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Review article

Regulatory T cell therapy for type 1 diabetes: May the force be with you

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A B S T R A C T

Type 1 diabetes mellitus (T1DM) results from autoimmune destruction of insulin producing beta cells. Regulatory T cells (Tregs) have been shown to be defective in this setting. Immuno-therapies targeting T cells, and resetting the balance between T effectors and Tregs, have had some initial success in preserving beta cell function. With a goal to use Tregs themselves as a novel therapeutic, we developed a technique to isolate and expand Tregs from patients with T1DM. These ex vivo expanded $CD4^{+}CD127^{lo/-}CD25^{+}$ cells exhibit improved function and retain their T cell receptor diversity. These cells have subsequently been used in phase I clinical trials in patients with recent onset T1DM. The infusions were well tolerated, with no safety concerns. The studies are too small to assess efficacy definitively, although some individuals exhibit stable beta cell function over intervals as long as 2 years. These efforts set the stage for a larger phase II effort in new onset T1DM, and combination studies with other drugs, as well as efforts in other autoimmune diseases.

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1. Diabetes background

Type 1 diabetes mellitus (T1DM) management remains a daunting clinical challenge. T1DM affects approximately 1.25

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million people in the Unites States [1]. The incidence is rising steadily, particularly for younger children, and projections suggest nearly a tripling of T1DM from 2010 to 2050 [2]. Despite on-going technological advances, the majority of affected patients are not able to achieve recommended glycemic targets [3]. As a result, patients remain at risk for the acute and chronic long term complications associated with the disease, including hypoglycemia, diabetic ketoacidosis, and microvascular disease. Given the lack of definitive clinical therapies to fully mimic beta cell function, one means to circumvent such concerns is via preservation of beta cell function in those at risk for T1DM, or following diagnosis, when 15–40% of beta cell mass remains [4,5].

T1DM occurs in individuals with underlying genetic risk, coupled with synergy from one or more heretofore poorly characterized environmental triggers [6]. The disease results from a breakdown in the mechanisms that mediate immune tolerance, resulting at least in part from expansion of islet-reactive T effector (Teff) cells and subsequent beta cell destruction. Broad immune modifiers, such as with cyclosporine, can extend the honeymoon phase in some patients, but carry the attendant risks of continuous immunosuppression [7,8]. Therapies directed more specifically against T-cells can alter the course of disease, as shown in both preclinical models and in clinical trials [6,9–13]. These trials have met with initial but limited success in most cases, with typical beta cell preservation for 6–12 months, with a subset of treated subjects responding, and no therapy offering robust, extended beta cell preservation over time. We have not yet found an agent that can induce tolerance, wherein a brief course of treatment can fundamentally reset the immune response, and result in long term beta cell preservation.

From the new onset T1D clinical trials efforts to date, two substantial findings have emerged: first, short-term immune regulation of T cells can have a long-term effect on disease progression; and second, immunomodulatory anti-T cell agents that have efficacy, in both animal and human studies, restore the balance between Teff and regulatory T cell (Treg), and induce a Treg subset that is likely to be responsible for the long lived efficacy. Current efforts are underway to develop individual and combination therapies that will eliminate the pathogenic effector cells while promoting Tregs in an effort to induce and sustain long term efficacy. To this end, we have pursued an alternate strategy for treatment of T1DM, utilizing a cell based approach with Tregs. Although applied in multiple pre-clinical settings, at the time that we initiated these studies, Tregs had been used only in graft versus host disease, requiring the use of unrelated cord-blood-derived Treg cells [14]. In this review, we summarize our efforts to isolate, expand and test the clinical effects of Tregs in the autoimmunity setting where the current safety and limited efficacy results suggest that this novel approach should be pursued in larger scale efforts to prevent and treat T1DM, and other autoimmune diseases.

2. Treg overview, and role in DM

The immune system is finely tuned to efficiently target a broad array of diverse pathogens and keep cancer cells in check, while avoiding reactions against self. To control autoimmunity, a number of suppressor cell populations have been defined in rodents and man, including regulatory B cells, dendritic cells and macrophages. However, regulatory T cells (Tregs) have emerged as the major cell subset maintaining tolerance, with the ability to potently suppress the activation and effector function of other immune cells, including CD4 and CD8 T cells, B cells, NK cells, macrophages, and dendritic cells [15,16]. Tregs encompass various subsets of $CD4^{+}$ and $CD8^{+}$ cells and may develop centrally in the thymus (tTregs) or in the periphery from conventional T cells (pTregs) in response to activation signals and exposure to cytokines such as TGFβ and retinoic acid (Fig. 1). Thymically derived Tregs are characterized by their expression of CD4, high levels of CD25, and low levels of CD127. Most importantly, these cells stably express the unique transcription factor Forkhead box P3, FOXP3, which controls the development and function of this small T cell subset. Importantly, these cells have emerged as the vital pathway in maintaining immune homeostasis [17,18]. The Treg T-cell receptor repertoire is highly skewed towards self-reactivity, which may be important in ensuring their ability to prevent the activation of autoreactive effectors, while preserving immune reactivity of effector T cells against foreign pathogens, including viruses, bacteria and immunogenic tumors. The pivotal role of FOXP3 in maintaining Tregs is noted by mutations in the FOXP3 gene, resulting in the *scurfy* mouse and in the IPEX syndrome ((Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) in humans [19]. The mice die from over-whelming inflammation and multi-organ autoimmunity within weeks, while humans may manifest disease as early as the fetal and neonatal period [20], and often die within the first 1–2 years of life unless reconstituted with a bone marrow transplant.

