Title
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Permalink
https://escholarship.org/uc/item/6664t6jz

Journal
Science Translational Medicine, 7(315)

ISSN
1946-6234

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Publication Date
2015-11-25

DOI
10.1126/scitranslmed.aad4134

Peer reviewed
**IMMUNOTHERAPY**

**Type 1 diabetes immunotherapy using polyclonal regulatory T cells**

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Type 1 diabetes (T1D) is an autoimmune disease that occurs in genetically susceptible individuals. Regulatory T cells (Treg) have been shown to be effective in the autoimmune disease setting. Thus, efforts to repair or replace Treg in T1D may reverse autoimmunity and protect the remaining insulin-producing β cells. On the basis of this premise, a robust technique has been developed to isolate and expand Treg from patients with T1D. The expanded Treg retained their T cell receptor diversity and demonstrated enhanced functional activity. We report on a phase 1 trial to assess safety of Treg adoptive immunotherapy in T1D. Fourteen adult subjects with T1D, in four dosing cohorts, received ex vivo-expanded autologous CD4+CD127lo/−CD25+ polyclonal Treg (0.05 × 10^8 to 26 × 10^8 cells). A subset of the adoptively transferred Treg was long-lived, with up to 25% of the peak level remaining in the circulation at 1 year after transfer. Immune studies showed transient increases in Treg in recipients and retained a broad Treg phenotype long-term. There were no infusion reactions or cell therapy–related high-grade adverse events. C-peptide levels persisted out to 2+ years after transfer in several individuals. These results support the development of a phase 2 trial to test efficacy of the Treg therapy.

**INTRODUCTION**

Type 1 diabetes (T1D) is an autoimmune disease that occurs in genetically susceptible individuals, influenced by the environment and stochastic events (1). These conditions result in immune dysregulation, leading to the generation of pathogenic T cells and destruction of β cells in the islets of Langerhans. T1D is one of the most prevalent chronic diseases of childhood. Despite advances in insulin formulations, insulin delivery systems, and glucose monitoring, less than one-third of patients meet clinical care targets needed to prevent secondary end-organ complications such as retinal, renal, and neurological disease (2, 3). Thus, it is not surprising that the past two decades of research have focused on developing new therapeutics to prevent and treat this devastating disease. Several immunomodulatory therapies, including anti-CD3 (teplizumab) (4), LFA31g (alafaccept) (5), and anti-thymocyte globulin (thymoglobulin) in combination with granulocyte colony-stimulating factor with or without cyclophosphamide (6), and bone marrow transplantation (6, 7), have shown some promise for the treatment of T1D, yet none has induced permanent immune tolerance (that is, nonresponsiveness to self-tissues or foreign tissues without the need for continuous immune suppression) or resulted in long-term insulin independence. However, one common finding that has emerged from these studies is that the major immunomodulatory effect was to induce, or preferentially support, a regulatory T cell (Treg) subset that is likely to be responsible for the drug efficacy (8). Indeed, this key discovery has provided increased emphasis on the development of Treg and Treg supportive therapies for the treatment of this challenging disease.

Treg were initially described as a population of CD4+CD25+ T cells that are critical for controlling autoimmunity and tolerance (9, 10). Treg inhibit effector T cell (Teff) responses both in vitro and in vivo through a variety of activities including cell-cell contact and soluble factors (11). The identification of the transcription factor FOXP3 as a lineage marker for Treg has been instrumental in advancing the field. Mutations or deficiency in the FOXP3 gene in scurfy mice or immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients results in a reduced and/or nonfunctional Treg compartment, leading to a fatal multiorgan autoimmune disease (12). FOXP3 controls many aspects of Treg biology, including their development, transcriptional program, and suppressive function in vitro and in vivo. Thus, CD4+CD25+FOXP3 Treg are an essential immunosuppressive cell population for the extrinsic control of immune homeostasis and control of autoimmunity and have a unique and highly robust therapeutic profile. Although Treg require specific T cell receptor (TCR)–mediated activation to develop regulatory activity, their effector function regulates local inflammatory responses through a combination of cell-cell contact and suppressive cytokine production (11, 13, 14). Thus, Treg specific for a limited number of antigens can efficiently suppress a polyclonal autoreactive response due to dominant antigen nonspecific immunoregulation termed “bystander suppression.” Moreover, activated Treg can recruit additional regulatory cell populations through a process termed infectious tolerance to achieve long-lasting disease protection (14). There is increasing evidence in mouse models that the adoptive transfer (AT) of Treg in multiple disease settings, including T1D, results in disease prevention and, in many cases, disease remission (15, 16). Recently, we and others have shown that Treg are defective in a wide variety of autoimmune diseases, including T1D (17). These defects are manifested by loss of Treg number in inflamed tissues, reduced signaling through the interleukin-2 (IL-2) receptor (based on reduced signal
transducer and activator of transcription 5 (STAT5) phosphorylation],
and instability of the suppressive activities of the cells in vitro and in vivo
(18). These observations have opened an important new concept of drug
intervention in autoimmunity, namely, Tregs as immunotherapeutics,
particularly if the abnormalities observed in Tregs in vivo can be corrected
through their expansion.

Unlike mice, isolation of Tregs based on the CD4 and CD25 markers
is not sufficient to isolate most of the FOXP3+ Tregs without the risk of
contamination with potentially autoreactive T eff. We have shown that a
combination of cell surface markers—CD4, CD25, and CD127—provides
a robust cocktail to isolate FOXP3+ Tregs by fluorescence-activated cell
sorting (FACS) from peripheral blood of subjects with T1D (19). On the
basis of this selection method, we developed a clinical-scale expansion
process for obtaining, in many instances, greater than 3 × 10^9 Tregs after
in vitro expansion of cells sorted from peripheral blood lymphocytes
from a single donor (20).

