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REVIEW

Genetic engineering of regulatory T cells for treatment of autoimmune disorders including type 1 diabetes

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Abstract

Suppression of pathogenic immune responses is a major goal in the prevention and treatment of type 1 diabetes. Adoptive cell therapy using regulatory T cells (Tregs), a naturally suppressive immune subset that is often dysfunctional in type 1 diabetes, is a promising approach to achieving localised and specific immune suppression in the pancreas or site of islet transplant. However, clinical trials testing administration of polyclonal Tregs in recent-onset type 1 diabetes have observed limited efficacy despite an excellent safety profile. Several barriers to efficacy have been identified, including lack of antigen specificity, low cell persistence post-administration and difficulty in generating sufficient cell numbers. Fortunately, the emergence of advanced gene editing techniques has opened the door to new strategies to engineer Tregs with improved specificity and function. These strategies include the engineering of FOXP3 expression to produce a larger source of suppressive cells for infusion, expressing T cell receptors or chimeric antigen receptors to generate antigen-specific Tregs and improving Treg survival by targeting cytokine pathways. Although these approaches are being applied in a variety of autoimmune and transplant contexts, type 1 diabetes presents unique opportunities and challenges for the genetic engineering of Tregs for adoptive cell therapy. Here we discuss the role of Tregs in type 1 diabetes pathogenesis and the application of Treg engineering in the context of type 1 diabetes.

Keywords Adoptive cell therapy · Chimeric antigen receptor · Gene editing · Regulatory T cell · Review · T cell receptor

Abbreviations

2.5HIP	2.5 hybrid insulin–chromogranin A peptide
APC	Antigen presenting cell
CAR	Chimeric antigen receptor
GVHD	Graft-versus-host disease
G6PC2	Glucose-6-Phosphatase Catalytic Subunit 2
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
MND	Myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted
NSG	NOD scid gamma
PPI	Preproinsulin
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Treg	Regulatory T cell

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Introduction

Type 1 diabetes is an autoimmune disease marked by the destruction of pancreatic islet beta cells by autoantigen-specific T cells, leading to the loss of insulin production and dysregulation of blood glucose. Suppression of this autoimmune response is a major goal in the prevention and treatment of type 1 diabetes. To this end, therapeutic approaches that target regulatory T cells (Tregs), defined by expression of the transcription factor FOXP3 and the high-affinity IL-2 receptor, CD25 [1], are a major area of focus. Unlike traditional immunosuppression, Tregs have the potential to potently inhibit immune responses in an antigen-specific manner without globally impacting beneficial immunity to infection and cancer. The immunomodulatory effects of Tregs are achieved through a broad arsenal of contact-dependent and -independent suppressive mechanisms,

including immunosuppressive cytokine production, inhibition of antigen presenting cell (APC) function, IL-2 consumption and direct effector T cell killing [1]. In this review, we first summarise the role of Tregs in type 1 diabetes pathogenesis and efforts to utilise unmodified cells for treatment, and then describe advanced genetic engineering approaches that aim to improve the efficacy of adoptive Treg therapy by enhancing their phenotype, antigen specificity, and/or function.

Regulatory T cells in type 1 diabetes

A number of functional and genetic studies of Tregs from individuals with type 1 diabetes point to a role for their dysfunction in pathogenesis. Although peripheral blood Treg numbers are normally not affected in type 1 diabetes, their suppressive function may be reduced [2–6] and their signature gene expression profile is altered [7, 8]. Impaired IL-2 signalling in Tregs likely contributes to these changes, as Tregs from people with type 1 diabetes exhibit reduced IL-2-stimulated phosphorylation of a downstream signalling molecule, signal transducer and activator of transcription 5 (STAT5) [9], and increased expression of protein tyrosine phosphatase N2 (PTPN2), a negative regulator of IL-2 signalling [9–11]. SNPs in regions of epigenetic regulation important for Treg identity have also been identified in type 1 diabetes [12]. Direct evidence linking Treg dysfunction to type 1 diabetes comes from the study of immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, wherein FOXP3 mutations lead to varying degrees of Treg dysfunction and multiorgan autoimmunity usually including type 1 diabetes [13].