Tregs mediate their effects through a variety of different mechanisms, including directly via cell-to-cell contact or indirectly via secreted factors (Fig. 1) [15,17]. Tregs may inhibit the proliferation or be directly cytotoxic for Teff and various other immune cells, or induce the production of factors directly and indirectly (such as IL-10, IL-35, indoleamine 2,3-dioxygenase 1 (IDO) and TGFβ), which can in turn lead to bystander suppression and promote the development of other regulatory cells in their vicinity, leading to so-called infectious tolerance and robust local regulation [21–24]. Finally, these cells can control the metabolic milieu of an ongoing pathogenic response by controlling amino acid metabolism through expression of CD39 and CD73, key receptors in the adenosine metabolism pathway [25]. It has been postulated, however, that both tTregs and pTregs and their suppression mechanisms can be tailored to the nature of the effector response they regulate, modulated by context and location [26]. Cell surface molecules that are expressed by Tregs, such as CTLA4, PD-1 and LAG3, are also important for their suppression in some settings [23,27–30]. CTLA-4 exhibits the unique ability to mediate the immune response via removal of co-stimulatory ligands from antigen presenting cells via trans-endocytosis [31].

Studies from mice and man indicate that Tregs play a central role in protection from T1DM. T1DM is accelerated in NOD mice depleted of $CD4^{+}CD25^{+}$ Tregs and NOD mice deficient in CD28, which is necessary for the development of Tregs, have high rates of diabetes at an early age [32,33]. Similarly, removal of proliferative signals necessary for Treg development or survival, such as IL-2, exacerbates diabetes in NOD mice [33]. Although the absolute number of Tregs does not appear to be different in NOD compared to control mice, or in the circulation of patients with T1D compared to healthy controls, there is evidence to suggest that Treg function is impaired, particularly in inflammatory settings such as in insulitis. Recently, we and others showed that the number and function of Treg cells was perturbed in NOD mice, and the ability to induce Tregs was reduced in response to TGFβ [34,35]. D'Alise et al. identified defects in Treg transcripts in locations tied to the pathogenesis of diabetes in NOD mice (lamina propria and pancreatic lymph node) [36]. In man, a number of studies have now evaluated whether Treg number or function is altered in T1DM. Although there are conflicting reports, more recent studies report that Treg number is normal, but pinpoint functional defects [37–39]. Individuals carrying the rs12722495 susceptibility gene, which encodes an IL-2RA haplotype, exhibit reduced proliferative responses to IL-2 and decreased suppressive function of Tregs in vitro, which

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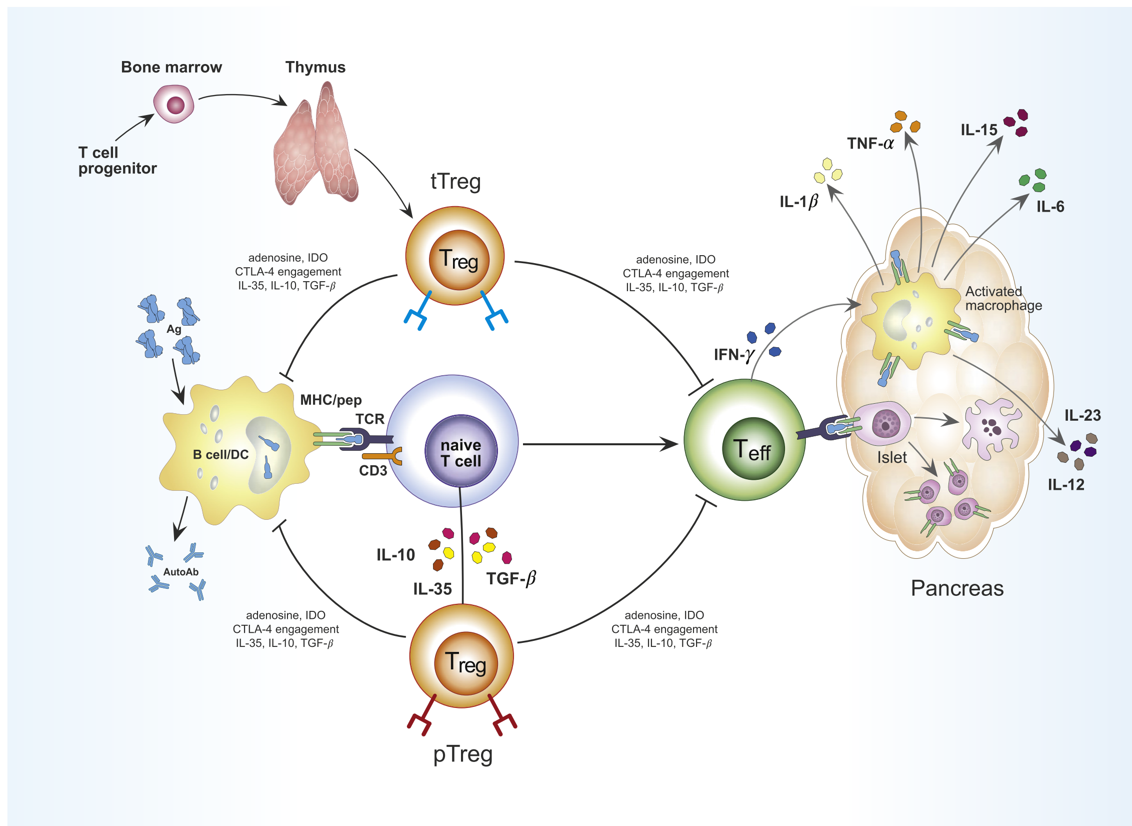