Here, we describe the results of a phase 1 trial in recent-onset T1D.
The study included four dosing cohorts (a total of 14 adult patients) that
received expanded polyclonal Tregs (polyTregs) ranging from ~5 × 10^6
to ~2.6 × 10^9 cells in a single infusion. The expansion process resulted
in Tregs with enhanced STAT5 phosphorylation in response to IL-2,
increased Treg suppressive activity in vitro, and long-term survival in
vivo (greater than 1 year). The cell therapy was well tolerated with no
evidence of short-term toxicities (including infusion reactions or cyto-
kine release syndrome), precipitous decline in endogenous insulin pro-
duction, or opportunistic infections.

RESULTS

Study design

The trial was a phase 1, two-center, open-label, dose-escalation study
conducted at the University of California, San Francisco (UCSF) and
Yale University in which participants with recent-onset T1D received
a single infusion of ex vivo–expanded autologous CD4+CD127lo/CD25+
polyTregs in four dosing cohorts (Fig. 1). The first subject in each cohort
was observed for 3 weeks after infusion, after which time the study team
met to review cumulative safety data. If no grade 3 or higher adverse
event was observed, subsequent subjects in that cohort could be treated.

After the 13-week follow-up visit of the last subject in each cohort, an
independent data and safety monitoring board (DSMB) reviewed
cumulative safety data for approval to escalate the dose and progress
to the next cohort. Primary outcome measures were adverse events, lab-
oratory abnormalities, and other signs of toxicity. Secondary diabetes-
related outcome measures included C-peptide response during mixed
meal tolerance tests (MMTTs), insulin use, and hemoglobin A1c
(HbA1c). Figure 1 highlights the schedule of events that includes rele-
vant time points for patient blood sampling for various assessments
described below. Table 1 describes the endpoints for the study as
defined. This study was approved by institutional review boards at
UCSF and Yale University and is registered with ClinicalTrials.gov
(NCT01210664).

Patient characteristics

Twenty-six patients were screened, 16 met eligibility criteria and
were enrolled, and 14 subjects received a single infusion of polyTregs.
Table 2 shows the demographic and baseline characteristics of the
treated subjects. Of the 14 treated subjects, 6 were female and 8 were

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**Fig. 1. Study design. (A) Dose escalation plan. Subjects were enrolled in four
cohorts with target doses ranging from 0.05 × 10^8 to 26 × 10^8, where the dose
was escalated by eightfold for each subsequent cohort. The first subject in each
cohort received a single infusion of polyTregs and was observed for a minimum
of 3 weeks for dose-limiting toxicities (DLT), after which time clinical data were
extracted from the database and study team safety review was conducted. If no
grade 3 or higher adverse event was observed, treatment of subsequent
subjects in that cohort could proceed. Otherwise, treatment would be
suspended for DSMB review. After treatment of the last subject in each cohort,
subjects were observed for a minimum of 13 weeks. The study team reviewed
cumulative data to assess for any grade 3 or higher related adverse event, any
related serious adverse event, undetectable C-peptide in an MMTT at week 13
in two subjects, or any other significant safety concerns based on other
considerations. The study team’s review decision was reported to the DSMB
for approval before proceeding to the next dosing cohort. (B) Subject schedule
of events. Blood (target of 400 ml) for Treg manufacturing was drawn at week
−2, and Treg infusion was given on day 0. Subjects were seen for follow-up as-
sessments on day 4, then weekly for the first 4 weeks, then every 13 weeks for
the first year, and then every 6 months for 2 years, and contacted by phone
every 6 months for years 3 to 5 to assess for adverse events.**
male. The mean age was 30.3 ± 8.7 years, and the mean disease duration was 39 ± 26.4 weeks at the time of screening. The mean follow-up at the time of data cutoff for this article was 124 weeks (cohort 1: 182 weeks; cohort 2: 156 weeks; cohort 3: 104 weeks; and cohort 4: 78 weeks). The two subjects who did not receive expanded Tregs were not included in the data analysis.

Table 1. Primary and secondary study objectives and endpoints.

<table>
<thead>
<tr>
<th>Objectives</th>
<th>Endpoints</th>
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<tr>
<td>Primary objective</td>
<td>Primary endpoints</td>
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<td>Assess the safety and feasibility of intravenous infusion of ex vivo-selected and ex vivo-expanded autologous polyTregs in patients with T1D</td>
<td>Adverse events</td>
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<tr>
<td>Laboratory abnormalities</td>
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<td>Signs of toxicity</td>
<td></td>
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<tr>
<td>Infusion reactions</td>
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<tr>
<td>Complications related to infection</td>
<td></td>
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<tr>
<td>Potential negative impact on the course of diabetes</td>
<td></td>
</tr>
<tr>
<td>Secondary endpoints</td>
<td></td>
</tr>
<tr>
<td>C-peptide response during MMTTs</td>
<td></td>
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<tr>
<td>Insulin use</td>
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<td>HbA1c</td>
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</table>

Secondary objectives

Effects on endogenous insulin secretion: Assess the effect of Tregs on β cell function and on other measures of diabetes severity
Surrogate markers of diabetes immune response: Measure the effect of Tregs on the pathologic autoimmune response underlying T1D and on general immune responsiveness

Table 2. Subject characteristics. Subjects are listed in order of enrollment.