This strong evidence for Treg dysfunction in type 1 diabetes, combined with their ability to inhibit immune responses in a variety of contexts, has led to multiple efforts to boost their function using autologous cell therapy approaches. Importantly, ex vivo expansion in IL-2 seems to reverse the deficit in IL-2-stimulated STAT5 [14], so infused cells from patients are expected to be more functional. Several phase 1 clinical trials testing autologous Treg administration in recent-onset type 1 diabetes have now been completed; no severe adverse events were observed, indicating that this therapy is safe and well-tolerated [15–18]. Similarly, administration of autologous Tregs together with intraportal allogeneic islet transplantation yielded no severe negative effects [19]. Promisingly, Marek-Trzonkowska et al showed that children who received Treg therapy maintained higher C-peptide levels and had lower insulin requirements 1 year after treatment, compared with a matched control group [16]. Likewise, although the study was not designed to assess efficacy, Bluestone et al found that C-peptide levels were maintained for over 2 years after treatment for some patients, but there were no obvious improvements in disease severity [17]. Importantly, both studies observed a rapid decline in the number of circulating Tregs within weeks of infusion [16, 17]. In addition to the initial rapid decline, Bluestone and colleagues observed a biphasic exponential decay in which the second phase was characterised by a longer-lived Treg subset making up approximately 10–25% of infused cells [17]. These cells, which were detected up to a year post-infusion, primarily bore a naive or memory stem-like phenotype marked by expression of CD45RA and CCR7, as opposed to the pre-infusion central memory-like phenotype characterised as $CCR7^{+}CD45RA^{-}CD45RO^{+}$. Thus, it is clear that even in the context of polyclonal Treg therapy, long-lived cells exist, and these may be instrumental to maintaining efficacy long-term. However, the overall low efficacy of these polyclonal Treg therapies suggests that there is a need to identify new strategies to enhance Treg function, survival and proliferation in vivo. Addition of low-dose IL-2 treatment to Treg therapy can increase Treg numbers in individuals with type 1 diabetes, but this may be accompanied by expansion of inflammatory cell subsets and, therefore, is not an ideal strategy [18]. Together, these results (excellent safety but yet-to-be determined efficacy) are similar to those from a number of other trials testing polyclonal Treg cell therapy in other disease contexts [20]. Thus, a variety of new genetic engineering approaches have emerged that aim to enhance therapeutic efficacy by targeting Treg phenotype, antigen specificity, and survival, with the hope that these modifications will enable Treg therapy to achieve its full potential (Fig. 1).

Engineering Tregs from conventional T cells

A critical barrier in the development of adoptive Treg therapies is achieving sufficient cell numbers without compromising lineage homogeneity. Therapeutic Tregs are generally obtained from peripheral blood by sorting using a combination of cell surface markers, including CD4, high CD25 expression and lack of CD127 [21]. Although the resulting cells are relatively pure immediately post-isolation, contaminating effector T cells, which may also express CD25, can outgrow Tregs over time [22]. Additional selection of the naive ($CD45RA^{+}CD45RO^{-}$) population can enhance purity by eliminating activated T cells that transiently upregulate CD25 [22], but this significantly reduces cell yield. These limitations may also be compounded when using cells from a person with an inflammatory/autoimmune disease.

An alternative to isolation of Tregs is conversion of $CD4^{+}$ T cells to Tregs. Although expression of FOXP3 and suppressive function can be induced by T cell receptor (TCR) stimulation in the presence of TGF-β, the effects are transient and reversed upon its removal [23]. Thus, a preferred

