

Review

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# Regulatory T cell-based therapies for type 1 diabetes: a narrative review

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## Abstract

Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of pancreatic insulin-secreting beta cells, resulting in hyperglycemia and a lifelong need for exogenous insulin therapy. Regulatory T cells (Tregs) are essential for maintaining immune tolerance and preventing autoimmune reactions. It has been shown that dysfunctional Tregs participate in the pathophysiology of T1D. Therapeutic approaches designed to enhance Treg stability, survival, and function have progressively emerged as a promising treatment strategy for T1D. This narrative review explores the potential of Treg cell-based therapy as a therapeutic tool to alter the natural history of T1D. It discusses different pharmacological strategies to enhance Treg stability and function, as well as the latest advances in Treg cell-based therapies, including adoptive Treg cell therapy and genetic engineering of Tregs. It also outlines current challenges and future research directions for integrating Treg cell-based therapy into clinical



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practice, aiming to provide a comprehensive overview of its potential benefits and limitations as an innovative therapeutic intervention for T1D.

**Keywords:** Regulatory T cells, type 1 diabetes, immune regulation, autoimmune diseases, Treg cell-based therapy, pancreatic beta cells, immunotherapy, T1D management, immune tolerance, cell-based therapy

## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of pancreatic insulin-secreting beta cells, resulting in hyperglycemia and a lifelong need for exogenous insulin therapy<sup>[1]</sup>. Globally, the incidence of T1D is progressively increasing: an average annual increase in T1D incidence of 3%-4% has been reported during the past three decades<sup>[2]</sup>. In 2021, there were about 8.4 million subjects affected by T1D worldwide<sup>[3]</sup>. The burden of T1D is vast and is expected to increase rapidly, particularly in resource-limited countries<sup>[3]</sup>. Patients with T1D require lifelong insulin therapy, continuous glucose monitoring, and regular follow-up visits to properly manage the disease and prevent chronic complications<sup>[4]</sup>. Moreover, patients with T1D have to meticulously balance their insulin doses with dietary carbohydrate intake and physical activity, a task that can be burdensome and is accompanied by a high risk of hypoglycemia<sup>[5]</sup>.

Despite the notable progress in insulin delivery and continuous glucose monitoring devices, many patients with T1D do not achieve adequate glycemic control<sup>[6]</sup>. T1D patients face an increased risk of both acute (such as hyperglycemia, diabetic ketoacidosis, and hypoglycemia) and chronic complications, including cardiovascular disease, neuropathy, nephropathy, and retinopathy, which continue to exert a high emotional and clinical burden in this population<sup>[6,7]</sup>. Individuals with T1D are also at higher risk of developing other autoimmune conditions, such as autoimmune thyroid diseases, Addison's disease, celiac disease, and vitiligo, which are frequently observed in the context of polyglandular autoimmunity<sup>[8-10]</sup>. This increased susceptibility underscores the systemic nature of immune dysregulation in T1D, supporting the rationale for investigation of therapeutic strategies targeting regulatory immune mechanisms. Notably, studies suggest that the age of T1D onset may influence the likelihood of comorbid autoimmune conditions, with a higher incidence observed in subjects diagnosed with T1D at an older age<sup>[9,10]</sup>. Addressing these coexistent autoimmune conditions is therefore critical in developing comprehensive therapeutic approaches for the management of T1D<sup>[8-10]</sup>.

Delayed diagnosis of T1D can result in severe hyperglycemia and life-threatening diabetic ketoacidosis<sup>[11]</sup>. Current treatment paradigms focus primarily on exogenous insulin administration, which, while lifesaving, does not change the underlying autoimmune processes driving the disease pathophysiology<sup>[12]</sup>. Consequently, there is a significant demand for therapeutic strategies that can effectively alter the progression of the disease by targeting its autoimmune etiology<sup>[13,14]</sup>. One promising avenue of research in T1D is the use of regulatory T cells (Tregs), a subset of CD4+ T cells that are essential for maintaining immune tolerance and preventing autoimmune reactions<sup>[15]</sup>. Tregs from T1D patients can be dysfunctional<sup>[16]</sup>, potentially contributing to disease progression. Thus, restoring Treg function may help suppress autoreactive T cells and modulate T1D-related aberrant immune responses, offering a novel approach to halting or reversing beta cell destruction in T1D<sup>[17]</sup>. This therapeutic strategy for T1D may be promising especially within the framework of combination therapy approaches. Indeed, as compared to monotherapies, combination therapies targeting different immune and metabolic alterations related to T1D (besides beta cell autoimmune destruction) are more likely to sustain beta cell function in T1D patients across various stages of the disease<sup>[18]</sup>.

Studies conducted in mouse models and T1D patients support the presence of Treg dysfunction in T1D. Hence, Treg cell-based therapies are at the forefront of research and development, holding the potential to deliver a successful immune-targeted treatment for T1D<sup>[19]</sup>. One of the main challenges in this research area is to determine if it is essential to assess Treg function directly at the pancreatic level or if it is sufficient to assess the function of Tregs collected from peripheral blood. Future research studies focused on Treg function in the pancreas and pancreatic lymph nodes may provide key insights into the ultimate mechanisms underlying Treg dysfunction and failure in T1D<sup>[19]</sup>.

This narrative review aims to examine the role of Tregs in the pathophysiology of T1D, and to evaluate the potential of Treg cell-based therapies as valid disease-modifying strategies for T1D.

## TREGS: AN OVERVIEW

Billingham *et al.* conducted animal studies on skin grafting, showing that newborn mice could tolerate allogeneic skin transplants that were rejected by adults<sup>[20]</sup>. It was later discovered that this tolerance is driven by suppressor cells, whose suppressive capacity can be transferred from one cell population to another. In the 1970s, Gershon and Kondo first coined the term “infectious immunological tolerance” to describe this phenomenon<sup>[21]</sup>. This process is now recognized as a crucial mechanism in the regulation of peripheral immune tolerance in tissues and organs<sup>[22]</sup>. In 2003, Hori *et al.* discovered that FOXP3 represents the main regulatory gene for the development of Tregs<sup>[23]</sup>. This gene encodes a transcription factor (Foxp3: Forkhead box P3) that is genetically defective in an inflammatory and autoimmune syndrome in mice and humans, and is specifically expressed in naturally arising CD4<sup>+</sup> Tregs<sup>[23]</sup>. Since then, the pivotal role of CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs in the regulation of immune tolerance has been increasingly recognized<sup>[24,25]</sup>.