<table>
<thead>
<tr>
<th>Subject ID</th>
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<th>Sex</th>
<th>Time from diagnosis at screening (weeks)</th>
<th>HbA1c at screening (%)</th>
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Treg isolation and expansion

We have established a robust selection and expansion method for polyTregs from individuals with T1D, where three cell surface markers—CD4, CD25, and CD127—were used to FACS purify the FOXP3+ Tregs present in the peripheral blood as described previously (20). Purified Tregs were cultured with clinical-grade Dynabeads coated with anti-CD3 and anti-CD28 plus recombinant IL-2. As seen in Table 3, a unit of blood yielded between 4.2 × 10^6 and 11.8 × 10^6 purified CD4+ CD127lo/− CD25+ Tregs, which on average expanded 554.7 ± 370.2–fold (SD) (ranging from 29.8-fold to 1366.8-fold), with each incremental cohort averaging a greater fold expansion as our experience progressed (Table 3). The expression of FOXP3 has been the most reliable marker of Tregs. On average, the expanded Treg preparations that were infused were 92.2% FOXP3+ (range, 76 to 96.9%), with only two preparations <90% positive. The Treg preparations met the additional release criteria of high viability (cutoff ≥85%, actual >98%), high CD4+ percentage (≥95%), and low CD8+ cell contamination (cutoff ≤5%, actual <2.5%) (Table 4). Of 16 attempted expansions, two preparations of expanded Tregs did not meet release criteria. The cell preparation for patient 002-011 had a population (7.36%) of CD4+CD8+ “double-positive” Tregs in the expanded population, which exceeded the release criterion of ≤5% CD8+ cells. However, follow-up analyses showed that the double-positive cells were FOXP3+, did not change during the culture period, and suppressed efficiently in vitro. Moreover, the percentage of double-positive cells did not change during the culture period, suggesting that these cells were indeed present in the circulation of this donor, perhaps representing a small population of tissue-derived Tregs as has been suggested for Teffs previously (21). Preparation for patient 007-014 contained a population of CD4+ Teffs in the expanded cultures (about 80%). Subsequent studies showed that the Teff “contamination” was due to a low level of expression of CD127 on Teffs making it difficult, with current clinical-grade anti-CD127 monoclonal antibodies, to completely separate the Teff from Treg even after CD4 and CD25 gating.
Table 3. Initial T\textsubscript{reg} purity and expansion. The number of T\textsubscript{reg} isolated from ~400 ml of whole blood, the T\textsubscript{reg} purity (%CD4\textsuperscript{+}CD127\textsuperscript{lo}/CD25\textsuperscript{+}) after FACS, total number of T\textsubscript{reg} after 14-day expansion, fold increase from number of T\textsubscript{reg} seeded, and the total number of T\textsubscript{reg} infused.

<table>
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<th>Cohort</th>
<th>Subject ID</th>
<th>Number of T\textsubscript{reg} before expansion (×10\textsuperscript{6})</th>
<th>T\textsubscript{reg} purity (%)</th>
<th>Number of T\textsubscript{reg} after expansion (×10\textsuperscript{9})</th>
<th>Fold expansion</th>
<th>Number of T\textsubscript{reg} infused (×10\textsuperscript{8})</th>
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<td>98.1</td>
<td>3.20</td>
<td>677.2</td>
<td>23.5</td>
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Table 4. Final T\textsubscript{reg} release specifications and results. Final cell product was assessed for identity (≥60% FOXP3\textsuperscript{+} and ≥95% CD4\textsuperscript{+} cells), purity (≤5% CD8\textsuperscript{+} cells, <100 beads per 3 × 10\textsuperscript{6} cells, and endotoxin ≤3.5 EU/ml), sterility (negative for mycoplasma, anaerobic and aerobic bacteria, gram stain, fungal culture, KOH exam), and viability (≥85%).

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<td>Percentage of FOXP3\textsuperscript{+} cells (≥60%)</td>
<td>76.0</td>
<td>92.0</td>
<td>95.6</td>
<td>82.6</td>
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<td>Percentage of CD4\textsuperscript{+} cells (≥95%)</td>
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<td>95.0</td>
<td>97.3</td>
<td>95.2</td>
<td>98.5</td>
<td>98.7</td>
<td>98.5</td>
<td>98.3</td>
<td>97.9</td>
<td>97.3</td>
<td>98.4</td>
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<td>Percentage of CD8\textsuperscript{+} cells (≤5%)</td>
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<td>1.9</td>
<td>0.8</td>
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<tr>
<td>Viability (≥85%)</td>
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<td>99.8</td>
<td>99.4</td>
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Phenotypic and TCR analysis of expanded polyT\textsubscript{reg}

Key cell surface markers, CD4 and CD127, used to isolate the T\textsubscript{reg} were largely unchanged after expansion, although CD25 expression increased, which reflected the feedback induction caused by culturing the cells with IL-2. Our previous data demonstrated that the naïve CD45RA\textsuperscript{+} T\textsubscript{reg} preferentially expand in the cultures; however, the CD45RA\textsuperscript{RO} cells down-regulate CD45RA and up-regulate CD45RO\textsuperscript{+} over the expansion period (20). An example is shown in Fig. 2A, where the percent CD45RO\textsuperscript{+} T\textsubscript{reg} went from 52% before culture to 97% after culture. In addition, the cell expansion led to up-regulation of the cell surface markers CCR7 and CD38 increasing from 67% and 8% fresh, to 98% and 97% in expanded populations, respectively (Fig. 2, B and C). CCR7 has been shown to enhance T\textsubscript{reg} trafficking to lymph nodes (22). CD38, a multifunctional enzyme that catalyzes the synthesis and hydrolysis of cyclic adenosine diphosphate (ADP)–ribose from nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) to ADP-ribose, is reported to be essential for the regulation of intracellular Ca\textsuperscript{2+} and associated with enhanced T\textsubscript{reg} function (23).
Functional analysis of expanded polyTregs

FOXP3 protein can increase transiently in Tefs in response to activation signals. However, the DNA methylation state of enhancer region of the FOXP3 locus [Treg-specific demethylated region (TSDR)] remains methylated in all but bona fide Treg (24). We analyzed methylation of each preparation. The FOXP3 TSDR remained demethylated in the expanded Treg and FOXP3 protein levels correlated with overall demethylation status at the FOXP3 TSDR, indicating overall purity and stability of the expanded Treg (Fig. 3A).