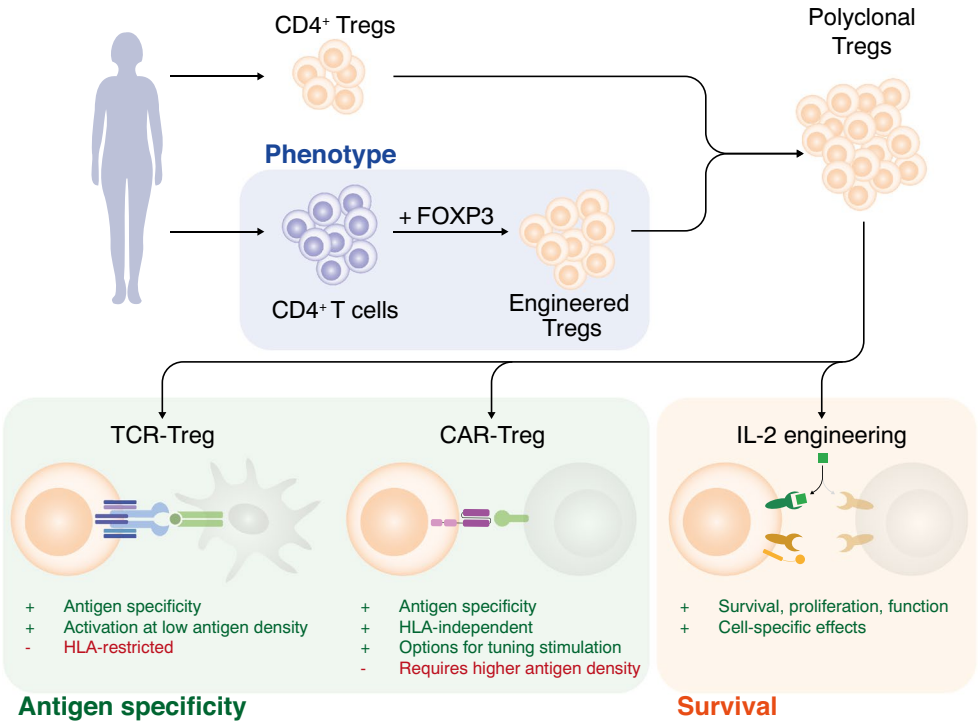

Fig. 1 Strategies to genetically engineer Treg phenotype, antigen specificity or survival for adoptive cell therapy. Tregs for adoptive cell therapy can be directly isolated from peripheral blood or produced from CD4$^+$ T cells by induced expression of FOXP3. Antigen specificity can be conferred on this polyclonal Treg population by expression of disease-relevant TCRs or CARs. In vivo survival of adoptively transferred Tregs can be improved through engineering of IL-2 signalling by expression of an orthogonal IL-2 receptor that only binds an exogenously administered ligand or by expression of surface-bound IL-2 capable only of cis-interactions with receptors. This figure is available as a downloadable slide

approach is viral transduction-mediated overexpression of FOXP3 (Fig. 1), which results in expression of Treg markers, including CD25 and CTLA-4, suppressed production of inflammatory cytokines and acquisition of suppressive function in vitro for both mouse [24–26] and human [27, 28] CD4$^+$ T cells. Notably, FOXP3 transduction conferred suppressive function onto mouse BDC2.5 CD4$^+$ T cells (specific for 2.5 hybrid insulin–chromogranin A peptide [2.5HIP]), which were able to stabilise or reverse recent-onset diabetes in NOD mice upon infusion (Table 1) [29]. The efficacy of polyclonal or antigen-specific converted cells has also been demonstrated in a number of other models of autoimmune and inflammatory conditions, including colitis, allergy, autoimmune dermatitis, haemophilia A and arthritis (reviewed in Tuomela et al [30]). Importantly, the FOXP3-transduction approach has now reached the stage of clinical testing, with active recruitment to a first-in-human trial of lentiviral-mediated transduction of FOXP3 into T cells in patients with IPEX syndrome (NCT05241444) [31].

The concept of induced FOXP3 expression has recently been further refined using recombinase-mediated homology-directed repair to introduce a strong promoter sequence (MND; myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted) upstream of the endogenous FOXP3 locus [32]. This approach reduces the possibility of genotoxicity, gene silencing and variability due to random integration of retro- or lentiviral vectors. When combined with lentiviral transduction of TCRs specific for diabetes-relevant peptide–MHC complexes, the resulting islet-antigen-specific engineered human Tregs reduced co-stimulatory molecule expression on APCs and suppressed effector T cells with the same or distinct TCR specificity (Table 1) [33]. In mice, knock in of the MND promoter into BDC2.5 TCR transgenic T cells resulted in antigen-specific engineered Tregs that could prevent diabetes induced by BDC2.5 T cells or polyclonal T cells from diabetic NOD mice [33].

Despite the observed success in mouse models, uncertainties remain concerning Treg production via induced FOXP3 expression. Although FOXP3 is the ‘master’ transcription factor mediating Treg phenotype, not all aspects of the Treg phenotype can be ascribed to FOXP3. Direct comparison of the gene expression signatures of naturally occurring mouse Tregs and FOXP3-transduced T cells revealed a significant number of genes not regulated by FOXP3 expression alone [34]. Moreover, in mice lacking functional FOXP3,

Table 1 Summary of key pre-clinical studies investigating antigen-specific Treg transfer, TCR-Tregs, CAR-Tregs or FOXP3 engineering in a type 1 diabetes context