Fig. 1. Current model of Treg development and function: Treg cells develop in the thymus and periphery. In the thymus, a subset of $CD4^{+}CD8^{+}$ T cells escape negative selection through IL-2, and TGF-β signals and become tTreg cells with a high affinity for self antigens. In the periphery, naïve $CD4^{+}$ T cells encounter antigen and differentiate into pTreg cells in the presence of TGF-β, IL-10 and IL-2. These cells also recognize self antigens but utilize a conventional TCR repertoire. Both Treg populations control naïve and ongoing immune responses through a number of independent pathways ranging from direct cell-cell interactions, such as utilized by CTLA-4, to indirect suppression mediated by soluble cytokines (IL-10, IL-35 and TGF-β), metabolic controls (Adenosine metabolism and IDO). The consequence of these activities is to reduce effector T cells function and promote immune tolerance.

presumably indicates impaired Treg function in these patients [40,41]. Notably, in response to IL-2, this defect appears to be reversible: in a previously conducted clinical trial of exogenous IL-2, the impaired STAT5 phosphorylation was improved [39,42].

Therapies that augment the number or function of Tregs have beneficial effects on the progression of T1DM. In pre-clinical studies, Tang et al. observed that IL-2/anti-IL-2 complexes could “repair” Tregs within the pancreas and alter the progression of the disease [34]. Strikingly, Grinberg-Bleyer et al. found that IL-2 reverses established diabetes in NOD mice by a local effect on pancreatic regulatory T cells [43]. Tang et al. showed that infusion of Tregs into NOD mice prevents disease onset and even reverses diabetes in mice with hyperglycemia [44]. Recently, we and our colleagues showed in a humanized mouse model that treatment with teplizumab causes migration of T cells to the lamina propria where they acquire the Treg phenotype and function, characterized by the production of IL-10 and TGF-β [45].

Clinical trials in new onset T1D also support the finding that restoring the balance between Tregs and Teffs preserves beta cell function. Treatment of patients with Fc receptor non-binding anti-CD3 monoclonal antibodies (teplizumab and otelixizumab) has appeared promising across a series of trials [46]: in one recent study, teplizumab preserved beta cell function in approximately half of treated subjects for at least 2 years [46]. Treated subjects had an increase in Treg function and IL-10 production, with a selective decrease in the pro-inflammatory cytokine IFN-γ. Murine studies indicate that the anti-CD3 mAb selectively depletes Teffs while preserving Tregs [47]. Promising findings were also noted in the TIDAL study, in which new onset T1D subjects treated with human leukocyte function antigen-3 (LFA-3)-Ig (Alefacept) exhibited depletion of T central and effector memory cells but preserved Tregs, with an increase in the Treg to Teff ratio, and with resultant preservation of beta cell function approximately 18 months after therapy was stopped [12]. Although anti-thymocyte globulin (ATG) alone was ineffective [48], lower dose ATG coupled with granulocyte colony stimulating factor (GCSF) appears promising in resetting the balance bewteen Tregs and Teffs in recent onset T1D, while preserving beta cell function [49].

Tregs may complement a multipronged approach that includes the initial broad modulation of Teff function by elimination or inducing non-responsiveness [50]. However, in most cases the treated patients are young and therefore can potentially regenerate the pathogenic T cell population that is postulated to account for loss of efficacy over time. In addition, we and others have postulated that the precursor pool of autoreactive cells may be resistant to the effects of immune modulators that have been tested to date and may serve as a continuous source of effector cells that differentiate and cause on-going beta cell damage. Therefore, to have persistent effects, therapies need to target both the effector cells as well as the precursor cells that are their source. We hypothesized that Tregs may fulfill this therapeutic need by offering a safe and effective means to block activation of precursor cells, utilizing essential growth factors needed by effector T cells, and acting via other mechanisms used to destroy autoreactive effector T cells.

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Infused Tregs would be expected to traffic to the areas of inflammation and autoimmunity, in the case of T1DM to the islets, and block local tissue destruction without requiring the generalized immunosuppression or T cell depletion that is utilized by current approaches. Immune modulation may be achieved at the site by bystander suppression and infectious tolerance even without survival of the infused cell product. With this rationale, we proceeded to develop a Treg product for clinical use.