Impaired function of Treg in patients with T1D has been ascribed to a reduced phosphorylation of STAT5 (pSTAT5) in response to IL-2. In the phase 1 trial demonstrated four- to eightfold greater suppressive activity than nonexpanded Treg, from the same individual (Fig. 3D). Overall, the expanded Treg from the 14 patients in the phase 1 trial demonstrated suppressive activity greater than 50% at ratios of 1:32 Tconv/Treg or lower, which suggested overall greater activity by the expanded versus nonexpanded Treg (Fig. S2). Finally, we examined a series of cell surface and functional markers on the nonexpanded versus expanded Treg (Fig. 3E). In addition to the increased suppressive activity, the expanded Treg showed significant increases in the expression of CD25, CTLA-4, and LAP, all of which have been shown to be involved in Treg function (11, 14, 29). Together, the phenotypic and functional data suggest that the expansion of the Treg increased not only the overall number of cells for AT but also the functional potential of these cells on a per-cell basis.

Safety

All patients received the target dose according to dose escalation cohort (Table 3). A tabulated cumulative summary of adverse events for all cohorts categorized by system organ class and severity is listed in Table S1. There were no infusion reactions. After a mean follow-up of 31 months, there were 11 grade 3 or 4 adverse events, which largely reflected metabolic abnormalities of underlying diabetes. Four serious adverse events were reported. One patient had three episodes of severe hypoglycemia 14, 248, and 463 days after Treg infusion in one subject and one episode of diabetic ketoacidosis 67 days after Treg infusion in a second subject.
T cell proliferation in these cultures was analyzed by flow cytometry (Fig. 3. Treg identity and function. (A) Percent of expanded cells expressing FOXP3 protein was determined by flow cytometry, and percent of DNA demethylated at the FOXP3 TSDR was determined by Epiontis as described in Materials and Methods. (B) Percent of STAT5 phosphorylation in response to IL-2 stimulation in CD4+CD25+ Treg, before and after expansion in healthy controls (n = 5) and subjects with T1D (n = 10). Staining and gating were done as previously described (25). Significance was determined by the Mann-Whitney test. (C) In an in vitro culture, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled Teffs were cultured for 4 days in the presence of anti-CD3/CD28 antibody-coated beads in the presence or absence of expanded/natural Treg of the same donor. T cell proliferation in these cultures was analyzed by flow cytometry for CFSE dilution as previously described (41). Each condition was set up in duplicate wells and compared to cultures with Treg alone. Data are means ± SEM for n = 4 healthy individuals. Statistical difference between nonexpanded and expanded Treg was determined by t test, where the difference seen at 1:2 is P = 0.0050, 1:4 is P = 0.0025, and 1:8 is P = 0.0354 (**P < 0.05 and ***P < 0.01). (D) Suppression assays using CFSE-labeled CD4+CD127+CD25− cells sorted from standard peripheral blood mononuclear cells (PBMCs) as Teffs cultured alone or activated with anti-CD3/anti-CD28-coated beads and/or co-incubated with Treg (from patients enrolled in this trial) show a consistent level of suppression. Comparison of mean of % proliferating CFSE+ cells with increasing ratio of Treg/Teff is shown for nonexpanded Treg as compared to expanded Treg (n = 3). Statistical difference between nonexpanded and expanded Treg from T1D patients was determined by t test, where the difference seen at 1:8 is P = 0.0195, 1:4 is P = 0.0333 (**P < 0.05), 1:2 is P = 0.0029, and 1:16 is P = 0.0289 (**P < 0.01). (E) Treg functional markers CD25, CD39, CD45RA, CD45RO, CTLA-4, and LAP were analyzed on expanded Treg cultured alone or activated with anti-CD3/anti-CD28 labeling and gating were done as previously described (25). E002-022 = 0.0050, 1:4 is P = 0.0001, and 1:8 is P = 0.0007. (F) Suppression assays using CFSE-labeled CD4+CD127+CD25− cells sorted from standard peripheral blood mononuclear cells (PBMCs) as Teffs cultured alone or activated with anti-CD3/anti-CD28-coated beads and/or co-incubated with Treg (from patients enrolled in this trial) show a consistent level of suppression. Comparison of mean of % proliferating CFSE+ cells with increasing ratio of Treg/Teff is shown for nonexpanded Treg as compared to expanded Treg (n = 3). Statistical difference between nonexpanded and expanded Treg from T1D patients was determined by t test, where the difference seen at 1:8 is P = 0.0112, and 1:32 is P = 0.0560.

No opportunistic infections or malignancies were observed. One subject developed grade 2 pharyngitis and had low-copy number cytomegalovirus (CMV) detected on day 7, but not detected at day 28, due to a presumed new infection with CMV occurring before receiving cells. There was no apparent relationship between adverse events and Treg dose (table S2). The full listing of adverse events is in tables S3 to S6.