Treatment	First…last authors, year	Key findings	Species	Antigen specificity
Antigen-specific Treg transfer				
	Tang…Bluestone, 2004 [40]	$CD4^+$ $CD25^+$ BDC2.5 Tregs expanded using anti-CD3/CD28. BDC2.5 Tregs prevented spontaneous diabetes in NOD.$CD28^{-/-}$ mice, diabetes induced by diabetogenic T cells in NOD.$RAG^{-/-}$ mice, rejection of syngeneic transplanted islets in chronically diabetic NOD mice and progression of new onset diabetes in NOD mice.	Mouse	2.5HIP (BDC2.5)
	Tarbell…Steinman, 2004 [41]	$CD4^+$ $CD25^+$ BDC2.5 Tregs expanded with peptide-pulsed DCs. Prevented diabetes induced by cyclophosphamide in NOD.BDC2.5 mice and diabetogenic splenocytes in NOD.scid mice.	Mouse	2.5HIP (BDC2.5)
	Masteller…Bluestone, 2005 [43]	WT Tregs expanded using p31 (a BDC2.5 TCR mimotope) presented on recombinant I-Ag7- and anti-CD28-coated beads. Reduced spontaneous diabetes in NOD.$CD28^{-/-}$ mice.	Mouse	2.5HIP (BDC2.5)
	Tarbell…Steinman, 2007 [42]	$CD4^+$ $CD25^+$ BDC2.5 Tregs expanded using peptide-pulsed DCs. $CD62L^+$ BDC2.5 Tregs delayed spontaneous diabetes in NOD mice. Reversed hyperglycaemia in recent-onset diabetic NOD mice.	Mouse	2.5HIP (BDC2.5)
	Spence…Tang, 2018 [57]	BDC2.5 Tregs or sorted islet-resident Tregs (thought to be enriched for insulin specificity), prevented diabetes in ~80% of NOD.$CD28^{-/-}$ mice.	Mouse	2.5HIP (BDC2.5) Insulin (indirect)
	Jing…Bettini, 2023 [56]	Tregs expressing a high-affinity but not low-affinity TCR can control diabetes induced by high-affinity effector cells in a NOD.$TCR\alpha^{-/-}$ model.	Mouse	Insulin
TCR-Tregs				
	Hull…Tree, 2017 [49]	Lentiviral transduction of islet-specific TCRs into human $CD4^+$ $CD25^{hi}$ $CD127^{lo}$ Tregs. Mediated bystander suppression of CD4/CD8 T cells in vitro.	Human	HLA-DR4 restricted IA2; HLA-DR3 restricted InsB:11–30
	Yeh…Brusko, 2017 [50]	Lentiviral transduction of islet-specific TCRs into human $CD4^+$ $CD25^+$ $CD127^{lo}$ Tregs. Mediated antigen-specific and bystander suppression in vitro.	Human	HLA-DR4 restricted $GAD_{555–567}$ (×2)
	Yang…Buckner, 2022 [33]	Lentiviral transduction of islet-specific TCRs into human $CD4^+$ T cells followed by induced FOXP3 expression. Mediated antigen-specific and bystander suppression in vitro.	Human	HLA-DR4 restricted G6PC2 (×4), GAD65 (×2), PPI
CAR-Tregs				
	Tenspolde, Zimmerman…Jaeckel, Hardtke-Wolenski, 2019 [63]	Insulin-specific CAR (CD8 hinge; CD8 TM; CD28.CD3ζ signalling) expressed in FOXP3-transduced T cells. Enhanced persistence but no protection against spontaneous diabetes in NOD mice.	Mouse	Insulin

Table 1 (continued)

Treatment	First…last authors, year	Key findings	Species	Antigen specificity
	Spanier, Fung…Fife, Levings, 2023 [66]	'TCR-like' CAR (CD8 hinge; CD28 TM; CD28.CD3ζ signalling) expressed in mouse Tregs. Protected against BDC2.5 T cell-induced diabetes in NOD.RAG$^{-/-}$ mice and spontaneous diabetes in NOD mice.	Mouse	I-Ag7 restricted InsB:10–23
	Obarorakpor…Zhang, 2023 [67]	'TCR-like' CAR (CD28 hinge; CD28 TM; CD28.CD3ζ signalling) expressed in mouse Tregs. Inhibited spontaneous diabetes in NOD.CD28$^{-/-}$ mice and diabetogenic NOD splenocyte-induced diabetes in NSG mice.	Mouse	I-Ag7 restricted InsB:9–23
	Muller, Ferreira…Tang, 2021 [73]	HLA-A2-specific CAR (IgG4 hinge; CD28 TM; CD28.CD3ζ signalling) expressed in human TCR-deficient Tregs. Mediated homing to A2-NSG islets and human A2^{+} islets in diabetic NSG mice.	Human	HLA-A2 (islet transplant)
	Pierini…Meyer, 2017 [74]	Modular CAR (CD28.CD3ζ signalling) binding separate targeting module (anti-H-2D^{d}). Mediated homing of mouse Tregs to MHC-mismatched islet transplant and enhanced graft survival.	Mouse	H-2D^{d} (islet transplant)
FOXP3 engineering				
	Jaeckel…Manns, 2005 [29]	Retroviral transduction of mouse BDC2.5 CD4^{+} T cells with *Foxp3*. Conferred suppressive function in vitro and prevented progression of recent-onset spontaneous diabetes in NOD mice.	Mouse	I-Ag7 restricted 2.5HIP (BDC2.5)
	Yang…Buckner, 2022 [33]	Induced expression of endogenous *FOXP3* gene in islet-specific mouse and human CD4^{+} T cells via introduction of a strong promoter (MND) by CRISPR-Cas9-based HDR editing. Mediated antigen-specific and bystander suppression in vitro. Foxp3-engineered BDC2.5 T cells protected against BDC2.5 T cell-induced or diabetogenic NOD splenocyte-induced diabetes in NSG mice.	Human; Mouse	*Human:* HLA-DR4 restricted G6PC2 (×4), GAD65 (×2), PPI; *Mouse:* I-Ag7 restricted 2.5HIP (BDC2.5)

DC, dendritic cell; HDR, homology-directed repair; TM, transmembrane; WT, wild-type

'Treg-like' cells bearing elements of a Treg genetic signature can develop [35, 36]. Further complexity is added by spliced isoforms of FOXP3, which exist in humans but not other species, particularly as their biological role and regulation is still being defined [37–39]. Overall, genetic engineering of FOXP3 expression represents a viable pathway to producing therapeutic Tregs, but with more research it is possible that the optimal approach to engineering Treg phenotype will progress beyond overexpression of FOXP3.

