cells	SCID mice reconstituted with CD45RB^{hi} cells	Reversal of established disease	29
	OVA-specific T_R1 cells	SCID mice reconstituted with CD45RB^{hi} cells	Prevention	2
	Caecal-bacteria-specific T_R1 cells from C3H/HeJBir mice	SCID mice reconstituted with pathogenic T_H1 cells from C3H/HeJBir mice	Prevention	93
Systemic lupus erythematosus	Thymus-derived T_{Reg} cells	NZB × NZW mice	Control of autoimmunity	94
Scurfy disease	T_{Reg} cells	FOXP3-deficient mice	Rescue of the lymphoproliferative syndrome in neonatal mice	95
Transplantation				
GVHD	T_{Reg} cells	Mice having received an allogeneic BMT	Inhibition of GVH lethality	96
	T_{Reg} cells	Mice having received an allogeneic BMT	Delay and prevention	35
	Donor T_{Reg} cells	Mice having received an allogeneic BMT	Inhibition of GVH lethality	36
	Alloantigen-specific T_R1 cells	Mice having received an allogeneic BMT	Inhibition of GVH lethality	38
Graft rejection	T_{Reg} cells expanded *in vivo*	Mice having received an allogeneic islet-cell transplant	Prevention of allograft rejection	39
	T_{Reg} cells expanded *ex vivo* with rapamycin	Mice having received an allogeneic islet-cell transplant	Prevention of allograft rejection	41
	T_R1 cells induced *in vivo*	Mice having received an allogeneic islet-cell transplant	Prevention of allograft rejection	42
	TCR-transgenic HA-specific T_{Reg} cells	Mice having received an allogeneic skin transplant (HA expressing)	Prolonged survival of established graft	40

BMT, bone-marrow transplantation; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; FOXP3, forkhead box P3; GAD65, glutamic-acid decarboxylase 65; GVH, graft-versus-host; HA, haemagglutinin; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; NOD, non-obese diabetic; NZB, New Zealand black mouse strain; NZW, New Zealand white mouse strain; OVA, ovalbumin; RAG, recombination activating gene; SCID, severe combined immunodeficient; TCR, T-cell receptor; T_R1 cell, T regulatory type 1 cell; T_{Reg} cell, regulatory T cell.

Table 3 | **Regulatory T-cell-based immunotherapy in humans**

Regulatory T-cell subset		Method		Potential application
Pros	**Cons**	**Selection**	**Expansion/induction *in vitro***	
FOXP3*$^+$*CD4*$^+$*CD25*$^+$ *regulatory T cells				
• Circulating cells • Defined surface markers • Proven efficacy in several preclinical models	• Low numbers in the circulation • Multiple antigen specificity • Risk of pan immunosuppression	• Two-step procedure with magnetic beads to enrich for CD4$^+$CD25$^+$ cells • Further selection for low CD127 expression (?)	• Beads coated with CD3- and CD28-specific antibody plus IL-2 and rapamycin • Antigen specific (?)	• GVHD (clinical trial ongoing), autoimmunity, organ transplantation (?), IPEX (?)
T regulatory type 1 cells				
• Inducible *ex vivo* • Antigen specific • Proven efficacy in several preclinical models	• Hard to purify	• Not feasible	• IL-10 with or without IFNα • IL-10-treated dendritic cells • Tolerogenic dendritic cells	• GVHD (clinical trial ongoing), organ transplantation, autoimmunity

GVHD, graft-versus-host disease; IFNα, interferon-α; IL, interleukin; IPEX, immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome.

the rejection of allogeneic solid-organ transplantation. Several studies indicate that T_{Reg} cells generated *in vivo* in transplanted animals by various approaches (such as by treatment with vitamin D_3 and mycophenolate mofetil[39]) do transfer tolerance in secondary transplant recipients. However, the lack of data proving efficacy after transfer of freshly isolated T_{Reg} cells might be due to unreported unsuccessful experiments and/or to the requirement of antigen-specific T_{Reg} cells for protection. Alloantigen-specific T_{Reg} cells may be absent in freshly isolated cells but may be generated upon *in vivo* expansion in the presence of specific alloantigens. Positive results generated with the transfer of antigen-specific transgenic T_{Reg} cells are consistent with this view[40]. By contrast, adoptive transfer of T_{Reg} cells polyclonally expanded *ex vivo* with rapamycin, which blocks the proliferation of effector T cells while sparing T_{Reg} cells, promotes tolerance to allogeneic pancreatic islet grafts[41], suggesting that the antigen specificity of T_{Reg} cells is dispensable in this model. At present, the lack of data clearly showing that transferred T_{Reg} cells protect from allograft rejection in several preclinical animal models represents a major hurdle to the use of T_{Reg}-cell-based immunotherapy in solid-organ transplantation.

We have shown that T_R1 cells generated *in vivo*, in mice transplanted with allogeneic pancreatic islets and treated with rapamycin and IL-10, transfer antigen-specific tolerance to secondary transplant recipients[42]. Antigen-specific T_R1 cells can therefore be envisaged as a possible approach for preventing allograft rejection in humans.

Therapy with regulatory T cells in humans

Despite the data generated in preclinical animal models that successfully show that regulatory T cells can prevent or cure several T-cell-mediated diseases, many questions remain to be addressed for the translation of this approach to the clinic. This section addresses which human regulatory T-cell subset should be used, which method for its selection, expansion and induction should be adopted, and in which disease might regulatory T cells function successfully (TABLE 3).

Which regulatory T-cell subset should be used for adoptive immunotherapy? Several subsets of regulatory T cells have been described so far and potentially each of these subsets can be considered for clinical trials. Here, we focus on T_{Reg} cells and T_R1 cells and indicate that their use has both advantages and disadvantages. T_{Reg} cells are circulating cells with a defined phenotype (CD4$^+$CD25$^+$CD127^{low}) that allows for their isolation and purification. Furthermore, T_{Reg} cells have been tested in several preclinical animal models and have been shown to provide active and specific regulation *in vivo*. The major hurdles in using T_{Reg} cells however is their low numbers in the circulation and their broad and still poorly defined antigen specificity, as this may cause pan immunosuppression when they are transferred *in vivo*. Both these shortcomings could be overcome by the strategies discussed below.

T_R1 cells have the advantage, over T_{Reg} cells, of being inducible *ex vivo* and therefore cells of the desired antigen specificity can be more easily generated. The major limitation of this subset is that it is difficult to purify T_R1 cells owing to a lack of specific cell-surface markers. T-cell lines that are highly enriched for T_R1 cells can be obtained but contamination with non-regulatory T cells might represent a significant caveat. Furthermore, it is unclear whether the phenotype of T_R1 cells generated *in vitro* is stable once they are transferred *in vivo* in humans. Importantly, T_R1 cells produce the immunosuppressive cytokine IL-10 only after activation with their specific antigen, but once activated, they can mediate some level of bystander suppression[43]. Bystander suppression would be limited to the site where T_R1 cells localize, as no pan immunosuppression has been observed in mouse models or patients with high numbers of circulating T_R1 cells[21,42]. Therefore, one could envisage generating T_R1 cells specific for an antigen that is not related to the disease, with the assumption that once T_R1 cells are administered to the patients they could be activated and suppress pathogenic T cells with different antigen specificity. This might represent an interesting strategy, especially when the antigen causing the disease is unknown. However, the presence of an antigen capable of activating regulatory T cells

Homeostatic proliferation
Spontaneous proliferation of T cells in lymphopenic conditions, caused by chronic diseases or treatments such as thymectomy or irradiation. Factors that support T-cell homeostatic proliferation include peptide–MHC–TCR interactions and cytokines such as interleukin-7 and interleukin-15.

Rapamycin
An immunosuppressive drug that, in contrast to calcineurin inhibitors (such as cyclosporin A and FK506), does not prevent T-cell activation but blocks interleukin-2-mediated clonal expansion by blocking mTOR (mammalian target of rapamycin). It does not interfere with the function and expansion of naturally occurring regulatory T cells.

Bystander suppression
Suppression in which responses to a second, unrelated antigen can be inhibited when it is presented together with the antigen towards which tolerance has been already established.

is required, as shown by preclinical studies in which mice receiving ovalbumin-specific T_R1 cells generated *ex vivo* were cured from inflammatory bowel disease or experimental autoimmune encephalomyelitis (a mouse model of multiple sclerosis) only after administration of ovalbumin[2,44].