Metabolic results
Metabolic function was evaluated by measuring the C-peptide area under the curve (AUC) during an MMTT (Fig. 4). The C-peptide responses were generally unchanged at 1 year and even after 2 years in dose cohorts 1 and 2. Three of four subjects in cohort 3 and three of the four subjects in cohort 4 showed a decline in C-peptide of more than 50% over 78 weeks of follow-up. Two of four subjects in cohort 4 also had a decline in C-peptide of more than 50% over 52 weeks of follow-up; however, the remaining two patients remained stable from week 13 to 52 weeks. The heterogeneity of diabetes progression and the dependence of progression on age and on duration of diabetes do not allow us to draw a conclusion from these findings in a small number of subjects. The HbA1c levels remained stable in all but one subject in cohort 4, 007-103, whose levels went from 6.2% at screening to 12.6% at week 13, with the individual's C-peptide diminishing from 0.49 at screening to 0.249 pmol/ml at week 13. Insulin use was generally stable. In summary, there were too few subjects to make a clear statement about stabilization or decay in C-peptide in treated subjects. However, we note that 7 of 14 patients had a C-peptide reduction of <10% of baseline C-peptide, predominantly in the lower-dose group, whereas 7 of 14 showed an increase in C-peptide decline at 1 year (predominantly in the higher-dose cohorts). Given the small number of subjects in each cohort, the overall changes in C-peptide in this study fall within the expected decline observed in the natural history of the disease (30).

T lymphocyte subsets
We compared T lymphocyte subsets among participants who had been treated with polyTreg before and after AT. Overall, consistent changes in T cell markers were not observed between
Tracking the adoptively transferred expanded polyT\textsubscript{reg}

Previous studies of allogeneic adoptive T\textsubscript{reg} immunotherapy have shown that T\textsubscript{reg} to be short-lived (32). Initial studies were performed to examine whether the adoptively transferred cells could be visualized on the basis of the cell surface expression profile of the expanded T\textsubscript{reg}. There was a short-term increase in the percentage of CD25 median fluorescence intensity (MFI) of the T\textsubscript{reg} population on day 7 after infusion (Fig. 5C). All of these changes most likely represent the detection of the transferred polyT\textsubscript{reg} population. There was also a significant decrease in CD56\textsuperscript{hi}/CD16\textsuperscript{lo} natural killer (NK) cells early after poly-T\textsubscript{reg} injection among all patients enrolled, consistent with the reduction of a more pathogenic type 1 IFN-\gamma tumour necrosis factor (TNF)-producing NK population (fig S3) (31). We did not find a statistically significant change in the titers of anti-GAD65 (glutamic acid decarboxylase 65) or anti-ICA512 (islet cell autoantibody 512) antibodies or differences between the treatment groups.

C-peptide AUC (pmol/ml)

HbA1c (%)

Insulin use (U/day/kg)

Fig. 4. Metabolic assessments. (Left column) C-peptide AUC. C-peptide AUC is reported for fasting 4-hour MMTT without carbohydrate restriction for 3 days preceding test. The target glucose level at the start of the test was between 70 and 200 mg/dl. Regular insulin or short-acting insulin analogs were allowed up to 6 and 2 hours before the test, respectively, to achieve the desired glucose level. The baseline blood samples (–10 and 0 min) were drawn, and then subjects drank Boost High Protein Nutritional Energy Drink (Nestle Nutrition) at 6 kcal/kg (1 kcal/ml) to a maximum of 360 ml. Blood was drawn at 15, 30, 60, 90, 120, 150, 180, 210, and 240 min after Boost dose. C-peptide AUC was calculated using the trapezoid rule. (Middle column) HbA1c. (Right column) Insulin use. Subjects self-reported insulin use for the 3 days immediately preceding the scheduled visit. The average total insulin (long acting + short acting) use per day normalized to weight is reported.

T\textsubscript{reg}-treated patients after AT when compared to pretreatment. A small, transient increase in CD38\textsuperscript{+} memory T cells was seen in the first week (Fig. 5A). A longer, more pronounced increase was seen in the percentage of CCR7\textsuperscript{+} T\textsubscript{reg} out to day 91 (Fig. 5B). In addition to these changes in T cell marker, an increase in CD25 median fluorescence intensity (MFI) of the T\textsubscript{reg} population was seen on day 7 after infusion (Fig. 5C). All of these changes most likely represent the detection of the transferred polyT\textsubscript{reg} population. There was also a significant decrease in CD56\textsuperscript{hi}/CD16\textsuperscript{lo} natural killer (NK) cells early after poly-T\textsubscript{reg} injection among all patients enrolled, consistent with the reduction of a more pathogenic type 1 IFN-\gamma tumour necrosis factor (TNF)-producing NK population (fig S3) (31). We did not find a statistically significant change in the titers of anti-GAD65 (glutamic acid decarboxylase 65) or anti-ICA512 (islet cell autoantibody 512) antibodies or differences between the treatment groups.

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There are several possible reasons for the decrease in the number of circulating Tregs, including cell death, migration to lymphoid tissues and inflammatory sites such as the pancreas, or a high degree of proliferation, thus diluting out the deuterium label. The current study design and data cannot distinguish among these possibilities. It also remained possible that the cells changed their Treg phenotype and the label was not captured in the sorting strategy for Tregs. To address this last possibility, we sorted all the CD4+ cells not in the Treg gate and separated them into naïve, central memory, and effector memory subsets based on the expression of CD45RO and CD62L to see whether deuterium label was found in CD4+ T cell other than Tregs (fig. S6). At no time, up to 365 days, was any deuterium observed in any other subset other than bona fide Tregs within the detection limit of 0.1% (Table 5). Because the labeling level of Tregs ranged from 2 to 8% early and 1 to 2% later, if some fraction of Tregs were converted to Teffs, then the relative contribution from Tregs to any other pool would have to be less than 1:20 to 1:80 early and less than 1:10 to 1:20 later, suggesting that conversion of Tregs into Teffs could have occurred as a rare event, below the level of detection. Overall, the data suggest that the infused Tregs did not transdifferentiate into Teffs.