3. Treg manufacture for clinical use

Tregs appear at relatively low frequency, comprising ~5–7% of $CD4^+$ T-cells in peripheral circulation. In order to develop a bona fide clinical therapeutic, we developed a procedure to isolate a pure population of Tregs that would reduce the potential for contamination with potentially pathogenic autoreactive T-cells. Next, we defined a set of growth conditions to expand the cells efficiently, and insure that the final product was equivalent to or better than the initial isolated cells. All of these steps needed to be adapted to a current good manufacturing compliant (cGMP) protocol for clinical application, within a GMP facility using FDA approvable tools and methodology. To that end, Putnam et al. devised a cGMP protocol for Treg isolation and expansion, first demonstrating the ability to produce Tregs from healthy individuals, and then showing that comparable outcomes are obtained from those with T1DM [51]. Following blood draw from the peripheral circulation and isolation of peripheral blood mononuclear cells, Tregs are purified using fluorescence-activated cell sorting (FACS)-based isolation. Three markers, $CD4^+$, $CD25^+$, and $C127^{lo/-}$, identify the majority of $FOXP3^+$ Tregs present in peripheral circulation, and the sorting strategy results in a very high percentage of $FOXP3^+$ cells that are then carried forward for the expansion protocol. The cells are placed in culture and activated and expanded using anti-CD3/anti-CD28-coated beads and IL-2 over a 14 day period, with mean fold expansion of 555 from a unit of blood from our phase I study described below [52].

Several phenotypic and functional assessments were performed to insure that the expanded cells reflected the desired product (Table 1) [51,52]. First, FOXP3 expression post-expansion was assessed by flow cytometry, where we found an average of 92% $FOXP3^+$ cells after expansion. Epigenetic modifications of the FOXP3 locus were also assessed to ensure that the FOXP3 expression was derived from a stable $FOXP3^+$ cell based on the demethylation of the $FOXP3^+$ Treg specific demethylation region (TSDR). Unlike in mice, activated human Teff cells can express lower levels of FOXP3 (about 30% the level of Tregs), however, the FOXP3 TSDR remains methylated and the transcription is generally extinguished over time. Typically, the level of TSDR de-methylation in our expanded cells was consistent with the high level of FOXP3 protein expression. We evaluated T-cell receptor (TCR) β-chain diversity prior to and after expansion, and found that polyclonality was maintained, without preferential expansion of a limited cell clonotypes, suggesting the expansion of a highly diverse Treg cell population. We noted that the expanded Tregs up-regulated several functional cell surface markers, including CCR7, CD38, CD25, CTLA-4, and LAP, which suggested enhanced activity of the expanded cells [16,17].

Finally, we employed an in vitro suppressor assay as a surrogate measure of Treg function [51,52]. Expanded Tregs were mixed with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled $CD8^+$ Tells (responders) that were stimulated with anti-CD3 and anti-CD28. In comparing the function of the expanded Tregs to that of the purified Tregs prior to expansion, we routinely noted 4–8-fold greater suppression by the expanded cells when compared to the matched pre-cultured Treg population. Overall, the patients in the phase I study demonstrated overall suppressive activity greater than 50% at ratios of 1:32 Tresponder/Tregs or lower in dilution. Thus, the expanded Tregs appeared to have greater function on a per cell basis.

Prior studies examining Tregs in a number of disease settings suggest that a subset of Tregs are unstable, with reduced TSDR demethylation and potential production of pathogenic cytokines, such as IFN-γ [53–55]. Thus, it was essential to determine that the expansion procedures did not preferentially expand these subsets, which could result in targeted effector function upon adoptive transfer back into the patient. Although IFN-γ^+ Treg cells were found following Treg expansion, these cells did not increase in frequency or stability when compared to baseline samples [51,52]. Importantly, the overall population of expanded Tregs retained a TSDR demethylation status and suppressive activity, while Teff cytokine secretion (IFN-γ and IL-17) did not increase after the ex vivo expansion.

A previous report noted that Tregs from subjects with T1D may have impaired function due to an IL-2 signaling defect, with reduced phosphorylation of STAT5 (pSTAT5) [39]. This transcription factor is essential for Treg expansion and survival. A prior clinical trial with IL-2 and rapamycin showed that this signaling pathway improved in the Tregs from the T1D patients at least one year post treatment [42]. We addressed whether the in vitro-expanded Tregs would have similar improvement following culture with exogenous IL-2. Indeed, STAT5 phosphorylation following stimulation with IL-2 was improved in the expanded Tregs from T1D patients when compared to that noted at baseline, and was comparable to that observed in healthy controls [52].

Table 1
Phenotypic and functional properties of Tregs after expansion as compared to baseline.

• Phenotypic Analysis	• Functional Analysis
– $CD4^+CD127^{lo/-}CD25^+$	– FOXP3 TSDR unchanged
– $FOXP3^+$	– ↑ pSTAT5 in response to IL-2
– ↑ $CD25^+$	– ↑ suppressive activity
– ↑ $CD45RO^+$	– Teff cytokines unchanged
– ↑ CCR7	
– ↑ CD38	
– ↑ CTLA-4	
– ↑ LAP	
– TCRβ repertoire unchanged	

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Collectively, these data show that a stable Treg cell population could be isolated from peripheral blood and rapidly expanded over a 2 week period to yield a product with improved phenotypic and functional properties when compared to freshly isolated Tregs from the patients (Table 1).