A combination of intracellular and surface markers [Foxp3, CD25, Nrp-1, glucocorticoid-induced tumor necrosis factor receptor (GITR), PD-L1, CTLA-4, LAG-3, and the transcription factor Helios] has been utilized to characterize Tregs<sup>[26-32]</sup>. According to their origin, Tregs can be divided into two main subpopulations, namely: “natural” Tregs (nTregs) cells that develop in the thymus; and “induced” Tregs (iTregs), which develop in the periphery from CD4<sup>+</sup> Foxp3(-) conventional T lymphocytes and can be generated *in vitro*<sup>[33]</sup>.

While nTregs can develop in the thymus in response to self-antigen (or strongly ligating peptides), iTregs develop in the periphery in response to exogenous antigens and weaker (suboptimal) T cell receptor (TCR) stimulation, in the presence of transforming growth factor beta (TGFβ)<sup>[34,35]</sup>. In particular, TCR stimulation and TGFβ released from the surface of activated thymic-derived Tregs promote Foxp3 expression in naïve T cells<sup>[36]</sup>.

Tregs can suppress T cell effector function via different mechanisms, such as inhibition of TCR-induced Ca<sup>2+</sup>, NFAT, and NF-κB signaling, IL-2 deprivation-mediated apoptosis, production of immunosuppressive cytokines (TGFβ, IL-35, IL-10), generation of adenosine, transfer of cyclic adenosine monophosphate (cAMP) through gap junctions, induction of effector cell death through granzyme and perforin, and downregulation of co-stimulatory molecules on antigen-presenting cells via CTLA-4<sup>[37]</sup>.

## PATHOPHYSIOLOGY OF T1D

T1D is a chronic autoimmune disease caused by the selective destruction of pancreatic insulin-secreting beta cells<sup>[38]</sup>. Many pathophysiological mechanisms have been implicated in the autoimmune destruction of insulin-producing beta cells, including those related to impaired Treg stability and function.

### Autoimmune destruction of pancreatic beta cells

T1D pathophysiology is characterized by aberrant immune responses directed against insulin-secreting pancreatic beta cells. T1D is considered a multifactorial disease characterized by a complex interaction between various susceptibility genes and environmental factors such as viral infections or dietary factors<sup>[39,40]</sup>. Both human leukocyte antigen (HLA) and non-HLA genes can contribute to the increased susceptibility to T1D<sup>[41]</sup>. Over the past few years, analysis of genome-wide association studies (GWAS) has detected more than 50 genomic regions (and related genes within these regions) implicated in the pathophysiology of T1D<sup>[42]</sup>. These genetic factors further support the hypothesis that Treg dysfunction contributes to initiating or exacerbating the autoimmune cascade that leads to T1D in genetically susceptible individuals<sup>[43]</sup>.

### Role of immune cells in T1D progression

Preclinical studies conducted on cell cultures and animals showed that several immune cells significantly contribute to the development and progression of T1D<sup>[44,45]</sup>. T1D is primarily considered a T cell-mediated autoimmune disease<sup>[46]</sup>. CD8+ cytotoxic T cells are the most abundant cells among the immune cells infiltrating the pancreatic islets, although CD4+ T cells, macrophages, and B lymphocytes are also found<sup>[47,48]</sup>. In particular, autoreactive CD8+ T lymphocytes recognize major histocompatibility complex (MHC) class I-restricted islet autoantigens on the surface of pancreatic beta cells and then carry out cytotoxic actions through various effector mediators, especially cytokines released by T helper 1 (Th1) cells, such as interferon-gamma (IFN- $\gamma$ )<sup>[46,47]</sup>. T helper 17 (Th17) cells and follicular helper T (Tfh) cells have also been associated with T1D pathophysiology<sup>[49,50]</sup>. Besides being the source of islet autoantibodies (which serve as the most reliable biomarkers for the disease)<sup>[51]</sup>, B cells contribute to the pathophysiology of T1D by producing pro-inflammatory cytokines and presenting autoantigens to T cells<sup>[52]</sup>.

### Immune dysregulation in T1D

Under physiological conditions, Tregs maintain immune tolerance and prevent autoimmunity. On the other hand, in T1D, there is a marked immune dysregulation characterized by altered Treg function<sup>[53]</sup>. Indeed, impairments in the frequency and activity of Tregs have been described in patients with T1D<sup>[54,55]</sup>. Additionally, defects in other immunoregulatory mechanisms further contribute to the dysregulation of immune tolerance in T1D [e.g., defective activity of tolerogenic dendritic cells (DC)]<sup>[56]</sup>.

The combination of increased autoreactive immune cell activation and defective immunoregulatory mechanisms promotes and aggravates beta cell destruction and loss<sup>[57]</sup>. Over time, the progressive loss of beta cells results in the clinical onset of T1D, which is characterized by hyperglycemia and its associated symptoms (e.g., polyuria, polydipsia, fatigue, unintentional weight loss, diabetic ketoacidosis)<sup>[58]</sup>. A better comprehension of the immune mechanisms underlying T1D-related beta cell destruction is essential for developing targeted therapies designed to preserve beta cell function and restore immune tolerance in patients with T1D<sup>[56]</sup>.