Engineering antigen specificity

Tregs, like other T cells, rely on the recognition of specific antigens via their TCRs for activation, proliferation and function. The premise of polyclonal Tregs for adoptive cell therapy, as tested in two type 1 diabetes trials [16, 17], depends on the natural existence of rare, disease-relevant TCRs in the cell population. As such, numerous studies in NOD mice have demonstrated that therapy using antigen-specific Tregs is far more effective than with polyclonal cells [40–43] (Table 1). Notably, one study found that as few as 5000 antigen-specific (BDC2.5 TCR transgenic) Tregs could prevent spontaneous autoimmunity in NOD mice, whereas 100,000 polyclonal Tregs failed to have an observable effect [42]. Similarly, transfer of 2 million antigen-specific (BDC2.5 TCR transgenic) Tregs blocked the rejection of a syngeneic islet transplant in NOD mice, but 5 million polyclonal Tregs had no effect [40]. Thus, the lack of antigen specificity may underlie the minimal effect seen in clinical trials of adoptive Treg therapy in type 1 diabetes.

Although antigen-specific Tregs are significantly more potent, isolating a sufficient number of these cells from peripheral blood represents a major barrier. In the mouse studies above, the majority took advantage of BDC2.5 TCR transgenic mice to make antigen-specific cells. In humans, although naturally occurring alloantigen-specific cells can be enriched and expanded from human blood [44, 45], a more feasible approach in type 1 diabetes is to confer Tregs with antigen specificity by introducing exogenous TCRs or chimeric antigen receptors (CARs) through genetic engineering (Fig. 1).

T cell receptors Because Tregs naturally recognise antigens via TCRs, this was the first approach taken to confer antigen specificity to a polyclonal population. Retroviral-mediated gene transfer of TCRs to Tregs has now been tested successfully in a number of mouse models of autoimmunity and transplantation, including arthritis, experimental autoimmune encephalitis, haemophilia A and heart allograft (reviewed in Tuomela et al [30]). Type 1 diabetes represents a uniquely promising area for the application of TCR-Tregs. First, a number of TCRs have already been isolated from islet-infiltrating pathogenic T cells and Tregs, offering multiple options for redirecting Treg specificity [46]. These TCRs are most commonly specific for epitopes derived from GAD65, glucose-6-phosphatase catalytic subunit 2 (G6PC2; also known as IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein), preproinsulin (PPI), proinsulin or insulin [46], and a growing collection of TCRs specific for hybrid peptides [47, 48]. Second, although TCRs are specific to particular HLA alleles, the close association between type 1 diabetes incidence and class II HLA haplotype means that a significant proportion of individuals could be covered by just a few TCRs [46].

The feasibility of using TCRs to redirect Treg specificity to type 1 diabetes-relevant antigens was first demonstrated by Hull et al, who transduced human Tregs with TCRs isolated from two islet-specific $CD4^+$ T cell clones (Table 1) [49]. Although expression of these TCRs, specific to tyrosine phosphatase (IA-2) or insulin presented in the context of HLA-DR3 and HLA-DR4, respectively, resulted in better antigen-specific suppression compared with polyclonal Tregs, function was suboptimal compared with control, influenza-specific Tregs [49]. Subsequently, Yeh et al showed that expression of TCRs specific for $GAD_{555–567}$ presented by HLA-DR4 enabled in vitro Treg suppression of conventional T cells specific for the same antigen, as well as $CD8^+$ T cells specific for MART-1 (melanoma antigen recognised by T cells 1), a model 'bystander' antigen [50]. As discussed above, TCR transduction has also been used to redirect FOXP3-engineered human and mouse T cells towards type 1 diabetes-relevant peptides (G6PC2, GAD65 or PPI for human and 2.5HIP for mouse) resulting in antigen-specific suppressive function [33].

A major finding of studies investigating islet-specific TCR-Tregs is their ability to mediate bystander suppression of T cells with different specificity in vitro [33, 49, 50]. There is also indirect in vivo evidence for bystander suppression by antigen-specific Tregs in NOD mouse models where diabetes occurs spontaneously [40], upon islet transplantation into diabetic mice [42], or as a result of NOD splenocyte transfer [33]. In these models, monospecific Tregs successfully suppress autoimmune diabetes induced by a polyclonal population of $CD4^+$ and $CD8^+$ T cells specific for a number of islet autoantigens, implying a role for bystander suppression. However, the nature and mechanism of bystander suppression in vivo is not well understood, and the potential risk of immune suppression towards pathogens or tumours is not well investigated. Existing evidence with FOXP3-engineered Tregs does show that anti-tumour, -fungal and -bacterial immunity is not impeded by administration [51], but more direct investigation is needed.