Which method should be used to select, expand and differentiate regulatory T cells? The isolation of human T_{Reg} cells from standard leukapheresis products by using a two-step magnetic cell-separation protocol performed under conditions of good manufacturing practice (GMP) has been shown to be feasible[45]. T_{Reg} cells represent only 5–10% of the peripheral-blood $CD4^+$ T cells and therefore, once isolated, they need to be expanded without the loss of their regulatory properties. The reduced ability of T_{Reg} cells to proliferate *in vitro* can be reversed by potent stimulation through the TCR in the presence of high doses of IL-2. Such expanded T_{Reg} cells maintain expression of relevant lymph-node-homing receptors, such as CD62 ligand (CD62L) and CC-chemokine receptor 7 (CCR7), and suppressive activity[46]. However, these cell-culture conditions are also highly advantageous for the expansion of effector T-cell populations, which can contaminate the purified T_{Reg}-cell population. In humans, even the $CD4^+CD25^{hi}$ T-cell subset contains ~50% of *in vivo* recently activated T cells, which ultimately outgrow T_{Reg} cells after prolonged culturing *in vitro*[47]. We believe that, to this day, the risk of co-expanding potentially harmful cells is the greatest concern regarding the clinical use of *ex vivo* expanded T_{Reg} cells. We have shown that the addition of rapamycin to the culture media significantly reduces the undesired expansion of effector T cells, as rapamycin selectively allows the proliferation of T_{Reg} cells but inhibits the proliferation of effector T cells[48]. Alternatively, a more sophisticated isolation procedure might limit contamination with effector T cells; the expression of CD127 can, for example, discriminate between $CD25^+$ activated ($CD127^+$) and regulatory ($CD127^{low/-}$) T cells[49,50] and might prove to be a useful marker for cell purification.

Although a polyclonal population of bona fide naturally occurring T_{Reg} cells can now be isolated and expanded, one of the remaining questions is whether their suppressive function would be antigen specific *in vivo*. Growth of antigen-specific T_{Reg} cells could be a preferable alternative to polyclonal expansion. However, alloantigen-specific human T_{Reg} cells have been described in only one study, in which human T_{Reg}-cell lines were generated by repetitive stimulation of $CD4^+CD25^+$ T cells with autologous dendritic cells (DCs) pulsed with HLA-derived allogeneic peptides[51]. Whether *ex vivo* expansion of human T_{Reg} cells specific for antigens other than alloantigens is possible is yet to be determined. Realistically, on the basis of the currently available data and technology, we believe that the use of polyclonal expanded T_{Reg} cells is the only approach that can currently be proposed for imminent clinical trials.

Several experimental protocols for the generation of antigen-specific human T_R1 cells have been published and are described elsewhere[52]. The only currently available GMP protocol to generate antigen-specific T_R1 cells for infusion after allogeneic BMT is the one described and used by our group. In this protocol, donor PBMCs are cultured *ex vivo* with host PBMCs (depleted of $CD3^+$ T cells and irradiated) in the presence of IL-10. After 10 days, donor T cells become anergic to the host antigens, while preserving the ability to proliferate in response to third party and recall antigens. Importantly, these IL-10-anergized T cells are enriched in alloantigen-specific T_R1-cell precursors that can be re-infused into the patients at the time of donor-cell engraftment to prevent GVHD (R. Bacchetta and M.-G.R., unpublished observations). We are now in the process of developing a more efficient GMP protocol in which monocyte-derived host DCs differentiated in the presence of IL-10 are used to stimulate donor $CD4^+$ T cells. The resulting anergic $CD4^+$ T-cell lines contain 5–10% alloantigen-specific T_R1 cells and are highly suppressive *in vitro* (S. Gregori and M.-G.R., unpublished observations).

For which disease should cellular therapy be used? Allogeneic BMT (or HSCT) is the first clinical setting in which cellular therapy with T_R1 cells has been applied and in which expanded T_{Reg} cells will be tested, with the aim of preventing acute GVHD. There are several reasons why acute GVHD has been selected as the initial clinical target for testing the feasibility and safety of regulatory T-cell immunotherapy. First, regulatory T cells expanded from peripheral blood have a sufficient number of alloreactive T cells. Second, regulatory T cells derived from a donor exhibit normal replicative potential as they have not been exposed to immunosuppressive treatment. Third, regulatory T cells can be administered before or at the same time as effector T-cell activation induced by alloantigens. Fourth, acute GVHD has a predictable time of onset, which is usually within 100 days after transplantation, and regulatory T-cell immunotherapy could therefore be timed to prevent or cure active disease. Fifth, the severe lymphopenia of the host following pre-transplant conditioning and post-transplant immunosuppressive treatment can favour the expansion of regulatory T cells, thereby reducing the number of cells required for a therapeutic effect. However, some caveats temper the enthusiasm for testing regulatory T cells in the prevention of acute GVHD. The alloresponse after transplantation is complicated by an intense inflammatory reaction with a 'cytokine storm' that might impede regulatory T-cell immunotherapy. Furthermore, most patients receive GVHD prophylaxis after transplantation and it is now well established that some immunosuppressive compounds interfere with regulatory T-cell activity[53]. The choice of drugs is therefore critical to avoid any *in vivo* antagonism of regulatory T cells. Rapamycin is of significant interest in this regard as it preserves the function of adoptively transferred T_{Reg} cells *in vivo*[54]. One can therefore envisage replacing the standard immunosuppressive treatment of GVHD with rapamycin, or presumably with any other non-calcineurin inhibitors, in conjunction with *ex vivo* expanded T_{Reg} cells.

Leukapheresis
A laboratory procedure for separating high numbers of leukocytes from peripheral blood.

Cytokine storm
A strong systemic immune response that results in the release of more than 150 inflammatory mediators (cytokines, oxygen free radicals and coagulation factors). Both pro-inflammatory cytokines (such as tumour-necrosis factor, interleukin-1 (IL-1) and IL-6) and anti-inflammatory cytokines (such as IL-10 and IL-1 receptor antagonist) are elevated in the serum of patients experiencing a cytokine storm.