### TREGS IN THE CONTEXT OF T1D

FOXP3+ Tregs are essential for maintaining immune homeostasis and preventing autoimmune diseases in healthy individuals<sup>[59]</sup>. These cells express high levels of CD25 [interleukin-2 (IL-2) receptor alpha chain]<sup>[60]</sup> and Foxp3<sup>[61]</sup>. Tregs develop primarily in the thymus (tTregs; thymus-derived Tregs), even though they can also be generated extrathymically at peripheral sites (pTregs; peripherally induced Tregs)<sup>[35,62]</sup> following encounters with antigens and activation of the IL-2 signaling pathway, which is critical for their function and survival<sup>[35,63]</sup>. Tregs act as “IL-2 sinks”, depriving pathogenic T cells of this interleukin that mediates effector T cell proliferation<sup>[55,64]</sup>. Additionally, Tregs produce immunosuppressive cytokines (i.e., TGF $\beta$ , IL-35, IL-10) and downregulate co-stimulatory molecules on antigen-presenting cells via CTLA-4<sup>[37]</sup>.

The essential role of Tregs in maintaining immune homeostasis and preventing autoimmune disorders is testified by the IPEX syndrome (Immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome), a monogenic disease characterized by early onset multi-organ autoimmunity due to loss-of-function mutations in the gene *FOXP3*, which encodes the transcription factor (Foxp3) essential for the development, maturation, and maintenance of Tregs<sup>[65]</sup>.

Treg dysfunction is a key contributor to T1D pathophysiology<sup>[55]</sup>. Indeed, changes in the frequency and activity (suppressive capacity) of Tregs have been described in patients with T1D<sup>[54,55]</sup>. Specifically, T1D-related Treg dysfunction appears to arise from a combination of factors, such as increased chronic inflammatory mediators, defective IL-2 signaling pathway, and decreased TCR diversity, resulting in altered Treg function within the pancreatic tissue<sup>[19]</sup>. Preclinical evidence also supports the involvement of the complement system in T1D-related Treg dysfunction, since complement C3 deficiency has been shown to prevent the development of streptozotocin (STZ)-induced autoimmune diabetes through the expansion of Tregs with increased suppressive capacity<sup>[66]</sup>.

Within the microenvironment of pancreatic islets, additional complexities emerge as metabolic factors (e.g., glucotoxicity, lipotoxicity), cytokine imbalance, and other infiltrating immune cells may further disrupt the immunoregulatory function of Tregs. Furthermore, a deeper understanding of the interindividual variability of Treg dysfunction across T1D subjects is essential for developing tailored immunotherapeutic strategies that can effectively prevent, restore, or improve Treg function in this population.

### Evidence from animal and human studies

Studies in non-obese diabetic (NOD) mice have revealed critical roles of Treg dysfunction in the pathophysiology of T1D. NOD mice represent a well-established model of T1D, in which diabetes develops through insulinitis<sup>[67]</sup>. Interventions aimed to restore Treg function in NOD mice led to promising results<sup>[55]</sup>. Of note, IL-2 administration has been shown to reverse established diabetes in NOD mice by increasing the number of Tregs in the pancreas and inducing the expression of Treg-related proteins such as Foxp3, CTLA-4, CD25, ICOS (inducible T-cell costimulator), and GITR in such cells<sup>[68]</sup>. It has also been shown that the immune dysregulation observed in NOD mice is due to an increased effector T cell resistance to regulation<sup>[69]</sup>.

It has been demonstrated that treatment with purified autologous CD4(+)CD62L(+) Tregs (co-cultured with human cord blood stem cells) can reverse diabetes in NOD mice, promoting beta cell regeneration, increasing insulin secretion, and reconstituting pancreatic islet architecture<sup>[70]</sup>. Preclinical evidence also showed that DC-expanded Tregs are effective in restoring normoglycemia and counteracting beta cell autoimmunity and insulinitis in NOD mice<sup>[71,72]</sup>. Another study demonstrated that the adoptive transfer of GM-CSF (Granulocyte-macrophage colony-stimulating factor)-exposed DCs to naive mice resulted in the expansion of Foxp3+ Tregs, which led to a significantly delayed development of autoimmune diabetes in NOD mice<sup>[73]</sup>.

Similarly, human studies demonstrated that T1D patients exhibit a defective Treg ability to inhibit the proliferation and activity of autoreactive CD4+ and CD8+ T cells<sup>[16,74,75]</sup>. Specific Treg alterations observed in T1D patients include increased Treg apoptosis<sup>[76,77]</sup>, decreased stability of FOXP3 expression<sup>[78,79]</sup>, and a rise in the proportion of Tregs that secrete pro-inflammatory cytokines like IFN- $\gamma$  and IL-17<sup>[80,81]</sup>. These findings emphasize the crucial role of Tregs in preventing aberrant immune responses and maintaining immune tolerance, thereby highlighting the remarkable therapeutic potential of targeted strategies aimed at restoring Treg function in T1D.

## THERAPEUTIC STRATEGIES UTILIZING TREGS

Treg cell-based therapies have garnered considerable attention in recent years as a novel therapeutic approach for T1D<sup>[82]</sup>. Various strategies leverage Tregs to modulate aberrant immune responses and restore immune tolerance, aiming to mitigate the T1D-related beta cell autoimmunity and preserve residual beta cell function. The therapeutic potential of Treg cellular therapies has been studied in various autoimmune diseases, in which abnormalities in the frequency and/or function of Tregs lead to aberrant autoimmune responses mediated by CD4+ and CD8+ T-cells<sup>[83-85]</sup>.

### Tregs isolation and expansion

Treg expansion methods often involve cytokines, such as IL-2, or immunomodulating agents that selectively promote Treg proliferation. Low-dose IL-2 is able to inhibit immune pathologies by promoting the expansion of Tregs that constitutively express the high-affinity IL-2R $\alpha$  subunit<sup>[86]</sup>. Yet, low-dose IL-2 may also result in the activation of pro-inflammatory non-Treg T cells via signaling through the IL2-R $\beta/\gamma$  complex<sup>[86]</sup>. Therefore, studies on the use of IL-2-based therapies in autoimmune diseases should also investigate novel strategies to enhance the specificity of IL-2 effects toward Tregs.