A critical factor governing the efficacy of TCRs is affinity or avidity: low-affinity TCR interactions may be insufficient to trigger effective signalling, whereas excessively

high-affinity may lead to cross-reactivity with other antigens [52]. Because higher-affinity TCR stimulation is thought to skew $CD4^+$ T cells towards a Treg phenotype during thymic development [53], it could be inferred that high-affinity TCRs confer superior Treg function. Some studies have suggested that this is indeed the case, with high-affinity or high-functional-avidity TCRs mediating enhanced suppressive function in response to alloantigens or islet-specific antigens [50, 54]. However, more recent work has revealed greater complexity in this area, demonstrating that low-affinity TCRs can, in some contexts, be more suppressive or mediate unique functional effects. In a study of human Tregs expressing islet-specific TCRs, functional avidity, measured by proliferation in response to peptide, was unexpectedly found to negatively correlate with suppression of polyclonal islet-specific effector T cells in vitro [33]. Furthermore, an in vivo comparison of TCRs with varying affinity against insulin showed that both high- and low-affinity Tregs are efficiently recruited to the pancreas but, importantly, act via different mediators [55]. Whereas low-affinity TCRs increased mRNA encoding amphiregulin and a subunit of IL-35, which are involved in tissue repair and protection against autoimmunity, respectively, high-affinity TCRs induced expression of classic Treg functional mediators, including CTLA-4, IL-10 and glucocorticoid-induced TNFR-related protein (GITR). Cells expressing high-affinity TCRs were also more enriched in the pancreatic lymph nodes compared to those with low-affinity TCRs. Nevertheless, both high- and low-affinity Tregs were simultaneously needed to delay the onset of diabetes in NOD mice, suggesting the need for Treg functional diversity in protection from autoimmune diabetes [55]. The efficacy of Tregs with high- or low-affinity TCRs may also vary depending on the TCR affinity of effector T cells, with a recent study finding that low-affinity TCR-Tregs can effectively suppress the infiltration of low-affinity but not high-affinity effector T cells [56]. It is important to note that differences between studies of TCR affinity and Tregs in type 1 diabetes may stem from differences in models and hence properties of the effector T cells being analysed. Overall, a greater understanding of how TCR affinity and avidity affects Treg localisation and suppressive function is needed to fully harness the potential of TCR-Tregs.

Additional factors to consider when designing TCR-Tregs for type 1 diabetes therapy are the source and the specificity of the TCR. Because type 1 diabetes-relevant conventional T cells are more abundant compared with Tregs, these cells provide a larger source of potentially effective TCRs. Use of TCRs from pathogenic islet-infiltrating conventional T cells may also have the benefit of specificity against antigens that are appropriately expressed and localised to trigger an immune response. As such, studies have most commonly used TCRs from islet-reactive conventional $CD4^+$ T cells [33, 49, 50, 55, 56], whereas islet Treg-derived TCRs have only been minimally explored [56, 57]. Extensive comparisons of the efficacy of Treg- vs conventional T cell-derived TCRs have not been carried out. Furthermore, it is not known whether targeting certain antigens, such as hybrid peptides, could be advantageous, as comparisons of TCR antigens are entirely lacking in vivo.

Altogether, TCR-Tregs can be successfully used to treat or prevent type 1 diabetes in mouse models. TCR-Treg products have not yet been tested clinically but considering the number of well-characterised disease-relevant antigens and the close association between incidence and HLA haplotype, type 1 diabetes is an obvious disease to target. Working towards this goal, Abata Therapeutics have recently announced the development of a therapeutic candidate, ABA-201, consisting of a type 1 diabetes-specific TCR-Treg [58]. Clinical studies are expected to begin in 2025. However, TCR selection (including antigen target, receptor source, affinity and HLA restriction) remains a major question in the field.

Chimeric antigen receptors An alternative approach to conferring antigen specificity to Tregs is via CARs, which comprise an extracellular antigen-binding domain linked via hinge and transmembrane domains to an intracellular signalling domain that triggers activation [59]. Unlike TCRs, CARs do not need to be MHC-restricted, permitting greater flexibility in applicability across patient populations with variable HLAs. CARs also offer the advantage of being a relatively modular system in which each component can be chosen to produce desired effect, an area that is being actively explored in Tregs [60, 61]. However, a notable disadvantage of CARs is the requirement for cell-surface-bound or oligomeric antigen, which is needed to cross-link the receptor, meaning that they cannot be used to target intracellular proteins or secreted soluble monomers. The antigen-binding domain is generally comprised of a single-chain variable fragment (scFv) derived from the variable regions of an antibody joined by a linker peptide [59]. First generation CARs primarily used a single CD3ζ signalling domain, but later generation CARs add one or more co-stimulatory domains derived from receptors such as CD28 and 4-1BB to increase potency [59]. The majority of CAR-Tregs in development utilise a CD28 co-stimulatory domain alongside the primary CD3ζ signalling domain [59]. However, systematic comparisons of co-stimulatory domains are still relatively limited [60, 61], and new CAR structures allowing cytokine signalling, safety switches or logic gating of responses are continually emerging [62]. The efficacy of CAR-Tregs has been demonstrated in a broad range of pre-clinical disease models, including experimental autoimmune encephalitis, inflammatory bowel disease, asthma, transplantation, GVHD and haemophilia A (reviewed in Tuomela et al [30]).