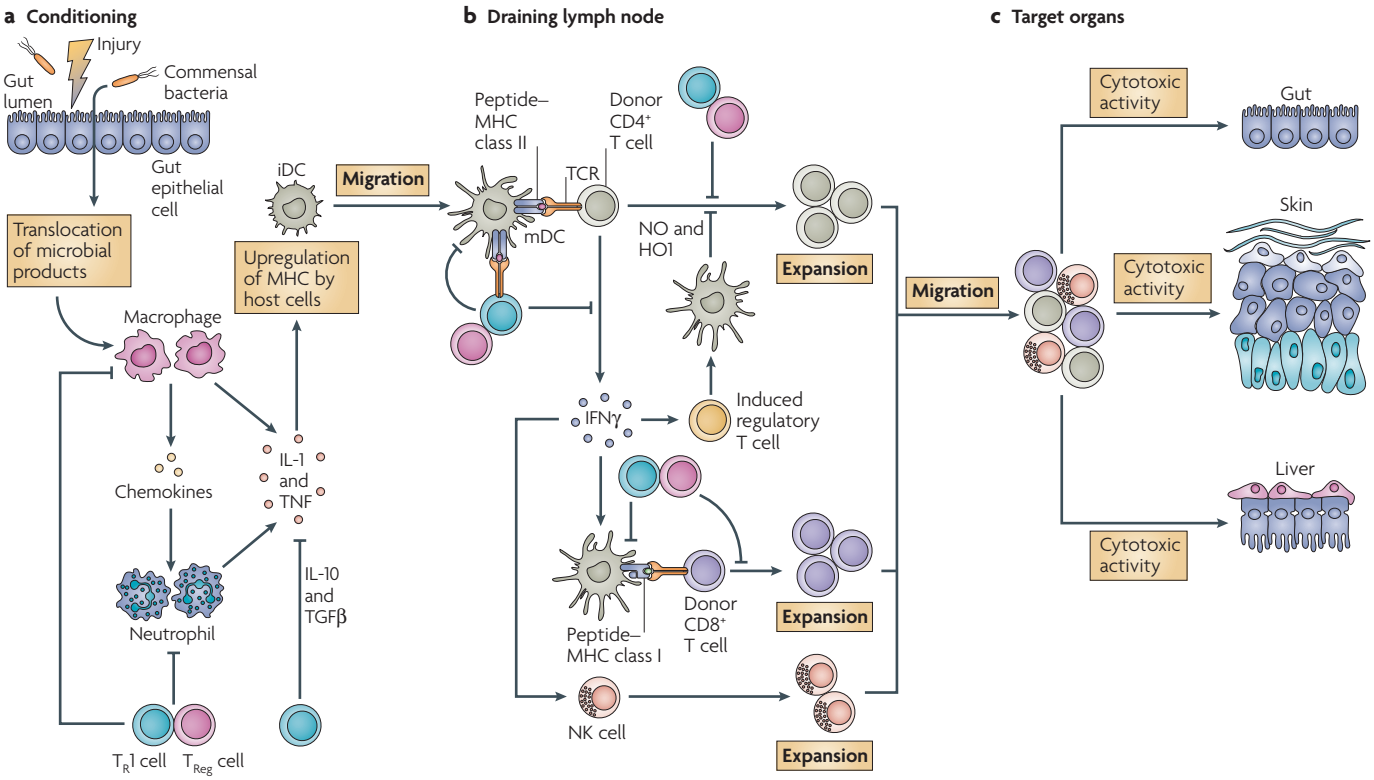

Figure 1 | **Pathogenesis of graft-versus-host disease and control by regulatory T cells.** The pathophysiology of acute graft-versus-host disease (GVHD) can be envisaged as a three step process. **a** | The first step occurs before donor-cell infusion. Prior to haematopoietic stem-cell transplantation (HSCT), the conditioning regimen (that is, irradiation and/or chemotherapy) leads to damage and activation of host tissues, especially the intestinal mucosa. This allows the translocation of microbial products, such as lipopolysaccharide, from the intestinal lumen to the circulation, which stimulates the secretion of pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumour-necrosis factor (TNF), from host tissues (particularly from macrophages). Activated macrophages produce chemokines that activate neutrophils, which further increases inflammation. The release of these pro-inflammatory cytokines increases the expression of MHC and adhesion molecules on host cells, enhancing their antigen-presenting capacity. **b** | The second step is characterized by activation of donor T cells, which occurs mainly in the lymph nodes that drain GVHD target organs, such as the gut, skin and liver. Donor T-cell activation results mainly in interferon-γ (IFNγ) production, which further increases the expression of MHC and adhesion molecules, chemokines and CD95 on antigen-presenting cells (APCs). This results in further increases in antigen presentation and recruitment and the expansion of host-specific cytotoxic $CD8^+$ and $CD4^+$ T cells, and natural killer (NK) cells. **c** | In the final step, effector cells then migrate to the target organs, where they mediate tissue injury that leads to multi-organ failure mediated mainly by the CD95–CD95 ligand and the perforin–granzyme pathways. Regulatory T cells can intervene at different stages to control GVHD. It is likely that regulatory T cells, like effector T cells, are first activated in the draining lymph nodes. Both naturally occurring forkhead box P3 $(FOXP3)^+CD4^+CD25^+$ regulatory T cells (T_{Reg} cells) and T regulatory type 1 (T_R1) cells, although through different mechanisms, block activation and expansion of effector T cells and/or modulate the functions of APCs, monocytes, macrophages and neutrophils. Consequently, activation and proliferation of alloreactive donor effector T cells is suppressed, resulting in diminished export from the lymph nodes to the target organs. In addition, T_R1 cells, through IL-10 and transforming growth factor-β (TGFβ) production, efficiently reduce the inflammatory state. IFNγ produced in the presence of inducible regulatory T cells leads to the expression of inducible nitric-oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO) with consequent production of NO and haem oxygenase 1 (HO1), which modulate effector T-cell functions. iDC, immature DC; mDC, mature DC; TCR, T-cell receptor.

Alternatively, regulatory T-cell immunotherapy could be carried out at later time points after transplantation to control chronic GVHD. An obvious drawback to this, however, is that the infused regulatory T cells would have to modulate an ongoing allogeneic T-cell response. A benefit, however, is that this application would circumvent the cytokine storm, which should be fully resolved in most of the patients, and this would presumably provide a more permissive environment for regulatory T-cell function.

Should studies carried out with the adoptive transfer of regulatory T cells to prevent GVHD prove to be safe, systemic autoimmunity is likely to be the next clinical setting in which the efficacy of regulatory T-cell immunotherapy will be tested. As described, preclinical models have clearly shown that polyclonal regulatory T cells can prevent autoimmune diseases, whereas only self-antigen-specific regulatory T cells can cure active autoimmunity. Therefore, antigen-specific regulatory

T cells can hypothetically be generated for the treatment of autoimmune diseases in which the target antigen has been identified. Insulin and GAD65 have been recognized as crucial target antigens in type 1 diabetes, myelin basic protein in multiple sclerosis, and type II collagen in rheumatoid arthritis. In type 1 diabetes, several preclinical models have shown that the transfer of regulatory T cells can block disease development. Individuals at risk of developing type 1 diabetes can now be efficiently recognized[55] and autologous regulatory T cells could be collected or differentiated before disease onset and stored until required. Early intervention when residual β-cell islet function is still present, could also be possible and may be beneficial.

Solid-organ transplantation might also benefit from a non-toxic regulatory T-cell-based immunotherapy for the induction of donor-specific tolerance and for the reduction of the requirement for constant immunosuppression. In patients with pancreatic islet transplants, cell therapy with alloantigen-specific T_R1 cells in combination with immunosuppressive regimens that are permissive for their *in vivo* expansion is currently under investigation at the San Raffaele Scientific Institute.

Finally, patients with IPEX possibly represent the best candidates for testing the efficacy of regulatory T-cell immunotherapy in humans. Indeed, scurfy mice, which develop a related syndrome as a result of a loss-of-function *Foxp3* mutation, can be rescued from lethality by injection of neonates with FOXP3-expressing T_{Reg} cells. However, contrary to mice, in humans the FOXP3 defect does not seem to be only restricted to T_{Reg} cells[25], and therefore the transfer of functional T_{Reg} cells alone might not represent a definitve curative therapeutic option. A better understanding of the immune mechanisms underlying IPEX needs to be gained before proposing T_{Reg}-cell-based immunotherapy for patients with this disease.

Possible mechanisms of regulatory T-cell therapy

To control complex immune-mediated diseases, such as acute GVHD after HSCT and autoimmune diabetes, the immune system needs to be modulated at different levels. Here we describe the known immunological events that occur in the two abovementioned pathological settings in an attempt to delineate, based on our present knowledge, the level at which regulatory T cells could intervene.

Regulatory T cells and the control of acute GVHD. The pathophysiology of acute GVHD after HSCT can be considered as a three step process in which both the innate and adaptive immune systems interact (FIG. 1). As draining lymph nodes are the sites of donor T-cell priming by host antigen-presenting cells (APCs), it seems probable that protection from GVHD by adoptively transferred regulatory T cells might also occur at these sites. Therefore, an important prerequisite for regulatory T cells to function is their ability to migrate to the correct site where they can encounter alloantigens presented by host APCs and be activated[34,56]. Early robust expansion of regulatory T cells in draining lymph nodes followed by their migration and localization to peripheral tissues was reported in mice[57], further supporting this hypothesis. After activation, T_{Reg} cells exert their suppressive function by inhibiting effector T-cell proliferation, cytokine production and migration. T_{Reg} cells also downmodulate DC function by preventing their maturation in a cell-contact-dependent manner[58]. T_{Reg} cells can also influence monocyte and macrophage function by inhibiting lipopolysaccharide-induced monocyte survival through the CD95–CD95 ligand apoptotic pathway[59]. Alternatively, T_{Reg} cells restrain monocytes by reducing their activation state, and this leads to reduced pro-inflammatory cytokine production and impaired APC function[60]. Interestingly, T_{Reg} cells also inhibit neutrophil activity and promote their apoptosis[61]. T_R1 cells, by producing IL-10, induce anergy in effector T cells and affect APC function by downregulating the expression of MHC and co-stimulatory molecules. In addition, IL-10 promotes the secretion of IL-1 receptor antagonist and soluble tumour-necrosis factor receptor rather than pro-inflammatory cytokines by APCs.