In this regard, de Picciotto *et al.* utilized mRNA to encode a half-life-extended human IL-2 mutein (HSA-IL2m) with mutations favoring reliance on IL-2R $\alpha$  (rather on the IL2-R $\beta/\gamma$  complex)<sup>[86]</sup>. Authors showed that subcutaneous delivery of IL-2 mutein as lipid-encapsulated mRNA nanoparticles leads to selective activation and proliferation of Tregs in mice and non-human primates, coupled with the decrease in disease severity in mouse models of experimental autoimmune encephalomyelitis and acute graft-versus-host disease. Thus, such findings highlighted a potential therapeutic application of mRNA-encoded HSA-IL2m in autoimmune disorders<sup>[86]</sup>.

Enhancement of Treg suppressive capacity is another important aspect to consider within the context of Treg cell-based therapies. Interestingly, Strauss *et al.* demonstrated the selective survival of naturally occurring human CD4+CD25+Foxp3+ Tregs that were cultured with rapamycin<sup>[87]</sup>. Of note, rapamycin-expanded T cells inhibited the proliferation and effector functions of autologous and allogeneic CD4+ and CD8+ T cells *in vitro*<sup>[87]</sup>.

Additionally, improving the efficacy of Treg cell-based therapies may also require enhancing Treg persistence or promoting migration of Tregs to secondary lymphatic tissues or sites of inflammation<sup>[88]</sup>. Therefore, further studies are certainly needed to establish which strategies are most effective in increasing the success of clinical Treg cell-based therapies.

### Adoptive Treg cell therapy

According to ethical guidelines, Tregs can be isolated from either umbilical cord blood or peripheral blood, while the thymus also represents an abundant source of Tregs in experimental animals<sup>[15]</sup>. Mononuclear cells, which include monocytes and lymphocytes, can be isolated using the standard cell isolation procedure with the gradient centrifugation technique. An additional purification and enrichment process is applied to sort out the specific T cell population from the isolated mononuclear cell population. The conventional method involves the depletion of CD19+ and/or CD8+ T cells, followed by the enrichment of the CD25+ fraction, thereby increasing the purity of FOXP3+ T cells to around 80%<sup>[15]</sup>.

Furthermore, flow cytometry and associated cell sorting represent cutting-edge techniques for the isolation of cells with high levels of cell purity. Flow cytometry-based cell isolation has constituted the foundation of immune cell differentiation and development, and it continues to evolve<sup>[89]</sup>. Purification technique based on



flow cytometry represents an advanced method of high-purity cell isolation. With flow cytometry-based purification technique, cells are selected and sorted out for maximum purity (above 99%) and sterility<sup>[15]</sup>. Given the low purity of Tregs obtained through conventional isolation strategies, the use of Treg purification methods based on flow cytometry has progressively increased. This method allows for Treg purification based on multiple surface markers in one step (such as CD25, CD127, CD62L, CD45RA and CD27)<sup>[90]</sup> and permits sub-gating to generate a Treg population with high purity or a specific subset of Tregs<sup>[91]</sup>.

Although the concentration of Tregs in the thymus and cord blood is high, post-enrichment Treg expansion in cell culture vessels is crucial to achieve the optimal Treg concentration for therapeutic applications<sup>[15]</sup>. *Ex vivo* polyclonal Treg expansion is frequently employed utilizing anti-CD3/CD28-coated beads, in the presence of IL-2<sup>[92]</sup>. Researchers have followed protocol modifications employing rapamycin and/or IL-2 to boost Treg yield with augmented suppressive capacity<sup>[15]</sup>. In this regard, another strategy consists of incorporating TGF $\beta$  and all-trans retinoic acid (ATRA) in the culture medium to enhance chemokine receptor expression on Tregs, thereby promoting Treg homing in the gut<sup>[15]</sup>.

Adoptive cell therapy using genetically engineered Tregs has been increasingly explored as a potential approach for treating transplant rejection and autoimmune diseases<sup>[93]</sup>. Tregs are the main orchestrators of maintaining peripheral tolerance, thereby preventing aberrant immune responses and autoimmunity<sup>[94]</sup>. Clinical investigations have demonstrated the efficacy of CD4+CD25+FOXP3+ Tregs in minimizing undesired or overshooting immune responses and in reducing the level of pharmacologic immunosuppression in transplant recipients<sup>[94]</sup>. However, the lack of standardized protocols and procedures for expanding and transducing Tregs from mice remains a major limitation of adoptive cell therapy based on genetically modified Tregs. Purification, expansion, and retroviral transduction of mouse Tregs with a vector encoding a chimeric antigen receptor as a model transgene has also been described to obtain notable improvements in genetically engineered Tregs<sup>[15]</sup>. Moreover, it has been documented that isolating Tregs with GFP expression leads to suitably pure cells<sup>[15]</sup>.

Adoptive transfer of autologous polyclonal Tregs has proven safe and partly effective in restoring immune tolerance and preserving beta cell function among T1D patients<sup>[95]</sup>. Although infused Tregs can be detected in the periphery for a prolonged period of time, the limited efficacy observed with polyclonal Tregs in T1D clinical trials has been attributed to several putative factors, including the lack of pancreatic islet specificity, *in vivo* Treg plasticity, restricted Treg proliferation, and local scarcity of IL-2<sup>[96,97]</sup>.

Novel gene engineering technologies are rapidly evolving to optimize the Treg functional properties for tailored applications (e.g., redirecting the antigen specificity)<sup>[15,98]</sup>. In the future, genetically modified Tregs may address the challenges associated with Treg expansion and purification, while concomitantly enhancing Treg suppressive capacity<sup>[15]</sup>.

### **Therapeutic strategies to enhance Treg function**

Another potential therapeutic approach for the management of T1D is represented by the use of pharmacological strategies aimed at increasing the frequency and/or improving the suppressive capacity of endogenous Tregs. Indeed, therapies that effectively target endogenous Treg cell function are highly desirable, particularly in light of the high costs and technical difficulties in harvesting cord blood stem cells or infusing antigen-specific Tregs<sup>[99]</sup>.

In this regard, the mTOR (mammalian target of rapamycin) inhibitor rapamycin<sup>[100]</sup> has proven effective in promoting the proliferation of functional Tregs (CD4+CD25+FOXP3+ Tregs) of both healthy subjects and T1D patients<sup>[101]</sup>. Furthermore, Monti *et al.* documented that administration of rapamycin prior to pancreatic islet transplantation in T1D patients enhanced the capacity of nTregs to inhibit the expansion of CD4(+)CD25(-) effector T cells<sup>[102]</sup>.