4. Initial clinical trials experience

The first-in-man study of these polyclonal Treg cells focussed on the safety and PK/PD following intravenous infusion of the ex vivo expanded autologous polyclonal Treg product in patients with T1D [52]. Secondary objectives included assessing effects on beta cell function, and surrogate markers of diabete immune responses. The open label, dose escalating phase I trial was conducted at University of California, San Francisco (UCSF) and Yale Universities (NCT01210664). Adult participants within 2 years of T1DM diagnosis received a single infusion of the ex vivo-expanded Treg product. Major eligibility criteria included positivity for at least one β-cell autoantibody; peak C-peptide levels of >0.1 pmol/ml during mixed meal tolerance test; positivity for Epstein-Barr antibody; and no evidence of chronic infection or other illnesses. Four dosing cohorts were established, starting at 0.05×10^8 cells and expanding 8-fold for each subsequent cohort to a maximum of 26×10^8 cells. A total of 14 subjects were enrolled, with 3 subjects in each of the first 2 cohorts and 4 subjects each in the last 2 cohorts.

The doses were selected based on data from murine models, and manufacturing feasibility. Animal studies have shown that as few as 100,000 Tregs can promote tolerance in certain autoimmune settings, but larger numbers of $5–10 \times 10^6$ polyclonal Tregs are needed to prevent or reverse T1D in the NOD mouse. WIth allometric scaling from mice to man, we estimated that 3×10^8 cells would deliver the minimum anticipated biological effect level (MABEL) in man and 150×10^8 cells would be needed for routine dosing. The initial dose was then set approximately 2 logs below MABEL, at 0.05×10^8 per person, for safety purposes, as we anticipated post-infusion expansion of the cells in the host and the potential that the infused cells might survive longer (with higher CD25 expression) than non-activated endogenous Tregs following adoptive transfer and suppress better on a per-cell basis. The upper limits of cells to be infused is near the upper limit of what we can routinely produce with current expansion protocols over this short period of time, and constitutes ~20% of the estimated total Treg number in man [56].

Sixteen of the 26 subjects screened met eligibility criteria, and 14 received a singled dose of Tregs. Two subjects had expanded Tregs that did not meet the release criteria, and were not included in the study. The mean age of the participants was 30 years, with mean duration of T1D of 39 weeks at the time of screening. The safety experience was excellent: no infusion reactions occurred, and the subjects have fared well in the subsequent follow-up, now 2–4 years from Treg infusion (the last subject was treated in October 2014). There has not been evidence for increased number of infections, or trouble resolving infections. Of note, one subject presented shortly after receiving the Treg infusion with evidence of primary cytomegalovirus infection, which resolved shortly thereafter, suggesting no significant generalized immunosuppression as a consequence of the Treg adoptive immunotherapy. The majority of adverse events have been mild to moderate.

We collected data on the effects of Treg infusions on β-cell function and diabetes management in the participants. Serial mixed meal tolerance tests were conducted to evaluate the change in C-peptide area under the curve over time (Fig. 2). Cohorts 1 and 2 were relatively stable over time, with follow-up out to 2 years at the time of this review. The course was more variable in the latter two cohorts, with 3 of 4 subjects in cohort 3 and 2 of 4 subjects in cohort 4 exhibiting a decline of 50% or greater within 78 weeks of follow-up. It is notable that 1 of 4 in each of these cohorts had sub-optimal glycemic control, which may have hastened β-cell decline in those particular subjects. We compared these responses to a cohort of subjects followed in new onset T1D trials in TrialNet, adjusting for age, sex, and duration of DM, and the subjects in cohorts 1–2 did better than expected, whereas subjects in cohorts 3 and 4 showed decline in C-peptide AUC that fell within the expected range (B. Bundy and J. Krischer, unpublished results). Given the limited

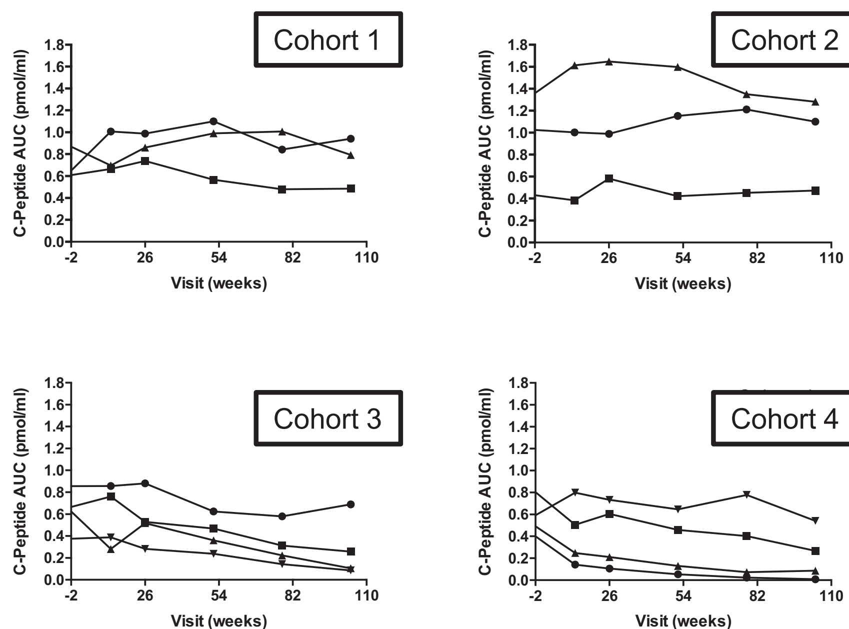

Fig. 2. Change in beta cell function over time after Treg infusion. C-peptide area under the curve (AUC) data from 4-h mixed meal tolerance tests are shown for each of the dosing cohorts (Cohort 1, 0.05×10^8; Cohort 2, 0.4×10^8; Cohort 3, 3.2×10^8, Cohort 4, 26×10^8 cells) from study entry up to 110 weeks later in NCT01210664. The studies and C-peptide AUC calculations were conducted as described previously [52].