Of particular interest is the observation that both interferon-γ (IFNγ) and nitric oxide (NO), which are produced at high levels during GVHD, have paradoxical functions[62]. The pathogenic role of IFNγ as a T helper 1 (T_H1)-cell-associated cytokine that is involved in the development of immune-mediated diseases has been well documented[63]. Similarly, NO is a cytotoxic molecule that may contribute to immune-mediated diseases. However, when regulatory T cells are present, these two molecules acquire immunoregulatory activity[62]. In the presence of regulatory T cells, IFNγ stimulates APCs to produce NO and haem oxygenase 1 (HO1) through increased expression of the enzymes inducible nitric-oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO), which degrades the essential amino acid tryptophan and markedly affects T-cell proliferation and survival. NO then can diffuse into neighbouring T cells, influencing their function and triggering apoptosis, whereas HO1 prevents T-cell proliferation and modulates T-cell activation through the degradation of the pro-oxidant haem into carbon monoxide, iron and biliverdin (reviewed in REF. 62).

Regulatory T cells and the control of autoimmune diabetes. Type 1 diabetes is a tissue-specific autoimmune disease in which inflammation plays a central role (FIG. 2). The mechanisms through which regulatory T cells can function *in vivo* to block the development of type 1 diabetes are under intense investigation. The model that we propose is based on data generated mainly in the mouse models, and its clinical significance is yet to be proved. T_{Reg} cells present in the pancreatic draining lymph nodes regulate the priming of autoreactive T cells by limiting their expansion and differentiation. Interestingly, T_{Reg} cells may function by interrupting the development of effector T cells through limiting the access of autoreactive T cells to DCs[64]. Thanks to the control of this crucial step in lymph-node priming, T_{Reg} cells limit the chance of a T cell becoming an effector cell. T_{Reg} cells inhibit the expression of CXC-chemokine receptor 3 (CXCR3) by T_H cells with a consequent lack of infiltration of these cells into the pancreatic islets[65]. Furthermore,

Scurfy mice
Loss-of-function mutations of *Foxp3* in scurfy mice inhibit the development of naturally occurring regulatory T cells, resulting in a highly dysregulated immune system and consequent aggressive autoimmunity. These mice show hyperproduction of cytokines and increased numbers of memory T cells.

CD95–CD95 ligand apoptotic pathway
CD95 ligand (also known as FAS ligand) binds to CD95 (FAS), which results in the formation of the death-inducing signalling complex and subsequent activation of caspases. Donor $CD4^+$ T cells with killing capability preferentially use this pathway during acute graft-versus-host disease.

Interleukin-1 receptor antagonist
(IL-1RA). A secreted protein that binds to IL-1R, thereby blocking IL-1R downstream signalling. IL-1RA inhibits the pro-inflammatory properties of IL-1α and IL-1β.

Nitric oxide
(NO). A small molecule synthesized, mainly in macrophages, from arginine by nitric oxide synthase (NOS) enzymes. Increased levels of NO are found in inflammatory and autoimmune diseases, and during allograft rejection. It is the effector cytotoxic molecule responsible for macrophage-mediated cytotoxicity but can also suppress T-cell proliferation.

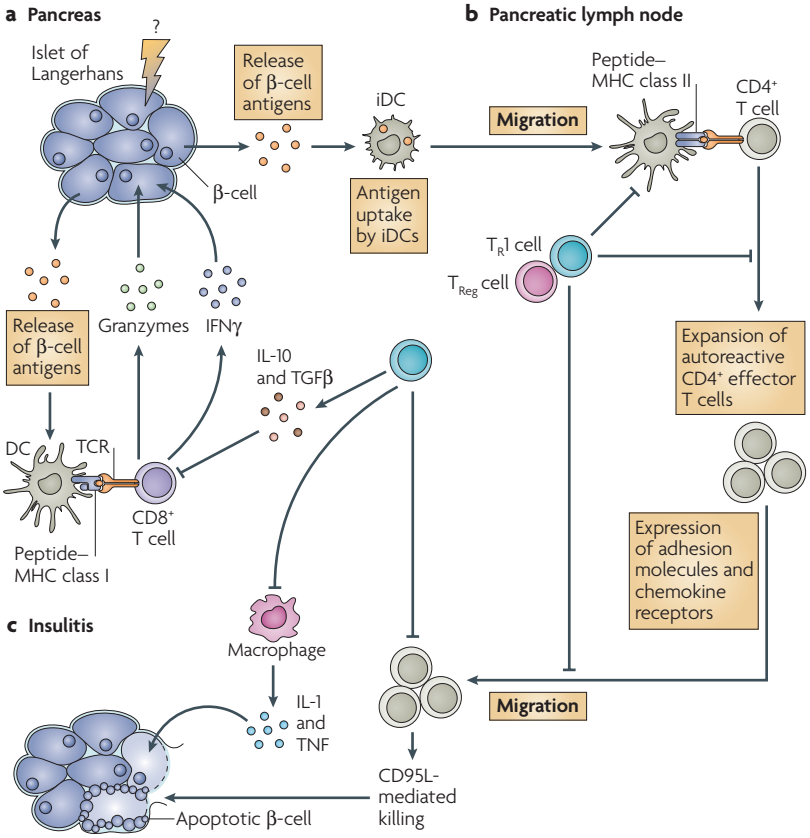

Figure 2 | **Pathogenesis of type 1 diabetes and control by regulatory T cells.** As for graft-versus-host disease, the pathophysiology of type 1 diabetes can be envisaged as a three step process. **a** | First, under still undefined pathogenic conditions, modified islet β-cell antigens are released and presented by MHC class I molecules. These previously 'cryptic' antigens are presented by tissue-resident antigen-presenting cells (APCs) and recognized by $CD8^+$ T cells that cause damage to MHC-class-I-expressing cells either through the release of cytotoxic cytokines (such as interferon-γ (IFNγ)) or through the perforin–granzyme pathway. **b** | The released islet β-cell components are taken up by immature dendritic cells (iDCs) in the pancreatic islets and transported to the draining pancreatic lymph nodes, where the antigens are processed and presented to $CD4^+$ T cells. Lymph-node priming is thought to be the second crucial step leading to expansion of low frequency circulating autoreactive T cells. After clonal expansion, $CD4^+$ effector T cells express adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and lymphocyte function-associated antigen 1 (LFA1), and chemokine receptors, such as CC-chemokine receptor 4 (CCR4), CCR5 and CXC-chemokine receptor 3. This allows the effector cells to home to the pancreatic islets, tracing antigen gradients and chemokines induced by the early $CD8^+$ T-cell-mediated inflammatory response. **c** | Once in the pancreas, the activated $CD4^+$ T cells recruit and activate inflammatory cells, causing insulitis. The effector phase of islet β-cell destruction is mediated by cytokines (mainly interleukin-1 (IL-1) and tumour-necrosis factor (TNF)) through the induction of pro-apoptotic signalling selectively in islet β-cells and/or by inducing the expression of CD95 by islet β-cells, which allows direct killing by CD95 ligand (CD95L)-expressing effector T cells. There is also evidence that the production of free radicals is involved in the pathogenic events leading to islet β-cell destruction (not shown). Regulatory T cells can intervene at different stages to control type 1 diabetes. As in graft-versus-host disease, it is likely that regulatory T cells are first activated in the pancreatic lymph nodes. Activated naturally occurring forkhead box P3 $(FOXP3)^+CD4^+CD25^+$ regulatory T (T_{Reg}) cells and T regulatory type 1 (T_R1) cells, through distinct regulatory mechanisms, block the activation and expansion of effector T cells either directly or indirectly through APCs. Expression of adhesion molecules and chemokine receptors by effector T cells is also suppressed by regulatory T cells, with consequent reduced effector T-cell migration to the target organ. The aggressiveness of insulitis is also directly inhibited in the pancreas by regulatory T cells. T_R1 cells, through IL-10 and transforming growth factor-β (TGFβ) production, can inhibit the onset of disease and reduce inflammation. TCR, T-cell receptor.