Preliminary clinical trials documented that short-term treatment with anti-CD3 monoclonal antibodies helps preserve residual beta cell function and enhances metabolic control in patients with newly diagnosed T1D<sup>[103,104]</sup>. More recently, a landmark randomized, placebo-controlled trial demonstrated that teplizumab (a humanized anti-CD3 monoclonal antibody) significantly preserves beta cell function and delays the progression to clinical T1D in individuals at high risk for development of clinical disease<sup>[105,106]</sup>. In the teplizumab group, the median time to diabetes was approximately 5 years, as compared to slightly over 2 years in the placebo group. Notably, 50% of the teplizumab-treated subjects were not diagnosed with T1D (vs. 22% of placebo-treated participants)<sup>[106]</sup>. This robust clinical evidence supported teplizumab as a valid treatment able to address the underlying autoimmune pathogenesis of T1D. Thus, in November 2022, the U.S. Food and Drug Administration (FDA) approved teplizumab (administered intravenously once daily for 14 consecutive days) as the first drug capable of delaying the onset of clinical T1D (stage 3 T1D) in individuals with pre-symptomatic T1D (stage 2 T1D) aged 8 years and older<sup>[107]</sup>. Mechanistically, teplizumab appears to forestall T1D-related autoimmune responses and preserve beta cell function through various mechanisms, including promotion of phenotypic changes in autoreactive CD8+ T lymphocytes, CD8+ T lymphocyte exhaustion, depletion of effector T cells, and preservation of Tregs<sup>[108]</sup>.

The successful outcomes demonstrated with early teplizumab therapy during the natural history of T1D emphasize the need for timely recognition of individuals at high risk of developing clinical T1D (e.g., subjects with multiple islet autoantibody positivity and dysglycemia) through advancements in T1D screening programs<sup>[109]</sup>. Additionally, there is a need for future studies to identify predictive biomarkers of response to teplizumab therapy in T1D patients (e.g., the presence of specific HLA variants or islet autoantibodies) in order to refine patient selection and optimize therapeutic outcomes.

Despite the recent FDA approval of teplizumab for individuals at high risk of developing clinical T1D, there are also some challenges to consider. In particular, the high costs of teplizumab for a single 14-day course of therapy<sup>[110]</sup> may significantly limit drug accessibility for many patients. Additionally, while teplizumab safety profile has proven favorable with regard to its current clinical indication in T1D, post-marketing surveillance remains essential to better assess the risk of adverse effects (e.g., infections, immune dysregulation, *etc.*) in the long term. Combining teplizumab with other drugs, such as other immunomodulators or beta cell protective agents, may further enhance its safety and its favorable impact on T1D progression. As research evolves, teplizumab's role may become a cornerstone in combination therapy approaches for the comprehensive management of T1D<sup>[18,111]</sup>.

It has been shown that autoantigen treatment relying on the administration of glutamic acid decarboxylase bound to aluminum hydroxide (GAD-alum) has the potential to preserve endogenous insulin secretion in patients with newly diagnosed T1D<sup>[112,113]</sup>. The immunomodulatory properties of GAD-alum are partly explained by its ability to induce GAD(65)-specific T lymphocytes with regulatory properties. In fact, GAD-alum treatment has been shown to induce GAD65-specific CD4(+)CD25(high)FOXP3(+) cells in patients with newly diagnosed T1D<sup>[114]</sup>.



Alefacept is a biologic agent approved for the treatment of moderate-to-severe chronic plaque psoriasis<sup>[115]</sup>. It consists of a fusion protein comprised of two LFA-3 molecules bound to the Fc portion of IgG1<sup>[116]</sup> and acts by binding to CD2, which is predominantly expressed on CD4+ and CD8+ effector memory T lymphocytes<sup>[117]</sup>. A randomized controlled trial showed that administering alefacept to patients with new-onset T1D preserved endogenous insulin secretion, reduced insulin requirements and hypoglycemic events, and induced beneficial immunological profiles by preserving Tregs and depleting CD4+ and CD8+ central memory T cells and effector memory T cells<sup>[118]</sup>.

IL-2 is crucial for the function and survival of Tregs, which, in turn, are highly sensitive to low levels of this interleukin within their environment<sup>[63]</sup>. Indeed, reduced IL-2 production is accompanied by decreased function of CD4(+) CD25(+) Tregs, which play an essential role in maintaining immune homeostasis<sup>[119]</sup>. In NOD mice and humans, genetic variation in *IL-2RA/CD25* and *IL-2-IL-21* gene regions can promote T1D susceptibility by influencing IL-2 and IL-2RA/CD25 expression at the protein level in various subsets of immune cells<sup>[120]</sup>. Accordingly, defective IL-2R signaling in CD4+ T cells of T1D patients contributes to the reduced persistence of FOXP3 expression that could affect the establishment of immune tolerance<sup>[78]</sup>.

Administration of IL-2 can reverse autoimmune diabetes in NOD mice via a local action on pancreatic Tregs<sup>[68]</sup>. A phase I/II randomized, double-blind, placebo-controlled, dose-finding study reported that low-dose IL-2 therapy [administered subcutaneously (at different doses) on a daily basis for a 5-day course, and then fortnightly for one year] in children with recent-onset T1D can induce a dose-dependent increase in the mean proportion of Tregs, which is associated with better maintenance of C-peptide secretion in Treg-high responders as compared to Treg-low responders<sup>[121]</sup>. Moreover, low-dose IL-2 therapy was not associated with serious adverse events<sup>[121]</sup>. The greater preservation of endogenous insulin secretion observed among Treg-high responders strongly supports the capacity of Tregs to regulate T1D-associated autoimmunity, and warrants the planning of future studies to better elucidate the role of IL-2 therapy in the prevention and treatment of T1D. Given the aforementioned findings<sup>[121]</sup>, future studies should also investigate the safety and efficacy profile of higher IL-2 doses in people with T1D.