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number of subjects in this study, the known heterogeneity in β-cell decline in those with T1D, and the lack of a contemporaneous placebo-treated control group, we are reluctant to draw any firm conclusions from these initial observations. Aside from the single subject in cohort 3 and 4, noted above, the HbA1C values remained within or near American Diabetes Association and study goals, and exogenous insulin use was relatively stable over time, with the exception of one subject in cohort 3. Thus, the clinical findings to date suggest that a single infusion of autologous ex vivo expanded polyclonal Treg product is safe and well tolerated. Further studies are now needed to obtain additional safety data, and determine efficacy.

A number of findings from this experience are noteworthy. First, it was feasible to obtain blood for isolation from subjects at a remote site, expand the Tregs at a central facility and then ship the cells back to the site for infusion. Therefore, a number of collaborative sites could be involved in future studies. Second, the Tregs expanded in vitro showed improved function using measures such as STAT5 phosphorylation to IL-2 and improved suppressor function in an in vitro assay, indicating that the impairments that have been described in Tregs from patients with T1D are reversible. We anticipate that these "repaired" cells will have improved survival and function in vivo. Third, we developed techniques to track the cells in vivo [52]. A transient rise in $CD4^{+}CD127^{lo/-}CD25^{+}$ Tregs was noted by FACS out to day 7, but was back to baseline by day 28. To better track the infused cells, we developed an approach to mark the expanded cells, labeling the Tregs with [6,6-$^{2}H_2$] glucose during their in vitro expansion, with $^{2}H_2$ label incorporated into replicating DNA. We labelled the cells from subjects in cohorts 3 and 4, and then evaluated peripheral blood at various time intervals after the initial infusion, sorting for Tregs and evaluating DNA enrichment by gas chromatography-mass spectrometry of genomic DNA. The maximal percentage of cells in the circulation was detected 7–14 days post-infusion. By 90 days post-infusion, approximately 25% of the labelled cells were still present in the peripheral circulation. Slower decline in label was noted over the ensuing 9 months, with prolonged presence of adopted Tregs still noted in the peripheral circulation even out to one year. This suggests a biphasic decay curve, with the initial half-life of ~20 days, and the second slow phase of 1 year or more in 4 of the 7 patients studied. One limitation of this assay is that we cannot determine why there has been a decrease in label, with formal possibilities including cell death; cell migration out of peripheral circulation and into lymph nodes or sites of inflammation (such as the pancreas); Treg proliferation, with dilution of the signal; or possible transformation of the Tregs into effector cells. To address the latter concern, we assessed various $CD4^{+}$ T cell subsets not in the Treg gated population, and failed to detect label in any population other than Tregs [52].

While our trial was being initiated, Marek-Trzonkowska and colleagues launched an open label non-randomized parallel matched group dose-escalation trial of expanded autologous $CD4^{+}CD127^{lo/-}CD25^{+}$ polyclonal Tregs in children with new onset T1D [57,58]. Ten subjects were enrolled initially in the treatment arm, but 2 were lost to follow-up and subsequently replaced; 10 untreated control subjects were used as comparators, matched for age, sex and disease duration. Two Treg cell doses were initially evaluated: a single dose of either 10×10^6/kg cells or 20×10^6/kg. The primary efficacy endpoint of the trial was the frequency of clinical remission, defined as daily dose of insulin (DDI) $\leq$0.5 IU/kg, and fasting C-peptide > 0.5 ng/ml one year after enrollment. During the conduct of the trial, 6 previously treated subjects were considered to have a waning clinical response over the ensuing 6–9 months, and a second dose of Tregs was given, up to a maximum total cell dose of 30×10^6 cells/kg. One year after study entry, eight of 12 treated subjects met the criteria of clinical remission, with 2 off of exogenous insulin; 2 of the 10 control patients remained in remission. Although the study interpretation was limited by small sample size, there was a suggestion that the higher dose of cells was better, as the 2 subjects off of supplemental insulin and 3 other subjects in remission received the higher 30×10^6/kg total Treg cell dose. Glucagon-stimulated C-peptide was assessed on a subset of treated patients and all of the controls at one year, suggesting more robust beta cell function in the treated group. As in our adult study, no significant safety issues were observed: no infusion reactions were noted, and there were no severe or serious adverse events reported. Taken together, the initial findings from these two phase I studies suggest that infusion of autologous ex vivo expanded Tregs is safe and well tolerated, and further study is necessary to explore efficacy.