However, there were indeed some changes in the phenotype of the infused Tregs. As mentioned above, the expanded polyTregs converted to a nearly uniform CD45RA-CD45RO-CCR7-CD38hi phenotype during ex vivo expansion (Fig. 2). To test the stability of these markers, CD4+CD127lo/−CD25+ Tregs were further divided into separate subsets based on CD45, CCR7, and CD38 expression at various time points before and after infusion of expanded Tregs. For all plots, comparisons were made to day 0 and significance was determined by paired t test.

Fig. 6. Survival of infused polyTregs. During ex vivo expansion, the 2H label from deuterated glucose ([2H2]glucose) contained in the cell culture medium is incorporated into the deoxyribose moiety in replicating DNA through the de novo purine nucleotide synthesis pathway. Three subjects (002-015, 007-102, and 002-017) were treated with a single dose of 2H-labeled Tregs at a target dose of 3.2 × 10^8 cells, and four subjects (002-018, 002-019, 007-103, and 002-022) were treated with a target dose of 26 × 10^8 cells that were about 60% enriched for the 2H label. Peripheral blood was collected on days 1, 4, 7, 14, 28, 91, 182, and 364 days after infusion, and Tregs were sorted from the peripheral blood. After isolation and hydrolysis of genomic DNA, the 2H isotopic enrichment of the purine deoxyribonucleosides in Tregs sorted from whole blood was assessed by gas chromatography–mass spectrometry (GC-MS). Background enrichment of unlabeled Tregs was ≤0.1% for each of the seven subjects.

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DISCUSSION

Several reports have described abnormalities in the functions of Tregs in patients with T1D, such as impaired responses to IL-2, a critical factor normally needed for Treg growth and survival. Our group also described instability manifest by their expression of pathologic cytokines, suggesting that ex vivo Treg expansion and repair might re-regulate the autoimmune process in individuals. Second, it will also be of interest to determine whether the relative proportion of Tregs that were short- or long-lived in the circulation, but this increased CD25 expression diminished rapidly, likely due to the reduced levels of IL-2 over time and perhaps leading to cell death (fig. S4). It should be pointed out that the increase in CD25 expression during expansion was transient in nature. However, it is noteworthy that although central memory T cells are CD45RO+CD45RA−, effector memory T cells express CD45RA− (33), suggesting that this change in phenotype for the AT Tregs may reflect further engagement of antigen in vivo and the development of a distinct Treg memory population.

In addition to demonstrating the long life span of a subset of Tregs, the kinetic modeling results may have clinical implications. First, the calculated whole body pool size of Tregs varied widely among T1D subjects. It will be of interest in future studies to determine whether this quantitative metric correlates with immune function or disease progression in individuals. Second, it will also be of interest to determine whether the relative proportion of Tregs that were short- or long-lived (range, 75 to 90% short-lived) has functional importance in Treg AT and whether this kinetic behavior is a feature of the host subject.

Our studies of the Treg pharmacokinetics, based on reinfusion of Tregs labeled ex vivo with stable isotopes, showed a delay in maximal AT Treg in the circulation and a two-phase decline curve that raises several questions. Other studies have suggested that the delay may be due to regulation of certain adhesion or homing receptors on the ex vivo-expanded Tregs that prevent the cells from immediately entering the circulation but rather promote homing and lodging in the liver or lung for some period of time (35). The apparent precipitous decline in circulating AT polyTreg number between the first and third month after transfer may be due to cell death, perhaps due to a decrease in IL-2 signaling when shifting from the in vitro milieu to in vivo. Consistent with this possibility, on day 1, we observed a significant increase in CD25+ Tregs in the circulation, but this increased CD25 expression diminished rapidly, likely due to the reduced levels of IL-2 over time and perhaps leading to cell death (fig. S4). It should be pointed out that the increase in the percentage of CD25 bright Tregs at day 1 may not fully reflect the presence of AT polyTregs entering the circulation because the relative percentages are higher than might have been expected based on the deuterium labeling. It would appear that the transfer of the ex vivo-expanded Tregs might have carried over some IL-2 bound to the receptor that induced IL-2 receptor (IL-2R) on the endogenous Tregs.

The Treg infusions were well tolerated, and the results of this safety study suggest that the infusions are safe over a more than 500-fold dose range. Cytokine release, infusion reactions, or infectious complications were not seen. We also showed that the infused Treg did not acquire pathologic phenotypes. The AT Treg reexpressed CD45RA. It is likely that the changes from CD45RA to CD45RO during expansion were transient in nature. However, it is noteworthy that although central memory T cells are CD45RO+CD45RA−, effector memory T cells express CD45RA− (33), suggesting that this change in phenotype for the AT Treg may reflect further engagement of antigen in vivo and the development of a distinct Treg memory population.

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Table 5. Stability of infused polyTregs. To address long-term stability of the infused expanded Tregs, samples collected on days 91 (in three of four patients only), 182, and 365 were sorted into Treg and non-Treg subsets as shown in fig. S6. Subsets were then analyzed by MS for 2H label, which was incorporated into the infused Treg during the expansion. Values shown are % enrichment for 4H and have an error of ±0.1.