### Gene therapy strategies to enhance the efficacy of Treg cell-based therapies

Gene therapy aims to correct defective (mutated) genes or create site-specific modifications to the human genome in order to treat a given disease or halt its progression<sup>[122]</sup>. In recent years, gene therapy has emerged as a promising therapeutic approach for treating T1D, although many studies on gene therapy for T1D have been conducted *in vitro* and in animal models<sup>[123,124]</sup>. Among the various gene-based interventions that are being explored for the treatment of T1D, there are the overexpression of genes and proteins that counteract the development and progression of T1D, transplantation of cells that express genes conferring protection against T1D, genetic vaccination, stem cell-based gene therapy, gene therapy relying on immune cell precursors, and gene therapy based on the use of viral and non-viral vectors<sup>[123]</sup>. For instance, *in vivo* beta cell-specific expression of the mouse *Klotho* gene [achieved through adeno-associated virus 2 (AAV-2) delivery of the *Klotho* gene driven by a beta cell-specific promoter] has been shown to improve glucose tolerance, reduce beta cell apoptosis, enhance insulin storage within beta cells, and increase plasma insulin levels in NOD mice, as a likely consequence of the mitigation of T cell infiltration within the pancreatic islets<sup>[125]</sup>. In another study involving NOD mice that were intraperitoneally injected with an adenovirus carrying IL-10 (Adv-IL-10), transgenic IL-10 expression in pancreatic beta cells led to a reduction in the expression of pro-inflammatory cytokines, attenuated insulinitis, increased the number of CD4+CD25+FoxP3+ Tregs, inhibited beta cell apoptosis, and lowered the incidence of diabetes<sup>[126]</sup>.

In the context of advanced gene editing techniques, novel approaches to designing engineered Tregs with enhanced function and specificity have also emerged<sup>[127]</sup>. Genetic engineering of Tregs has been investigated for the treatment of various autoimmune conditions, including T1D<sup>[127]</sup>. Such strategies involve the engineering of FOXP3 expression to generate a large pool of suppressive cells for subsequent infusion, expressing TCRs or chimeric antigen receptors to produce antigen-specific Tregs, and enhancing Treg survival by targeting specific cytokine pathways. While these approaches are being explored in various autoimmune and transplant contexts, T1D offers unique opportunities and challenges for the genetic engineering of Tregs for adoptive cell therapy<sup>[127]</sup>. Interestingly, Uenishi *et al.* recently described a cell engineering approach using bulk CD4<sup>+</sup> T cells to produce a dual-engineered Treg therapy (called GNTI-122) developed for the treatment of T1D and characterized by tissue specificity, enhanced stability, and selective IL-2 signaling<sup>[96]</sup>. This Treg therapy stably expresses FOXP3, incorporates a chemically inducible signaling complex (CISC), and targets the pancreas and draining lymph nodes. GNTI-122 cells exhibited an expression profile consistent with Tregs phenotype and function. In response to the cognate antigen, GNTI-122 demonstrated both direct and bystander suppression of polyclonal effector (islet-specific) T lymphocytes from T1D patients. Additionally, a mouse-engineered Treg analog of GNTI-122 migrated to the pancreas, decreased the severity of insulinitis, and prevented the progression to diabetes in an adoptive transfer mouse model of T1D<sup>[96]</sup>. Altogether, these findings support further development of GNTI-122 as an autologous antigen-specific engineered Treg cell-based therapy for T1D.

Transplantation of cells expressing genes that confer protection against T1D development is another therapeutic approach that is being investigated. In this regard, Li *et al.* demonstrated that transplantation of Aire-overexpressing bone marrow-derived DCs delayed the onset of diabetes in recipient STZ-induced T1D mice by reducing the production of insulin autoantibodies, mitigating insulinitis, inducing clonal anergy and clonal deletion of autoreactive CD4<sup>+</sup> T cells, inhibiting Th1 and Th17 cell production, and inducing Treg production<sup>[128]</sup>.

Furthermore, genetic vaccination has attracted attention as a potential strategy for the prevention and treatment of T1D. Different approaches to genetic vaccination have been investigated to prevent and/or halt beta cell autoimmunity, including plasmid DNA-based vaccines, viral vector-based vaccines, and the use of antisense oligonucleotides<sup>[129,130]</sup>. In particular, genetic-based vaccination can offer a therapeutic strategy to re-establish beta cell-specific tolerance within the compartment of T cells<sup>[129]</sup>. In animal models of T1D, the transfer of genes encoding beta cell autoantigens, immunomodulatory proteins and/or anti-inflammatory cytokines has proven effective in preventing or halting the diabetogenic response<sup>[129]</sup>.

Ideal gene therapy for T1D should aim to reduce beta cell autoimmunity, restore immune tolerance to beta cells and regenerate or preserve beta cells at the same time<sup>[124]</sup>. For example, Xia *et al.* demonstrated that treatment with lentiviral vector-encoding Reg3g (Regenerating islet-derived protein 3 gamma) promoted beta cell regeneration and protected beta cells from autoimmune damage in NOD mice by inducing tolerogenic DCs and increasing Treg differentiation<sup>[131]</sup>.

However, the safety and efficacy of the aforementioned gene-based therapies for T1D still need to be fully established in humans<sup>[123]</sup>.

### **Role of combination therapies in Treg differentiation and function**

Several immunotherapies have been investigated for the treatment of T1D, although most of them only showed limited or partial efficacy in preserving residual beta cell function<sup>[132]</sup>. In this context, given the multifaceted pathophysiology of T1D, combination therapies based on the simultaneous use of distinct

immunomodulatory and beta cell-protective agents targeting various immune and metabolic dysfunctions are more likely to be effective in attaining substantial preservation of beta cell function or even the insulin independence in T1D patients<sup>[18,133]</sup>. By allowing for the use of lower doses of each single agent and limiting drug toxicity, combination therapies for T1D may also be characterized by a better safety and tolerability profile, besides being associated with greater therapeutic effectiveness as compared to monotherapies. Moreover, such combination therapies for T1D should be investigated in the context of individualized patient-tailored therapeutic approaches and early intervention programs<sup>[134]</sup>.