T_{Reg} cells might restrain autoimmune aggression directly in the islets by controlling the inflammatory reaction (insulitis)[66]. T_R1 cells produce IL-10 and TGFβ, which both have an important immunoregulatory role in type 1 diabetes[67]. Transient expression of TGFβ in the islets during the priming phase of diabetes inhibits the onset of disease and stimulates the expansion or generation of intra-islet FOXP3-expressing T_{Reg} cells[68]. IL-10 modulates APC function, reduces inflammation and decreases T-cell activation. Furthermore, IL-10 produced by T_R1 cells downregulates the expression of intercellular adhesion molecule 1 (ICAM1) on effector T cells, which prevents their migration to the target organ[69] (M.B. and M.-G.R., unpublished observations). Finally, the paradoxical effects of IFNγ and NO that are thought to be important for the immunomodulatory effects of regulatory T cells during GVHD might also have a role in autoimmune diabetes[70].

Ongoing clinical trials with regulatory T cells

At present, clinical trials of immunotherapy based on regulatory T cells are ongoing in BMT for the prevention or cure of GVHD. A cellular therapy clinical trial that involves the transfer of IL-10-anergized T_R1 cells to patients with haematological cancers who are treated with HLA-haploidentical HSCT is underway. In this trial, PBMCs are collected from both the donor (before stem-cell mobilization) and the host (before conditioning). Subsequently, high doses of purified T-cell-depleted $CD34^+$ HSCs are infused into the myeloablated host. Once there are signs of neutrophil engraftment, donor PBMCs are thawed and cultured *ex vivo* in the presence of irradiated host PBMCs and IL-10. IL-10-anergized donor T cells are then infused at increasing doses (starting from 10^5 per kg of body weight) into the host with the ultimate goal of providing immune reconstitution without GVHD. The cell preparation contains donor T cells that are anergic to host antigens and that are enriched in precursors of host-specific T_R1 cells, but it also includes memory T cells that can respond to infectious agents and possibly provide a graft-versus-leukaemia (GVL) effect[71]. This clinical trial is currently being carried out in patients undergoing HLA-haploidentical HSCT but it has the potential to be extended to include patients undergoing allogeneic unrelated BMT or pancreatic islet transplantation, in which there is high risk of GVHD and graft rejection, respectively.

The first clinical trial with T_{Reg} cells is ongoing in Regensburg, Germany, and is led by M. Edinger. In this study, patients with high risk of cancer relapse or molecular relapse after allogeneic HSCT receive a pre-emptive donor lymphocyte infusion (DLI) after cessation of treatment with the immunosuppressant cyclosporin A. Eight to ten weeks after immunosuppressive treatment is withdrawn, patients receive between 1×10^6 and 5×10^6 freshly isolated donor T_{Reg} cells per kg of body weight followed by DLI of equal T-cell numbers. The data generated so far in five treated patients indicate that the isolation of sufficient numbers of T_{Reg} cells from donor blood is feasible, and no acute toxicities or other adverse events or complications (such as opportunistic

infections, GVHD and disease relapse) have been reported (M. Edinger, personal communication). This trial will provide crucial information on the purification procedure for T_{Reg} cells and on the safety of adoptive transfer with unmanipulated T_{Reg} cells. Another trial, designed by B. Blazar and C. June (University of Minnesota, USA), will start this year. In this study, donor T_{Reg} cells expanded with beads coated with CD3- and CD28-specific antibodies and high doses of IL-2 will be infused into the transplant recipient together with the HSCT (B. Blazar, personal communication). This trial will provide valuable information on the transfer of polyclonal T_{Reg} cells expanded *ex vivo*. Hopefully, these three clinical studies will pave the way for the future of regulatory T-cell-based immunotherapy in other diseases.

Strengths and pitfalls of regulatory T-cell therapy

Strengths. The administration of immunosuppressive drugs is the most common approach for treating immune-mediated diseases. However, the long-term administration of non-antigen-specific agents that cannot distinguish between beneficial and destructive immune responses is a major drawback. Furthermore, immunosuppressive treatment must be lifelong, as, if withdrawn, there is high risk of disease relapse due to lack of tolerance induction. By contrast, regulatory T cells are physiological components of the immune system and their adoptive transfer should re-establish the immunological homeostasis altered under pathological circumstances. Immunotherapy with T_R1 cells can, for example, be envisaged as an *in vivo* transfer of a 'biological source' of IL-10, which, if administered exogenously as a recombinant protein, would probably not have the same therapeutic effects. Indeed, we have shown that administration of exogenous IL-10 alone in a preclinical model of allogeneic pancreatic islet transplantation does not block allograft rejection, whereas adoptive transfer of antigen-specific T_R1 cells, which produce IL-10 upon antigen encounter, prevents rejection and induces long-term tolerance[42].

Importantly, regulatory T-cell-based therapy is customized to the patient and can therefore be created to satisfy the specific needs of the patient with limited side effects. This is, however, a double-edged sword, as T-cell products cannot be manufactured and distributed easily and freely as is the case for standard medicinal products and can be prohibitively expensive in their current experimental phase.

Pitfalls. The obstacles that limit regulatory T-cell-based immunotherapy at present are mainly technical and relate to cell manipulation. The regulatory T cells must be collected from the peripheral blood, for example by leukapheresis, and immediately processed or cryopreserved when necessary. Circulating T_{Reg} cells can be directly isolated from the circulating pool of $CD4^+$ T cells but because of their limited number they need to be further expanded *in vitro*. T_R1 cells are generated from $CD4^+$ T cells, which need first to be isolated and primed *in vitro*. Cell isolation and manipulation are therefore prerequisite for regulatory T-cell-based immunotherapy. Several

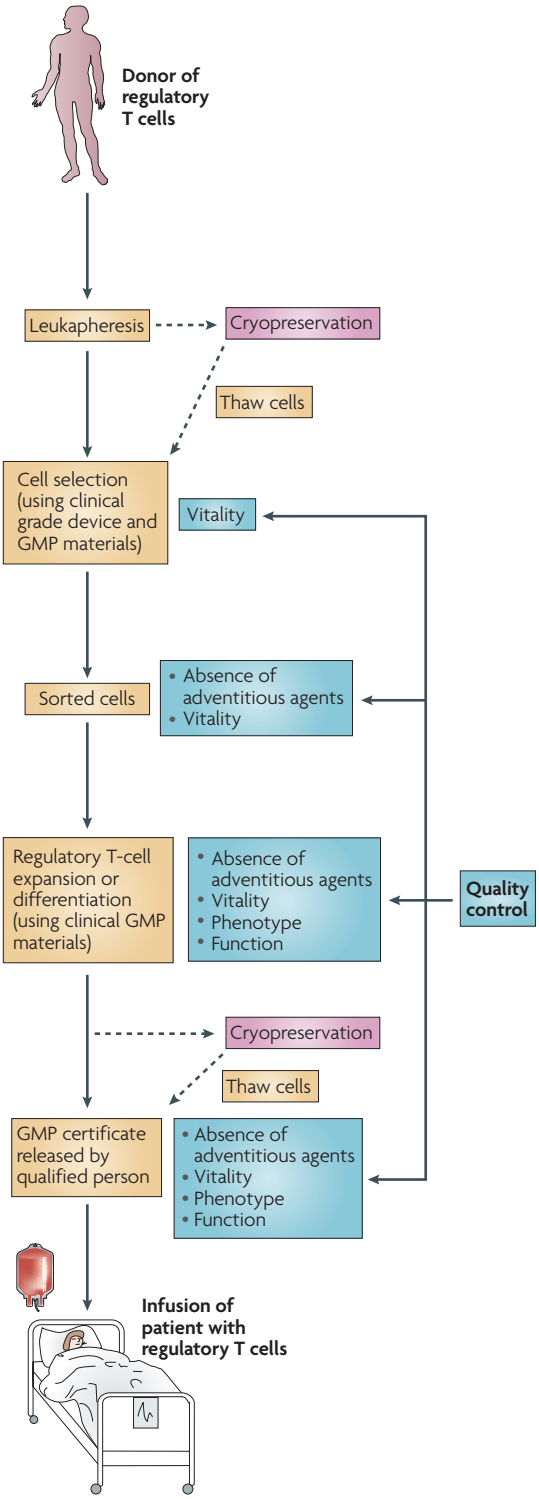

Figure 3 | **Development and assessment of human cell therapy products.** The steps necessary for the development of regulatory T cells as a medicinal product are shown. From cell collection to cell processing, expansion and differentiation, and final infusion into the patient, clinical good manufacturing practice (GMP) procedures need to be performed. Various quality controls are required in each manufacturing step and the final product can be released and infused into the patient only after approval by a qualified person.