5. Next steps

5.1. Optimizing the protocol for Treg isolation and expansion

There are several aspects of our current protocol for Treg isolation and expansion that can be optimized. Two of the 16 subjects in our current trial had expanded Tregs that did not meet our release criteria for infusion. Improved reagents may help optimize sorting and selection of cells while minimizing the risk for contamination. The starting number of Tregs used for our expansion protocol requires a significant blood volume to be obtained at baseline, and thus we are currently limited to participants of ~40 kg or greater. However, the incidence of T1DM is highest for younger children, and if the therapy is proven safe and effective in adolescents and adults, we would ideally like to offer such a therapy to smaller subjects. Thus, we need to find alternate means to more efficiently derive the necessary cell product. Considerations include starting with a smaller number of cells but devising means to increase the growth rate, or employing a longer culture time, or even utilizing leukapheresis. We are currently unable to freeze expanded cells and use them at a later date, but if this is established, a series of smaller blood draws could be used for Treg isolation and expansion, with a pooled sample administered at a later date. In addition, under our current operating procedures, expanded Tregs must be administered within 28 h after harvesting, yet the patient may have acute issues such as infection that preclude Treg administration within that window. Thus, those cells would need to be discarded, and the patient would need to return at a later date for a new blood draw to re-initiate Treg isolation and expansion, at significant expense and inconvenience, and they may not meet timelines in the context of a clinical research trial; the ability to archive expanded Tregs would circumvent these potential issues.

5.2. Identifying the optimal dose and duration of therapy

Based on the limited number of subjects evaluated to date, the optimal Treg dose has not yet been established. It is estimated that the total number of Tregs in a human is ~13×10^9 [56]. Therefore the infusion of 26×10^8 cells used in our highest dosing cohort represents 20% of the total number of cells. It is also possible that a lower cell number may be optimal - an anecdotal observation in our current phase I clinical trial but a focus for validation in a subsequent phase II study in which investigators will evaluate 2.5×10^6 versus 20×10^6 cells/kg administered to adolescents with recent onset T1DM (NCT02691247). Marek-Trzonkowska et al. suggested that the effect from Treg infusion may have waned in some children by 6–9 months [58], but approximately half of the treated adult subjects had stable β-cell function for at least 24 months [52]. Our future studies, using labelled Tregs, will provide information on the

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duration of the cells in the peripheral circulation.

5.3. Identifying subjects most likely to respond

Treg studies to date have involved patients with new onset T1D. However, recent data from our group suggests that the majority of beta cell killing occurs in the period prior to diagnosis with hyperglycemia [59]. If safe and effective in new onset T1DM, we anticipate pursuing use of Tregs for T1D prevention, targeting those found to be at risk through screening programs for relatives of affected individuals, such as offered via TrialNet [60].

5.4. Combination therapy

More robust success may hinge on combination therapy [61]. We have considered what might be used in combination with the Tregs to optimize the response. We posit that one successful approach would be to augment the Treg pool with an ex vivo expanded Treg infusion, while debulking the Teff population with drugs such as anti-CD3 monoclonal antibody or LFA3-Ig, which have already shown some promise in new onset T1D trials [11,12].

Another consideration is coupling the Treg cell infusion with IL-2. In vivo, at low doses, IL-2 serves as a vital growth and survival factor for Tregs [62], and it is an essential component in our ex vivo Treg expansion protocol [51]. Low dose IL-2 has been used to boost endogenous Tregs, with prevention and reversal of diabetes in the NOD mouse [34,43]. Early clinical studies with low dose IL-2 have resulted in selective Treg increases with salutary clinical responses in alopecia areata, HCV-induced vasculitis, and graft versus host disease [63–65]. A clinical trial with IL-2 plus rapamycin showed transient worsening in beta cell function [42], likely due to either the relatively higher dose of IL-2 employed and/or the rapamycin, with a dramatic increase in natural killer cells and eosinophils. However, early T1D studies with lower IL-2 doses have shown no acute worsening in β-cell function [66,67], and several studies are underway now to further assess safety and efficacy. In an upcoming phase I trial with autologous ex vivo expanded Tregs followed by low dose IL-2, we will evaluate if low dose IL-2 enhances the survival and function of the infused Tregs in vivo.

5.5. Source of Tregs

Umbilical cord blood has been considered as an alternate source of Tregs, as it contains limited numbers of $CD25^+$ antigen-experienced memory T cells, reducing the likelihood of contamination with antigen-primed Teff cells, and may have greater potential for expansion [68]. Cord blood is increasingly stored at birth, and thus potentially available for use as an autologous therapy. Brunstein and colleagues have utilized ex vivo expanded Tregs from HLA-matched umbilical cord blood for treatment of graft versus host disease [14,69]. Two smaller studies have evaluated the effect of non-expanded autologous umbilical cord blood transfusions into children with recent onset T1D [70,71]. Although well tolerated, neither demonstrated an impact on decline in beta cell function, likely due to an insufficient number of Tregs or loss of function with long-term storage of the cells. Nonetheless, improvements in culture methods may enable investigators to use umbilical cord blood as a source for Tregs.