One of the potential benefits of combination therapies for T1D is the higher efficacy in favoring the differentiation and suppressive capacity of Tregs. In this regard, Manirarora and Wei<sup>[135]</sup> demonstrated that combination therapy based on IL-2/IL-2 monoclonal antibody complexes, islet autoantigen peptides, and rapamycin determined a significant *in vivo* proliferation of CD4(+)CD25(+)Foxp3(+) Tregs with enhanced *in vitro* suppressive activity, and protected NOD mice against the spontaneous development of diabetes. Importantly, the protection from T1D development was transferrable by Tregs, and was attributed to the decreased islet immune cell infiltration and the skewing of immune responses toward a Th2 cytokine profile<sup>[135]</sup>. Based on these findings, authors suggested this novel method of peripheral immune regulation as a potential immunotherapeutic strategy for preventing T1D development or promoting immune tolerance to islet allografts without using immunosuppression<sup>[135]</sup>, which is one of the main goals of the research focused on pancreatic islet transplantation for the treatment of T1D<sup>[136,137]</sup>.

Nonetheless, a phase 1 clinical trial previously tested the safety and immunological effects of short-term rapamycin/IL-2 combination therapy in nine patients with T1D (autoantibody-positive adult patients between 4 and 48 months from disease diagnosis)<sup>[138]</sup>. Although Tregs increased during the first month of therapy, clinical and metabolic data showed a transient worsening in all patients. Additionally, the increase in Tregs was temporary, paralleling the IL-2 therapy. An increase in eosinophils and natural killer cells was also noted with IL-2 therapy. Overall, rapamycin/IL-2 combination therapy led to transient beta cell dysfunction despite the observed increase in Tregs<sup>[138]</sup>, although it was not clearly established whether these findings were entirely or partly mediated by the beta cell toxic effects of rapamycin<sup>[138,139]</sup>. Therefore, there is a great need for further intervention studies to determine the safety and efficacy of different combination therapies aimed at preserving beta cell function in T1D patients by partly relying on enhanced Treg differentiation and survival as one of the beneficial mechanisms in this population.

**Table 1** summarizes the potential and established advantages and disadvantages/limitations of using Treg cell-based therapy as a therapeutic strategy for T1D, highlighting its potential benefits and associated challenges.

## CHALLENGES AND CONSIDERATIONS

### Technical and logistical challenges in Treg cell-based therapy

The use of Tregs as a therapeutic strategy for the treatment of T1D is associated with many technical and logistical hurdles. The primary challenge lies in the isolation and expansion of a sufficient number of Tregs. Indeed, Tregs represent only a small fraction of the whole CD4+ T-cell population. In particular, nTregs account for about 6%-10% of the entire CD4+ T-cell subset<sup>[140]</sup>. With regard to Treg manufacturing for clinical trials, many questions remain unanswered and further studies are needed to establish which are the optimal Treg source and protocols for cell isolation and expansion to generate the most suitable Tregs for therapeutic applications<sup>[141,142]</sup>.

**Table 1. Potential and established advantages and disadvantages/limitations of using Treg cell-based therapies in T1D**

Aspect	Advantages	Disadvantages/Limitations
Efficacy	Suppression of autoreactive T cells; reduction of beta cell destruction; potential preservation of residual beta cell function and decrease in exogenous insulin requirements	Possible interindividual variability in the clinical efficacy of Treg cell-based therapies; possible need for personalized therapeutic approaches
Mechanism of action	Targeting one of the main pathophysiological mechanisms of T1D (Treg dysfunction) and promoting long-term immune tolerance	Limited stability of Tregs within an inflammatory microenvironment, resulting in an increased risk of Treg dysfunction
Safety	Treg cell-based therapies have proven generally safe in animal models of autoimmune diabetes	Potential systemic immunosuppression and increased risk of infections and malignancies; potential reprogramming of Tregs to pathogenic effector T cells (in the context of chronic inflammation); long-term safety of Treg cell-based therapy in T1D remains to be tested in clinical trials
Tregs source	Autologous Tregs can avoid immune rejection issues	Techniques used for the isolation and expansion of Tregs are complex and expensive
Evidence	Preclinical studies show prevention, delayed onset or reversal of autoimmune diabetes in animal models	Need for large clinical trials to establish the efficacy profile of Treg cell-based therapies in T1D
Ethical and regulatory aspects	The use of patient's cells aligns with ethical standards, reducing donor-related concerns	Complex regulatory landscape; possible ethical issues regarding equitable access and distribution
Innovation potential and genetic engineering of Tregs	The use of engineered and antigen-specific Tregs may represent a valid precision therapy for T1D	Need for advances in gene editing techniques and delivery systems allowing for the clinical application of genetic engineering of Tregs

T1D: Type 1 diabetes; Tregs: regulatory T cells.

It is also essential to characterize human Tregs by their phenotype and function, since the phenotype alone does not unambiguously define these cells<sup>[143]</sup>. Moreover, ensuring the stability and function of expanded Tregs is another critical aspect for their therapeutic use<sup>[142]</sup>, since these cells must maintain their phenotype and suppressive capacity in order to exert significant therapeutic actions *in vivo*. The choice of the most appropriate delivery method for Treg cell-based therapies is another important aspect to consider, since Tregs need to be administered through innovative and sophisticated methods that maximize their survival, function, and trafficking to target tissues *in vivo*.

**Potential risks and side effects of Treg cell-based therapy**

Treg cell-based therapy is not devoid of potential risks. One significant concern related to Treg cell-based therapies is the potential risk of prolonged systemic immunosuppression, which may lead to increased susceptibility to infections and malignancies in the long term. In particular, the use of a high number of polyclonal Tregs without any selection for antigen specificity may increase the risk of systemic immunosuppression<sup>[144]</sup>. Additionally, there is a risk that the infused Treg cells might lose their regulatory function or even convert to pro-inflammatory cells, exacerbating the autoimmune response rather than mitigating it. Given the instability and plasticity of pTregs in inflamed tissues<sup>[145]</sup>, the reprogramming of Tregs to pathogenic effector T cells can occur in the presence of chronic inflammation<sup>[146]</sup>. Another potential side effect of Treg cell therapies based on the use of (donor-derived) allogeneic Tregs is the recipient's production of antibodies recognizing the allogeneic Tregs, resulting in reduced Tregs effectiveness or even adverse immune reactions.