Graft-versus-leukaemia (GVL). An immune response mounted by the transplanted cells against the tumour cells of the host and it is one of the reasons that allogeneic transplants can be curative for cancer.

Donor lymphocyte infusion (DLI). Delayed administration of peripheral donor lymphocytes after T-cell-depleted bone-marrow transplantation in cancer patients. DLI plays a central role in both attacking the tumour cells and providing immune reconstitution. However, its use is limited by the risk of severe acute and chronic graft-versus-host disease.

other points need to be considered for the development of regulatory T cells as a medicinal product and many different quality controls are needed (FIG. 3).

Cell isolation, manipulation, expansion and re-infusion into the patients are all procedures that need to be performed under GMP conditions. As a result, this therapeutic approach is extremely expensive in its experimental phase and also requires certified GMP facilities. It is therefore unavoidable that only a few institutions can provide all the necessary infrastructure to make regulatory T-cell-based therapy a reality. However, should this immunotherapy meet its therapeutic targets, we might envisage that not only academic institutions but also pharmaceutical companies will be interested in adopting this therapeutic approach.

Safety of the infused *ex vivo* manipulated product is clearly a high priority. The risk of uncontrolled cell proliferation, pan immunosuppression and consequent tumour development should be carefully monitored. Furthermore, superior efficacy of regulatory T-cell-based immunotherapy over conventional therapy should be clearly demonstrated.

Current picture and future perspectives

Preliminary results from clinical trials with freshly *ex vivo* isolated T_{Reg} cells and *ex vivo* generated T_R1 cells in patients undergoing BMT show feasibility and suggest a good safety profile. Efficacy data will hopefully be generated in the coming years. These proof-of-principle clinical trials in HSCT will pave the way for further clinical studies in patients with genetic and acquired autoimmune diseases. However, these diseases may intrinsically have a higher level of complexity: they are not life threatening and therefore the risk–benefit assessment should be more stringent; the patients are not lymphopenic but instead may have a high number of circulating activated effector T cells; and the regulatory T cells will need to suppress memory autoreactive T cells in an inflammatory environment.

In some clinical settings, *ex vivo* expansion of T_{Reg} cells will be necessary to achieve a sufficient number of cells per kg of body weight. So far, sufficiently high numbers of T_{Reg} cells can be generated for preclinical studies but it remains to be determined whether the procedure can be scaled up under GMP conditions. In addition, the need for a patient-customized cell product prepared in GMP conditions may severely hamper a broad application of this cellular drug. However, proof of robust efficacy of this cellular product would generate increasing interest and investments to overcome these hurdles.

Regulatory T-cell-based immunotherapy should not be envisaged as an all-or-nothing approach to re-establish immunological tolerance on its own. As mentioned, pathological conditions such as GVHD and type 1 diabetes are multifactorial diseases and monotherapy risks being of limited success. We therefore propose that combining regulatory T-cell therapy with other therapeutic approaches might result in better efficacy. The selected compound should allow survival, expansion and function of regulatory T cells. Furthermore, it should prove of superior efficacy over monotherapy with regulatory T cells. Rapamycin has been shown to be effective in inducing the expansion of T_{Reg} cells and to be permissive for T_R1-cell induction by IL-10 (REF. 69). Similarly, other immunosuppressive compounds that do not act through the TCR signalling and calcineurin pathways may be combined with regulatory T-cell immunotherapy to downregulate inflammation and effector T-cell function.

In the future, *ex vivo* gene transfer with genes encoding proteins that induce or enhance regulatory T-cell function and confer regulatory activity to effector T cells can also be envisaged. Evidence that human T_{Reg} cells constitutively express high levels of FOXP3 and that mutations in *FOXP3* results in severe autoimmunity[25] clearly indicates that the expression of this transcription factor has a key role in regulatory T-cell function. It is therefore possible that ectopic expression of FOXP3 in non-regulatory T cells may represent an alternative therapeutic approach for the generation of stable and functional T cells with regulatory properties. Furthermore, the transformation of antigen-specific pathogenic T cells into regulatory T cells upon forced expression of FOXP3 might overcome all of the current limitations in generating antigen-specific T_{Reg} cells *ex vivo*. Engineering of antigen-specific pathogenic T cells with genes that encode immunosuppressive cytokines such as IL-10 and TGFβ could also be an interesting possibility. However, solid preclinical data supporting the safety and efficacy of these gene therapy approaches are required to propel it from the bench to the clinic.

1. Shevach, E. M. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* **25**, 195–201 (2006).
2. Groux, H. *et al.* A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389**, 737–742 (1997).
In this paper, both human and mouse IL-10-producing T_R1 cells were characterized for the first time.
3. Walker, M. R. *et al.* Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4$^+$ CD25$^-$ T cells. *J. Clin. Invest.* **112**, 1437–1443 (2003).
4. Sakaguchi, S., Setoguchi, R., Yagi, H. & Nomura, T. Naturally arising Foxp3-expressing CD25$^+$CD4$^+$ regulatory T cells in self-tolerance and autoimmune disease. *Curr. Top. Microbiol. Immunol.* **305**, 51–66 (2006).
5. Taams, L. S. *et al.* Antigen-specific T cell suppression by human CD4$^+$CD25$^+$ regulatory T cells. *Eur. J. Immunol.* **32**, 1621–1630 (2002).
6. Wing, K. *et al.* CD4 T cell activation by myelin oligodendrocyte glycoprotein is suppressed by adult but not cord blood CD25$^+$ T cells. *Eur. J. Immunol.* **33**, 579–587 (2003).
7. Danke, N. A., Koelle, D. M., Yee, C., Beheray, S. & Kwok, W. W. Autoreactive T cells in healthy individuals. *J. Immunol.* **172**, 5967–5972 (2004).
8. Kitani, A., Chua, K., Nakamura, K. & Strober, W. Activated self-MHC-reactive T cells have the cytokine phenotype of Th3/T regulatory cell 1 T cells. *J. Immunol.* **165**, 691–702 (2000).
9. Arif, S. *et al.* Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J. Clin. Invest.* **113**, 451–463 (2004).
This is the first study providing evidence that patients with type 1 diabetes have reduced numbers of circulating islet-specific T_R1 cells compared with HLA-matched healthy controls.
10. Baecher-Allan, C. & Hafler, D. A. Human regulatory T cells and their role in autoimmune disease. *Immunol. Rev.* **212**, 203–216 (2006).
11. Yudoh, K., Matsuno, H., Nakazawa, F., Yonezawa, T. & Kimura, T. Reduced expression of the regulatory CD4$^+$ T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis.*Arthritis Rheum.* **43**, 617–627 (2000).
12. Miura, Y. *et al.* Association of Foxp3 regulatory gene expression with graft-versus-host disease. *Blood* **104**, 2187–2193 (2004).
This study shows for the first time a significant reduction of *FOXP3* mRNA levels in PBMCs from patients with GVHD compared with those without GVHD.
13. Rezvani, K. *et al.* High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. *Blood* **108**, 1291–1297 (2006).