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>DNA enrichment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C012 (002-018)</td>
</tr>
<tr>
<td>Tregs</td>
<td></td>
</tr>
<tr>
<td>CD4+CD25+CD127lo</td>
<td>2.0 1.0 0.0 0.0</td>
</tr>
<tr>
<td>Non-Tregs</td>
<td></td>
</tr>
<tr>
<td>CD45RO+</td>
<td>0.0 0.0 0.0 0.0</td>
</tr>
<tr>
<td>CD45RO+CD62Lhi</td>
<td>0.0 0.0 0.0 0.0</td>
</tr>
<tr>
<td>CD45RO+CD62Llo</td>
<td>0.1 0.0 0.0 0.0</td>
</tr>
<tr>
<td>CD45RO+CD62Lhi</td>
<td>0.0 0.0 0.0 0.0</td>
</tr>
<tr>
<td>CD45RO+CD62Llo</td>
<td>0.0 0.0 0.0 0.0</td>
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<tr>
<td>CD45RO+CD62Llo</td>
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</tbody>
</table>
The improvement in \( T_{reg} \) function and reversibility of the reduced pSTAT5 suggest that this aspect of \( T_{reg} \) function may be amenable to repair at least in the short term and in the context of in vitro expansion, consistent with what was observed in the IL-2/rapamycin study (25). The reduction in pSTAT5 seen in T1D CD4+ T cells reflects several polymorphisms found in the IL-2R and PTPN2, both of which are risk alleles for T1D. However, it may also in part reflect the absence of availability of IL-2 in patients at the time of diagnosis, possibly by consumption by other \( T_{eff} \). Longer-term studies and analyses of the time of onset of this abnormality may help to resolve these questions. Despite the improvement in pSTAT5, we did not find that the cell culture process increased production of cytokines, such as IL-10, by the expanded \( T_{reg} \), suggesting that some \( T_{reg} \) functions are not enhanced by in vitro expansion. In the future, further studies of the functional phenotype of the \( T_{reg} \) in vivo will address whether these additional functional activities have been enhanced.

Finally, there are several additional conclusions that can be drawn from the studies that have important implications. First, we have demonstrated that the expanded \( T_{reg} \) can be exported to other clinical sites, increasing the feasibility of developing this cell therapy into a true therapeutic. Second, although there were some phenotypic changes that occurred after \( T_{reg} \) infusion (such as CD38 and CD45R0 expression), the \( T_{reg} \) were overall quite stable for key parameters such as FOXP3, CD25, CD127 expression, and lack of \( T_{eff} \) cytokine production. However, it should be noted that we are only able to sample the cells in the circulation and there may be changes at the site in inflammation in the islets that are not evident when examining the blood. Finally, the enhanced suppressive activity seen in vitro under conditions of \( T_{reg} \) activation and expansion with IL-2 might suggest that there will be increased efficacy if the \( T_{reg} \) were combined with IL-2 therapy in vivo. This combination therapy is currently under development. In summary, we have shown that autologous \( T_{reg} \) can be expanded and are well tolerated in patients with recent-onset T1D. The expansion in vitro improves functional defects that have been identified in these cells in patients. Further adequately powered studies will be needed to determine whether the improvement in function and number leads to restoration of immunologic tolerance and prevention of disease progression.

There are several limitations to the study. As a phase 1 study, it was not powered to detect improvement in metabolic function, and therefore, we are unable to assess whether the improvement in the \( T_{reg} \) that we observed in vitro or the greater number of cells after AT will prevent progression of the autoimmune disease. However, the precipitous decline in C-peptide that had been described with high doses of IL-2, which expanded \( T_{reg} \) and NK cells in vivo, was not seen, and several of the patients in this phase 1 study had prolonged C-peptide production, especially in the lower-dose cohorts. Recently, Marek-Trzonkowska et al. reported initial findings of the infusion of \( T_{reg} \) in children with new-onset T1D (36, 37). Their approach to expansion of the cells had many similarities to ours, but they did not assess the effects of the culture on \( T_{reg} \) function. Moreover, the dosing that was used in that study was not fixed but given per kilogram, and additional doses were administered on the basis of deterioration in metabolic function—a late occurrence in disease progression. Nonetheless, these investigators suggested that the autologous \( T_{reg} \) might be able to maintain C-peptide responses. As with our study, they reported that the infusions were well tolerated without additional safety concerns, most notably no significant risk for infection. Another caveat in these studies is that analyses of the AT poly\( T_{reg} \) were limited to sampling of the peripheral blood, rather than the sites of autoimmune inflammation. Thus, issues such as local immune suppression, \( T_{reg} \) instability, and alteration of \( T_{eff} \) cannot be adequately addressed in this disease setting. Future studies in other
autoimmune diseases and organ transplantation should allow for tissue biopsies that can determine the local effects of polyTreg administration.

Thus, in summary, this study reports on the successful isolation, expansion, and reinfusion of polyTregs derived from patients with T1D. This provides a platform for additional clinical trials in this and other autoimmune diseases. The current efforts to use Treg-promoting therapies, such as low-dose IL-2 (38), are likely to be complementary to this current AT effort, potentially resulting in a robust combination therapy, which, when combined with Treg-depleting agents such as teplizumab and afelicept, may lead to durable remission and tolerance in this disease setting (39). Finally, efforts are under way to develop islet antigen–specific Tregs using genetic engineering (chimeric antigen receptors and TCR transduction), which we and others have shown to be even more efficacious to treat autoimmune diabetes in animal models (16).