**Ethical and regulatory considerations**

Ethical and regulatory considerations are paramount in the research field of Treg cell-based therapies<sup>[147]</sup>. Ensuring patient safety through rigorous clinical trials is essential, and this requires navigating a complex regulatory landscape. Regulatory agencies must balance the need for thorough clinical trials, which ensure safety first, with the urgency of providing new treatments for T1D patients. From an ethical perspective,

sourcing Tregs, whether autologous or allogeneic, raises questions about donor consent and the potential for therapeutic exploitation. Moreover, if Treg cell-based therapies prove effective for the treatment of T1D in clinical trials, it will be essential to consider major issues related to equitable distribution of and access to such therapies. In this regard, policies for equitable access to health should ensure that these advanced cell therapies are accessible to diverse populations, not just those with greater financial resources.

## REGULATORY T CELL-BASED THERAPY FOR T1D AND FUTURE RESEARCH DIRECTIONS

The future of Treg cell-based therapies for T1D looks promising, driven by significant advancements in Treg-focused research. Recent advancements in genome editing technologies, such as CRISPR/Cas9, have made it possible to make precise modifications to enhance the stability and function of Tregs<sup>[148]</sup>. Researchers are also investigating the possibility of generating antigen-specific Tregs from induced pluripotent stem cells (iPSCs)<sup>[149]</sup>, a strategy that would potentially provide an unlimited source of these cells. Furthermore, advancements in single-cell RNA and TCR sequencing are offering more profound insights into the heterogeneity and plasticity of Tregs<sup>[150]</sup>, potentially facilitating the identification of Treg subpopulations with superior therapeutic potential.

Innovative therapeutic approaches include genetic engineering techniques to generate tailor-made antigen-specific Tregs that can protect tissues from immune-mediated attacks without causing systemic suppression<sup>[151,152]</sup>. Thanks to the recent biotechnological advances, the use of advanced biomaterials and innovative nanoencapsulation strategies may represent an attractive and supportive strategy for Treg cell-based treatment of autoimmune diseases, including T1D<sup>[153]</sup>. Biomaterial-enhanced Treg cell-based immunotherapy may maximize the therapeutic impact of Tregs by generating Tregs with defined antigen specificity and by improving Treg migration, stability, and suppressive capacity in target tissues<sup>[153]</sup>.

In addition, the therapeutic impact of Treg cell-based interventions in T1D may be significantly enhanced when employing combination therapy approaches involving the use of other anti-inflammatory, immunomodulatory and/or beta cell protective agents capable of acting synergistically with antigen-specific Tregs<sup>[18]</sup>. In this regard, it has been demonstrated that combined therapy with Tregs plus the monoclonal anti-CD20 antibody rituximab is consistently superior to Tregs monotherapy in preserving endogenous insulin production and maintaining clinical remission in patients with newly diagnosed T1D<sup>[154]</sup>.

Treg cell-based therapies may significantly contribute to expanding the current applications of precision medicine in the management of T1D. In this regard, tailored Treg cell-based therapies may be investigated in different subgroups of T1D patients based on their specific genetic background and immunological profile. This precision medicine approach may help optimize the safety and efficacy profile of Treg cell-based therapies, reducing the risk of adverse effects and improving clinical outcomes. Further studies are needed to identify novel biomarkers and advanced diagnostic tools that allow clinicians to monitor patients' responses to Treg cell-based therapies and make treatment protocol adjustments to achieve optimal therapeutic effects. While substantial progress has been made in understanding Treg biology and function, current Treg cell-based therapies still face limitations, such as the need to maintain Treg stability and function *in vivo*. Moreover, there is a need for further studies aimed at better understanding the genetic and epigenetic factors influencing Treg responses, in order to develop tailored Treg cell-based therapies for T1D. Additionally, studies comparing the effects of these therapies in T1D and other autoimmune disorders may uncover mechanisms to optimize Treg function, stability, and survival specifically in patients with T1D.

## CONCLUSION

The investigational use of Tregs as a therapeutic strategy for T1D represents a considerable advancement in the pursuit of a more targeted and effective treatment for this autoimmune disease. Tregs are critical in maintaining immune homeostasis and preventing autoimmune responses by inhibiting effector T cells that drive the destruction of pancreatic beta cells. The promising results from preclinical studies and early-phase clinical trials underscore the potential of Treg cell-based therapies to fundamentally alter the course of T1D. Current investigational therapeutic approaches include adoptive Treg cell therapy (based on the use of autologous Tregs) and the use of pharmacologic agents able to enhance Tregs function *in vivo*. Genetic engineering of Tregs represents another promising therapeutic avenue that could lead to durable and robust Treg effects *in vivo*.

However, some challenges need to be overcome before Treg cell-based therapies can be employed in clinical settings. These include the need to develop strategies to optimize the function, stability, and survival of Tregs within inflamed tissues and avoid potential off-target effects or undesired immunosuppression. Developing scalable manufacturing processes for clinical-grade Tregs. Regarding Treg manufacturing for clinical trials, further research is required to define the optimal Treg source and protocols for cell isolation and expansion. Furthermore, personalized approaches may be necessary to account for the heterogeneous immunopathology observed across different T1D patients<sup>[47,155]</sup>.

In conclusion, Treg cell-based therapies are promising therapeutic tools that have the potential to alter the natural history of T1D. Therefore, future randomized controlled trials are warranted to clearly demonstrate and validate the safety and efficacy profile of different types of Treg cell-based therapies used as disease-modifying agents in distinct subgroups of patients with T1D.

## DECLARATIONS

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### Authors' contributions

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All authors approved the final version of the manuscript.

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Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.



**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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