14. Zorn, E. *et al.* Reduced frequency of FOXP3$^+$CD4$^+$CD25$^+$ regulatory T cells in patients with chronic graft-versus-host disease. *Blood* **106**, 2903–2911 (2005).
15. Clark, F. J. *et al.* Chronic graft-versus-host disease is associated with increased numbers of peripheral blood CD4$^+$CD25^{high} regulatory T cells. *Blood* **103**, 2410–2416 (2004).
16. Meignin, V. *et al.* Numbers of Foxp3-expressing CD4$^+$CD25^{high} T cells do not correlate with the establishment of long-term tolerance after allogeneic stem cell transplantation. *Exp. Hematol.* **33**, 894–900 (2005).
17. Rieger, K. *et al.* Mucosal FOXP3$^+$ regulatory T cells are numerically deficient in acute and chronic GvHD. *Blood* **107**, 1717–1723 (2006).
18. Meloni, F. *et al.* Monocyte chemoattractant protein-1 levels in bronchoalveolar lavage fluid of lung-transplanted patients treated with tacrolimus as rescue treatment for refractory acute rejection. *Transplant Proc.* **35**, 1523–1526 (2003).
19. Demirkiran, A. *et al.* Low circulating regulatory T-cell levels after acute rejection in liver transplantation. *Liver Transpl.* **12**, 277–284 (2006).
20. Salama, A. D., Najafian, N., Clarkson, M. R., Harmon, W. E. & Sayegh, M. H. Regulatory CD25$^+$ T cells in human kidney transplant recipients. *J. Am. Soc. Nephrol.* **14**, 1643–1651 (2003).
21. Bacchetta, R. *et al.* High levels of interleukin 10 production *in vivo* are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J. Exp. Med.* **179**, 493–502 (1994).
This study describes the successful isolation of CD4$^+$ host-reactive T-cell clones from SCID patients transplanted with allogeneic HSCs, which produce high amounts of IL-10 in the absence of IL-4 after antigen-specific stimulation *in vitro*. The presence of these IL-10-producing CD4$^+$ T cells correlated with the absence of GVHD and long-term tolerance.
22. Baker, K. *et al.* High spontaneous IL-10 production in unrelated bone marrow transplant recipients is associated with fewer transplant-related complications and early deaths. *Bone Marrow Transplant.* **23**, 1123–1129 (1999).
23. Weston, L. E., Geczy, A. F. & Briscoe, H. Production of IL-10 by alloreactive sibling donor cells and its influence on the development of acute GVHD. *Bone Marrow Transplant.* **37**, 207–212 (2005).
24. VanBuskirk, A. M. *et al.* Human allograft acceptance is associated with immune regulation. *J. Clin. Invest.* **106**, 145–155 (2000).
25. Bacchetta, R. *et al.* Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J. Clin. Invest.* **116**, 1713–1722 (2006).
26. Marangoni, F. *et al.* WASP regulates suppressor activity of human and murine CD4$^+$CD25$^+$FOXP3$^+$ natural regulatory T cells. *J. Exp. Med.* **204**, 369–380 (2007).
27. Kriegel, M. A. *et al.* Defective suppressor function of human CD4$^+$ CD25$^+$ regulatory T cells in autoimmune polyglandular syndrome type II. *J. Exp. Med.* **199**, 1285–1291 (2004).
28. Bleesing, J. J. *et al.* Immunophenotypic profiles in families with autoimmune lymphoproliferative syndrome. *Blood* **98**, 2466–2473 (2001).
29. Mottet, C., Uhlig, H. H. & Powrie, F. Cutting edge: cure of colitis by CD4$^+$CD25$^+$ regulatory T cells. *J. Immunol.* **170**, 3939–3943 (2003).
30. Tang, Q. *et al. In vitro*-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J. Exp. Med.* **199**, 1455–1465 (2004).
31. Tarbell, K. V. *et al.* Dendritic cell-expanded, islet-specific CD4$^+$ CD25$^+$ CD62L$^+$ regulatory T cells restore normoglycemia in diabetic NOD mice. *J. Exp. Med.* **204**, 191–201 (2007).
32. Hanash, A. M. & Levy, R. B. Donor CD4$^+$CD25$^+$ T cells promote engraftment and tolerance following MHC-mismatched hematopoietic cell transplantation. *Blood* **105**, 1828–1836 (2005).
33. Joffre, O., Gorsse, N., Romagnoli, P., Hudrisier, D. & van Meerwijk, J. P. Induction of antigen-specific tolerance to bone marrow allografts with CD4$^+$CD25$^+$ T lymphocytes. *Blood* **103**, 4216–4221 (2004).
34. Taylor, P. A. *et al.* L-selectin^{hi} but not the L-selectin^{lo} CD4$^+$25$^+$ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood* **104**, 3804–3812 (2004).
35. Cohen, J. L., Trenado, A., Vasey, D., Klatzmann, D. & Salomon, B. L. CD4$^+$CD25$^+$ immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J. Exp. Med.* **196**, 401–406 (2002).
36. Hoffmann, P., Ermann, J., Edinger, M., Fathman, C. G. & Strober, S. Donor-type CD4$^+$CD25$^+$ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Exp. Med.* **196**, 389–399 (2002).
References 35, 36 and 96 are the first to show that the adoptive transfer of CD4$^+$CD25$^+$ regulatory T cells significantly delays or even prevents GVHD in preclinical mouse models of BMT.
37. Trenado, A. *et al.* Recipient-type specific CD4$^+$CD25$^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J. Clin. Invest.* **112**, 1688–1696 (2003).
38. Zeller, J. C. *et al.* Induction of CD4$^+$ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-β. *J. Immunol.* **163**, 3684–3691 (1999).
39. Gregori, S. *et al.* Regulatory T cells induced by 1α,25-dihydroxyvitamin D_3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J. Immunol.* **167**, 1945–1953 (2001).
40. Lee, M. K. 4th. *et al.* Promotion of allograft survival by CD4$^+$CD25$^+$ regulatory T cells: evidence for *in vivo* inhibition of effector cell proliferation. *J. Immunol.* **172**, 6539–6544 (2004).
41. Battaglia, M., Stabilini, A. & Roncarolo, M. G. Rapamycin selectively expands CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells. *Blood* **105**, 4743–4748 (2005).
In this study, we showed, for the first time, that rapamycin has the ability to allow the selective proliferation of T_{Reg} cells.
42. Battaglia, M. *et al.* Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* **55**, 40–49 (2006).
43. Waldmann, H., Adams, E., Fairchild, P. & Cobbold, S. Infectious tolerance and the long-term acceptance of transplanted tissue. *Immunol. Rev.* **212**, 301–313 (2006).
44. Barrat, F. J. *et al. 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.* **195**, 603–616 (2002).
45. Hoffmann, P. *et al.* Isolation of CD4$^+$CD25$^+$ regulatory T cells for clinical trials. *Biol. Blood Marrow Transplant.* **12**, 267–274 (2006).
46. Hoffmann, P., Eder, R., Kunz-Schughart, L. A., Andreesen, R. & Edinger, M. Large-scale *in vitro* expansion of polyclonal human CD4$^+$CD25^{high} regulatory T cells. *Blood* **104**, 895–903 (2004).
47. Levings, M. K. *et al.* Human CD25$^+$CD4$^+$ T suppressor cell clones produce transforming growth factor β, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J. Exp. Med.* **196**, 1335–1346 (2002).
48. Battaglia, M. *et al.* Rapamycin promotes expansion of functional CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J. Immunol.* **177**, 8338–8347 (2006).
49. Liu, W. *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4$^+$ T reg cells. *J. Exp. Med.* **203**, 1701–1711 (2006).
50. Seddiki, N. *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* **203**, 1693–1700 (2006).
51. Jiang, S., Camara, N., Lombardi, G. & Lechler, R. I. Induction of allopeptide-specific human CD4$^+$CD25$^+$ regulatory T cells *ex vivo*. *Blood* **102**, 2180–2186 (2003).
References 50 and 51 show that CD4$^+$CD25$^+$ T_{Reg} cells are CD127$^{low/-}$ and open new venues for a better isolation and purification of T_{Reg} cells.
52. Roncarolo, M. G. *et al.* Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol. Rev.* **212**, 28–50 (2006).
53. Battaglia, M. & Roncarolo, M. G. Induction of transplantation tolerance via regulatory T cells. *Inflamm. Allergy Drug Targets* **5**, 157–165 (2006).
54. Zeiser, R. *et al.* Inhibition of CD4$^+$CD25$^+$ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* **108**, 390–399 (2006).
55. Achenbach, P., Bonifacio, E. & Ziegler, A. G. Predicting type 1 diabetes. *Curr. Diab. Rep.* **5**, 98–103 (2005).
56. Ermann, J. *et al.* Only the CD62L$^+$ subpopulation of CD4$^+$CD25$^+$ regulatory T cells protects from lethal acute GVHD. *Blood* **105**, 2220–2226 (2005).
57. Nguyen, V. H. *et al. In vivo* dynamics of regulatory T cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood* **109**, 2649–2656 (2007).
58. Misra, N., Bayry, J., Lacroix-Desmazes, S., Kazatchkine, M. D. & Kaveri, S. V. Cutting edge: human CD4$^+$CD25$^+$ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J. Immunol.* **172**, 4676–4680 (2004).
59. Venet, F. *et al.* Human CD4$^+$CD25$^+$ regulatory T lymphocytes inhibit lipopolysaccharide-induced monocyte survival through a Fas/Fas ligand-dependent mechanism. *J. Immunol.* **177**, 6540–6547 (2006).
60. Taams, L. S. *et al.* Modulation of monocyte/macrophage function by human CD4$^+$CD25$^+$ regulatory T cells. *Hum. Immunol.* **66**, 222–230 (2005).
61. Lewkowicz, P., Lewkowicz, N., Sasiak, A. & Tchorzewski, H. Lipopolysaccharide-activated CD4$^+$CD25$^+$ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. *J. Immunol.* **177**, 7155–7163 (2006).
62. Wood, K. J. & Sawitzki, B. Interferon γ: a crucial role in the function of induced regulatory T cells *in vivo*. *Trends Immunol.* **27**, 183–187 (2006).
63. Farrar, M. A. & Schreiber, R. D. The molecular cell biology of interferon-γ and its receptor. *Annu. Rev. Immunol.* **11**, 571–611 (1993).
64. Tang, Q. *et al.* Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nature Immunol.* **7**, 83–92 (2006).
65. Sarween, N. *et al.* CD4$^+$CD25$^+$ cells controlling a pathogenic CD4 response inhibit cytokine differentiation, CXCR-3 expression, and tissue invasion. *J. Immunol.* **173**, 2942–2951 (2004).
66. Chen, Z., Herman, A. E., Matos, M., Mathis, D. & Benoist, C. Where CD4$^+$CD25$^+$ T reg cells impinge on autoimmune diabetes. *J. Exp. Med.* **202**, 1387–1397 (2005).
67. Roncarolo, M. G., Battaglia, M. & Gregori, S. The role of interleukin 10 in the control of autoimmunity. *J. Autoimmun.* **20**, 269–272 (2003).
68. Peng, Y., Laouar, Y., Li, M. O., Green, E. A. & Flavell, R. A. TGF-β regulates *in vivo* expansion of Foxp3-expressing CD4$^+$CD25$^+$ regulatory T cells responsible for protection against diabetes. *Proc. Natl Acad. Sci. USA* **101**, 4572–4577 (2004).
69. Battaglia, M. *et al.* Induction of tolerance in type 1 diabetes via both CD4$^+$CD25$^+$ T regulatory cells and T regulatory type 1 cells. *Diabetes* **55**, 1571–1580 (2006).
70. Chen, C., Lee, W. H., Zhong, L. & Liu, C. P. Regulatory T cells can mediate their function through the stimulation of APCs to produce immunosuppressive nitric oxide. *J. Immunol.* **176**, 3449–3460 (2006).
71. Battaglia, M., Gregori, S., Bacchetta, R. & Roncarolo, M. G. Tr1 cells: from discovery to their clinical application. *Semin. Immunol.* **18**, 120–127 (2006).
72. Sakaguchi, S. Naturally arising Foxp3-expressing CD25$^+$CD4$^+$ regulatory T cells in immunological tolerance to self and non-self. *Nature Immunol.* **6**, 345–352 (2005).
73. Allan, S. E. *et al.* The role of 2 FOXP3 isoforms in the generation of human CD4$^+$ Tregs. *J. Clin. Invest.* **115**, 3276–3284 (2005).
74. Singh, B. *et al.* Control of intestinal inflammation by regulatory T cells. *Immunol. Rev.* **182**, 190–200 (2001).
75. Shevach, E. M. *et al.* The lifestyle of naturally occurring CD4$^+$ CD25$^+$ Foxp3$^+$ regulatory T cells. *Immunol. Rev.* **212**, 60–73 (2006).
76. Scheffold, A., Hühn, J. & Höfer, T. Regulation of CD4$^+$CD25$^+$ regulatory T cell activity: it takes (IL-) two to tango. *Eur. J. Immunol.* **35**, 1336–1341 (2005).
77. Levings, M. K. *et al.* Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25$^+$CD4$^+$ Tr cells. *Blood* **105**, 1162–1169 (2005).
78. Levings, M. K. *et al.* IFN-α and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* **166**, 5530–5539 (2001).
79. Ziegler, S. F. FOXP3: of mice and men. *Annu. Rev. Immunol.* **24**, 209–226 (2006).
80. Khattri, R., Cox, T., Yasayko, S. A. & Ramsdell, F. An essential role for Scurfin in CD4$^+$CD25$^+$ T regulatory cells. *Nature Immunol.* **4**, 337–342 (2003).

81. Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S. & Noelle, R. J. Cutting edge: contact-mediated suppression by $CD4^+CD25^+$ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J. Immunol.* **174**, 1783–1786 (2005).
82. Zhao, D. M., Thornton, A. M., DiPaolo, R. J. & Shevach, E. M. Activated $CD4^+CD25^+$ T cells selectively kill B lymphocytes. *Blood* **107**, 3925–3932 (2006).
83. Grossman, W. J. *et al.* Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **21**, 589–601 (2004).
84. Chen, D. *et al.* $CD4^+$ $CD25^+$ regulatory T-cells inhibit the islet innate immune response and promote islet engraftment. *Diabetes* **55**, 1011–1021 (2006).
85. Ding, Q. *et al.* B7H1-Ig fusion protein activates the $CD4^+$ IFN-γ receptor$^+$ type 1 T regulatory subset through IFN-γ-secreting Th1 cells. *J. Immunol.* **177**, 3606–3614 (2006).
86. Tarbell, K. V., Yamazaki, S., Olson, K., Toy, P. & Steinman, R. M. $CD25^+$ $CD4^+$ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J. Exp. Med.* **199**, 1467–1477 (2004).
87. Masteller, E. L. *et al.* Expansion of functional endogenous antigen-specific $CD4^+CD25^+$ regulatory T cells from nonobese diabetic mice. *J. Immunol.* **175**, 3053–3059 (2005).
88. Chen, C., Lee, W. H., Yun, P., Snow, P. & Liu, C. P. Induction of autoantigen-specific Th2 and Tr1 regulatory T cells and modulation of autoimmune diabetes. *J. Immunol.* **171**, 733–744 (2003).
89. You, S. *et al.* Presence of diabetes-inhibiting, glutamic acid decarboxylase-specific, IL-10-dependent, regulatory T cells in naive nonobese diabetic mice. *J. Immunol.* **173**, 6777–6785 (2004).
90. Hori, S., Haury, M., Coutinho, A. & Demengeot, J. Specificity requirements for selection and effector functions of $CD25^+4^+$ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc. Natl Acad. Sci. USA* **99**, 8213–8218 (2002).
91. Kohm, A. P., Carpentier, P. A., Anger, H. A. & Miller, S. D. Cutting edge: $CD4^+CD25^+$ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J. Immunol.* **169**, 4712–4716 (2002).
92. Morgan, M. E. *et al.* Effective treatment of collagen-induced arthritis by adoptive transfer of $CD25^+$ regulatory T cells. *Arthritis Rheum.* **52**, 2212–2221 (2005).
93. Cong, Y., Weaver, C. T., Lazenby, A. & Elson, C. O. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. *J. Immunol.* **169**, 6112–6119 (2002).
94. Scalapino, K. J., Tang, Q., Bluestone, J. A., Bonyhadi, M. L. & Daikh, D. I. Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of *ex vivo* expanded regulatory T cells. *J. Immunol.* **177**, 1451–1459 (2006).
95. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nature Immunol.* **4**, 330–336 (2003).
96. Taylor, P. A., Lees, C. J. & Blazar, B. R. The infusion of *ex vivo* activated and expanded $CD4^+CD25^+$ immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* **99**, 3493–3499 (2002).

Acknowledgements
The authors thank R. Bacchetta and S. Gregori (HSR-TIGET) for scientific discussions. M.-G.R. is supported by grants from the Telethon Foundation, RISET and the Juvenile Diabetes Research Foundation (JDRF). M.B. is supported by grants from Telethon and the JDRF.

Competing interests statement
The authors declare no competing financial interests.

DATABASES
The following terms in this article are linked online to:
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
CD4 | CD25 | CD127 | FOXP3 | TGFβ

FURTHER INFORMATION
Maria-Grazia Roncarolo's homepage: http://www.sanraffaele.org/EN_home/Research/Departments-Institutes_e_Research_Programs/The_San_Raffaele_Telethon_Institute_for_Gene_Therapy_(HSR-TIGET)/index.html
RISET transplantation tolerance: http://www.risetfp6.org
The European Agency for the Evaluation of Medicinal Products: http://www.emea.europa.eu/pdfs/human/bwp/4145098EN.pdf
U.S. Food and Drug Administration: http://www.fda.gov/cber/gene.htm
Access to this links box is available online.