Excitingly, the first human CAR-Tregs designed to target the commonly mismatched HLA-A2 are now being tested clinically in kidney transplantation (NCT04817774) and liver transplantation (NCT05234190).

In comparison, broad investigation of CAR-Treg use in the context of type 1 diabetes is limited, likely due to difficulties in identifying a suitable and specific CAR target (Table 1). One attempt at creating mouse CAR-Tregs specific for insulin was unable to prevent spontaneous diabetes in NOD mice, despite long-term persistence after transfer and effective in vitro activity [63]. The cause of this failure is unclear but one possibility is antigen availability, since the CAR could only be activated by immobilised insulin or soluble hexameric insulin. CARs generally rely on greater antigen density relative to TCRs due to reduced engagement of accessory receptors at the immune synapse [64, 65]. However, new approaches are emerging that overcome the lack of type 1 diabetes-specific CAR targets. Two recent studies developed a 'TCR-like' CAR derived from an antibody that recognises an insulin B peptide presented in the context of I-Ag7 MHC class II [66, 67]. NOD Tregs transduced with an InsB-I-Ag7-specific CAR proliferated and activated in response to tetramer or peptide-pulsed splenocytes/APCs [66, 67]. Critically, InsB-I-Ag7 CAR-Tregs also reduced proliferation and IL-2 production by BDC2.5 T cells in vitro, demonstrating effective bystander suppression [66]. Upon in vivo administration, InsB-I-Ag7 CAR-Tregs prevented the development of diabetes induced by BDC2.5 T cells in NOD.RAG$^{-/-}$ mice and significantly reduced the development of spontaneous diabetes in NOD mice [66] or NOD.CD28$^{-/-}$ mice [67]. A consideration is that, as with other CARs, TCR-like CARs may be more dependent on high antigen density compared with TCRs targeting the same peptide–MHC complexes [64, 65]. Another consideration is where the antigen is encountered, i.e. on APCs and/or beta cells. Beta cells themselves can express HLA class I and II under inflammatory conditions [68, 69], although likely at lower levels than on APCs, and so TCR-like CARs may be best suited for APC-based activation. Overall, this new CAR design combines the high specificity of TCRs for peptide–MHC ligands with the powerful and adaptable signalling of CARs to produce a cell therapy with highly promising results in vivo.

Alternative strategies that avoid the difficulty of choosing effective CAR antigens are also available. One approach that could be effective in islet transplantation for type 1 diabetes is targeting HLA mismatch using HLA-A2-specific CAR-Tregs, which have already shown efficacy in delaying skin graft rejection and xenogeneic GVHD in mice [60, 70–72]. In a model of islet transplantation, human HLA-A2-specific CAR-Tregs transferred into NOD scid gamma (NSG) mice rapidly homed to HLA-A2-expressing islets [73]. Similarly, mouse Tregs expressing a modular CAR system, in which the CAR binds modified monoclonal antibodies against the target of interest rather than the target antigen itself, homed to transplanted pancreatic islets expressing a mismatched MHC-I molecule, reduced CD8+ T cell infiltration and prolonged graft survival [74]. Such a modular system could relatively easily be used to target diverse antigens in individuals as an 'off-the-shelf' therapy. In addition, CARs that recognise ligands present in inflammatory environments [75] may also offer an alternative to type 1 diabetes-specific CARs.

Overall, CAR technology is relatively unexplored in the context of type 1 diabetes treatment, particularly due to difficulties in identifying good targets. However, strategies targeting other ligands, such as peptide–MHC, mismatched HLA on transplanted islets or inflammatory markers, offer a promising alternative. In general, the advantage of TCRs in the context of type 1 diabetes is that they can be relatively easily isolated from islet-infiltrating T cells. However, if effective CARs can be designed for appropriate antigens, they offer far greater design flexibility to hone the functional effects and targeting of Tregs.

Supporting Treg survival

Following adoptive transfer of Tregs, it is critical that function is maintained long-term to prevent relapse of chronic disease. However, trials testing adoptive Treg transfer for type 1 diabetes treatment have observed rapid decline of Treg numbers in the blood after infusion, which may contribute to the poor efficacy [16, 17]. Although antigen-specific Tregs generally persist far longer than polyclonal Tregs in mouse models, numbers still progressively decline over time [60]. Understanding why this decrease occurs, and whether or not long-term survival is needed, is critical for improving the efficacy of Treg therapy.