5.6. Antigen specific Tregs

The Tregs that we administered in our initial T1DM study did not express TCRs that were specific for diabetes antigens. There were a number of reasons for the choice of polyclonal Tregs for administration. First, their efficacy has been shown in animal models, and the animal data from studies with exogenous IL-2 that enhance the number of Tregs indicate that polyclonal cells are efficacious. Unlike other cellular events, the mechanisms of Treg-mediated suppression are not antigen-specific and therefore polyclonal T cells should be capable of regulating cells with specificities for diabetes antigens. Finally, we have shown the safety of the polyclonal cells, a primary consideration in designing a clinical research program. Pre-clinical studies suggest that antigen-specific Tregs are more effective in controlling autoimmune-mediated beta cell destruction compared to polyclonal Tregs [44]. Although in T1DM target antigens have been defined [72], a major challenge lies in isolating these rarer cells from peripheral circulation, and then expanding them for clinical use. Thus, one may be best served in generating antigen-specific Tregs de novo. Chimeric antigen receptor (CAR) engineered Tregs have been successful in pre-clinical models of autoimmunity [73–76], and may be feasible for clinical use, although the particular antigen profile may need to be adjusted for each affected patient.

5.7. Other indications

As the efforts with Tregs in T1DM have been evolving, other indications are being explored, and these may serve to further inform our efforts in T1DM. Treg therapy was first evaluated in graft versus host disease following allogeneic hematopoietic stem cell transplantation, with 6 published studies employing $FOXP3^+$Tregs or Tr1 cells [14,69,77–81]. No safety issues have been reported, and a decreased incidence of graft versus host disease has been reported in many of the studies. Further efforts are underway in other autoimmune settings (Table 2), including the same ex vivo expanded polyclonal Treg cell product utilized in our adult T1D

Table 2
Treg trials in autoimmunity.

Indication, PI/Institution	Treg source	Dose	Study design	Status
Type 1 Diabetes				
Bluestone/UCSF	polyclonal expanded	$0.05–26 \times 10^8$	Phase I	Published [52]
• Marek-Trzonkowska/Medical University of Gdansk	polyclonal expanded	$10–30 \times 10^6$/kg	Phase I	Published [57,58]
• Gitelman/UCSF; Griffin/Sanford	polyclonal expanded	2.5×10^6, 20×10^6/kg	Phase II	Recruiting
• Bluestone/UCSF	polyclonal expanded + IL-2	3×10^6, 20×10^6/kg	Phase I	Planning
Active cutaneous lupus				
• Dall'Era/UCSF	polyclonal expanded	$0.05–3.2 \times 10^8$	Phase I	Recruiting
Inflammatory Bowel Disease				
• Desreumaux/Hospital Huriez,Lille	Ovalbumin-specific Tregs	$10^6–10^9$	Phase I/IIa	Published [82]
Uveitis				
• Bodaghi/Hopitaux de Paris	polyclonal expanded (intravitreous)	$0.4–3.6 \times 10^6$	Phase I	Recruiting
Pemphigus vulgaris				
• Haley/UCSF	polyclonal expanded	2.5×10^8, 1×10^9	Phase I	Planning

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study for a phase I study with active cutaneous lupus (NCT02428309) and pemphigus vulgaris. An ovalbumin-specific Treg phase I/IIa trial has been conducted in inflammatory bowel disease [82], and polyclonal Tregs are also being considered for a future study [83]. In mice, pre-activated polyclonal Tregs injected into the vitreous control autoimmune uveitis, and this approach is now being evaluated in a phase I study (NCT02494492) [84]. Efforts are also underway to utilize Tregs in solid organ transplantation to induce tolerance to allo-antigens. Of particular interest is The ONE study, a multi-center effort to systematically evaluate various immuno-modulatory cell products in order to determine the optimal means to induce tolerance (NCT02244801) [85]. Biopsy of the affected organ will likely afford unique insights into Treg therapeutics, as pancreatic lymph node and pancreas biopsy is not feasible in our T1D trials. As Treg therapy for organ transplantation evolves, one can envision utilizing Tregs in conjunction with beta cell replacement therapy, either whole pancreas, islet transplantation, or in combination with beta cells derived from stem cells.

6. Summary

In summary, Tregs offer a promising novel cell-based approach to curtailing autoimmune destruction of beta cells in T1DM. Initial phase I studies in adults and children have shown that it is possible to isolate and expand a relatively pure population of Tregs, and the final product appears better than that derived at baseline, based on in vitro assays. The infusions are well tolerated, with no safety concerns noted to date. The studies are too small to assess efficacy, although some individuals exhibit stable beta cell function over intervals as long as 2 years. A phase II study in adolescents with recent onset T1D will offer further insights into safety and efficacy, and a phase I study coupling Treg infusion with low dose IL-2 will be a first attempt at a combination therapy to enhance Treg efficacy. These represent early efforts in a rapidly emerging field that includes other trials in both autoimmunity and alloimmunity. Open questions include: defining the optimal dose of cells to infuse; determining duration of effect, and whether re-dosing is necessary; what is the best source of Tregs; and how can the expansion protocol be optimized. Insights from Treg trials in other settings may help optimize the approaches in T1DM, and may lead to Treg use in disease prevention and in beta cell replacement.

Disclosures

Dr. Gitelman reports receiving personal fees from Caladrius Biosciences, Sanofi, and Tolerion. Dr. Herold reports receiving personal fees from Caladrius. Dr. Bluestone reports receiving personal fees from Caladrius, grants and personal fees from Juno, and non-financial support from Becton Dickinson.

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