A potential mechanism for poor Treg persistence is lack of sufficient IL-2 signalling. IL-2 has long been recognised as a critical cytokine for Treg survival and function, since Tregs cannot produce it themselves [76]. Furthermore, reduced IL-2 signalling has been identified as a cause of Treg dysfunction and apoptosis in individuals with type 1 diabetes [9, 77]. Therefore, to boost endogenous Treg function, multiple clinical trials have tested the administration of low-dose IL-2 for the treatment of type 1 diabetes [18, 78–82]. Through the use of very low doses of IL-2, this strategy aims to provide Tregs expressing the high-affinity IL-2 receptor, CD25, with IL-2 without activating other immune cells expressing lower-affinity receptors. However, results in type 1 diabetes have been mixed; although most trials observe dose-dependent expansion of Treg numbers, several trials have also reported expansion of inflammatory immune subsets, including CD8$^+$ T cells, natural killer cells, mucosal-associated invariant T cells and neutrophils, which

could contribute to type 1 diabetes progression [18, 78–83]. A number of alternative strategies have been developed to target Tregs more specifically, including IL-2 muteins, agonist antibodies or IL-2-antibody fusions [84–86]. However, even with this increased specificity, CD25 can be expressed on a range of other immune cells apart from Tregs, which could contribute to side effects [87].

To avoid activation of inflammatory immune subsets, several gene editing strategies have been taken to enable the specific targeting of Tregs (Fig. 1). One such approach is the engineering of a synthetic orthogonal receptor–ligand pair, in which T cells are transduced with an orthogonal IL-2 receptor that can only be activated by an exogenously administered synthetic ligand [88]. Transduction of mouse Tregs with this orthogonal IL-2 receptor improved in vivo proliferation and survival upon ligand administration, resulting in long-term heart allograft tolerance [89] and inhibition of GVHD [90]. Alternatively, Tregs can be transduced with a membrane-bound form of IL-2, in which IL-2 is tethered to the membrane by a short linker that only allows cis-interactions between IL-2 and its receptors on the same cell [91]. Co-expression of membrane-bound IL-2 with a CAR resulted in enhanced survival in low IL-2 conditions, as well as prolonged in vivo survival [91]. Yet another strategy is to incorporate the intracellular IL-2 signalling domain into a CAR. In conventional T cells, inclusion of STAT3 and STAT5 binding domains in the cytoplasmic domain of an anti-CD19 CAR resulted in enhanced in vivo persistence and antitumour efficacy [92]. Overall, these approaches could be particularly effective in the context of type 1 diabetes, in which IL-2 signalling is impaired in Tregs, but this has not yet been tested.

In addition to IL-2 signalling, work is ongoing to identify other pathways that could be targeted to enhance Treg survival following infusion. These include strategies to ameliorate host responses against allogeneic Tregs through MHC knockout to produce an ‘off-the-shelf’ cell therapy [93]; overexpression of immunosuppressive cytokines, such as IL-10 [94]; or genetic engineering of metabolic pathways [95]. Overall, as our understanding of Treg function and in vivo survival progresses, it is clear that a myriad of other strategies will continue to emerge with the potential to enhance therapeutic efficacy.

Conclusion and future perspective

As a disease driven by T cell-mediated inflammation and cytotoxicity, type 1 diabetes represents a clear opportunity for application of adoptive Treg therapy. Polyclonal Treg therapy has already been proven to be safe and well-tolerated in the context of type 1 diabetes, but efficacy remains unclear. As such, new genetic engineering strategies are emerging to enhance Treg phenotype through FOXP3 engineering, antigen specificity through TCRs and CARs, and survival through targeting the IL-2 signalling pathway. Since the first patients have now received CAR-Tregs in the context of transplantation (NCT04817774), it is likely that these therapies will begin to reach clinical trial in the context of type 1 diabetes as well. Indeed, the ABA-201 TCR-Treg product is expected to reach clinical trial by 2025 [58].

As genetic engineering strategies progress, more attention will be needed in combining Treg therapy with complementary treatment strategies, such as islet replacement, beta cell protective treatments or other immunomodulatory therapies. Notably, as the anti-CD3 therapy, teplizumab, has achieved United States Food and Drug Administration (FDA) approval for recent-onset type 1 diabetes, understanding its interaction with Treg therapies is critical. A study in NOD mice showed that anti-CD3 therapy prior to polyclonal Treg administration did not enhance donor Treg engraftment and no additive or synergistic efficacy was observed in vivo, but engraftment could be improved using cyclophosphamide prior to Treg injection [96]. Investigation of anti-CD3 therapy with antigen-specific Treg administration has not been carried out. In addition, stem cell-derived islets offer an opportunity to genetically tailor both islet cells and Tregs for synergistic function through, for example, expression of synthetic CAR ligands, co-stimulatory molecules or cytokines to support Treg suppressive function.

Overall, the progress made in the last two decades has been rapid, from the identification of Tregs to the development of engineered antigen-specific cells capable of preventing autoimmune diabetes in mice. Together, these advancements represent significant progress in the process of developing a cure for type 1 diabetes.

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