**MATERIALS AND METHODS**

**Participants**

This study enrolled male and female subjects diagnosed with T1D within 3 to 24 months of screening who were 18 to 45 years of age with peak C-peptide >0.1 pmol/ml during MMTT challenge, were positive for Epstein-Barr antibody, and were positive for at least one of the following antibodies: tyrosine phosphatase–related islet antigen 2 (IA-2), ICA, GAD65, insulin, and zinc transporter 8 (ZnT8). Subjects also had to have adequate venous access to support draw of 400 ml of whole blood and infusion of investigational therapy. Subjects were determined to be ineligible if they had hemoglobin <10.0 g/dl; leukocytes <3000/μl; neutrophils <1500/μl; lymphocytes <800/μl; platelets <100,000/μl; Tregs <10/μl; evidence of active infection [HIV-1/HIV-2, hepatitis B, hepatitis C, Epstein-Barr virus (EBV) or CMV genomes, or positive purified protein derivative (PPD) skin test]; chronic use of systemic glucocorticoids or other immunosuppressive agents or biologic immunomodulators within 6 months before study entry; history of malignancy except adequately treated basal cell carcinoma; or any chronic illness or previous treatment that, in the opinion of the investigator, should preclude participation in the trial. Pregnant or breastfeeding women were excluded from the study, as well as any female who was unwilling to use a reliable and effective form of contraception for 2 years after Treg dosing, and any male who was unwilling to use a reliable and effective form of contraception for 3 months after Treg dosing. All participants provided written informed consent before participating in any study procedures.

**Treg isolation and expansion (40)**

About 400 ml of fresh peripheral blood was collected into blood pack units containing citrate phosphate dextrose (Fenwal) and processed within 24 hours for isolation of PBMCs via Ficoll density gradient (GE Healthcare Bio-Sciences). For subjects enrolled at Yale, blood was shipped to UCSF for processing in the good manufacturing practice (GMP) laboratory, and the expanded polyTregs were shipped back to Yale for infusion.

Tregs were isolated on a BD FACSaria II high-speed cell sorter housed in a class 10,000 clean room with the following GMP-grade lyophilized antibodies: CD4-PerCP (peridinin chlorophyll protein) (L200), CD127-PE (phycoerythrin) (40131), and CD25-APC (allophycocyanin) (2A3) (BD Biosciences). The sorted CD4+CD127<sup>lo</sup>-CD25<sup>−</sup> T cells were collected into 3 ml of X-VIVO 15 medium (Lonza, catalog no. 04-418Q) containing 10% human heat-inactivated pooled AB serum (Valley Biomedical). Treg populations were analyzed for purity after sort and determined to be 98.4% (range, 96.3 to 99.6%) CD4+CD127<sup>lo</sup>-CD25<sup>−</sup> T cells.

FACS-isolated cells were plated at ~2.5 × 10<sup>6</sup> T<sub>reg</sub> per well in multiple wells of a 24-well plate (Nunc) and activated with Dynabeads CD3/CD28 CTS anti-CD3/anti-CD28–coated microbeads (Life Technologies) at a 1:1 bead/cell ratio. Cells were cultured either in X-VIVO 15 or in X-VIVO 15 customized by Lonza by substituting 100% of the glucose in the base medium with d-glucose (66.7 g/L), 99% (Cambridge Isotope Laboratories, catalog no. DLM-349-MPT) supplemented with 10% human heat-inactivated pooled AB serum. At day 2, the culture volume was doubled and IL-2 was added (Prolifektin, 300 IU/ml; Prometheus).

Cells were resuspended, fresh medium and IL-2 were added at days 5, 7, 9, and 12, and the cells were transferred to cell culture plates and flasks (Nunc), and/or bags (Saint-Gobain) of increasing size to maintain a seeding density of ~2 × 10<sup>6</sup> to 3 × 10<sup>6</sup> cells/ml in plates or flasks and a concentration of 500,000/ml in bags. On day 9, cells were restimulated with fresh anti-CD3/anti-CD28–coated beads at a 1:1 ratio. On day 14, cells were consolidated and debanded using a MaxSep magnet, and bead removal was verified via flow cytometry. Briefly, Dynabeads CD3/CD28 CTS (Invitrogen, catalog no. 402.03D) and Spherobeads (BD, catalog no. 556291) were used as controls for determining instrument settings and defining Dynabeads gate based on forward scatter (FSC) versus side scatter (SSC) followed by FL2 versus FL3 channels on FACSCalibur. Triplicate samples of expanded CD4+CD127<sup>lo</sup>-CD25<sup>−</sup> T<sub>reg</sub> at ~5 × 10<sup>6</sup> cells/ml were analyzed, and a number of cells and Dynabeads in each sample were collected to determine cell number and bead number contained within each sample. The average bead count and average cell count were used to calculate the bead/cell ratio.

The product was prepared as a cell suspension of fresh, noncryopreserved cells in sterile infusion solution composed of 1:1 PlasmaLyte A/5% dextrose, 0.45% NaCl (Baxter) containing 0.5% human serum albumin (HSA) (Grifols), all supplied as U.S. Food and Drug Administration–approved drugs (PlasmaLyte A and dextrose/NaCl) or licensed products (HSA) for injection and conforming to U.S. Pharmacopeial Convention (USP) standards.

**Administration and follow-up**

Results of blood chemistry and hematology were reviewed, and a history of any recent illness or fever was obtained before infusion. Patients received premedication with acetaminophen and diphenhydramine. PolyTregs were infused via a peripheral intravenous line over 10 to 30 min. Vital signs were taken before and after infusion, then every 15 min for at least 1 hour, then every hour for the first 4 hours, and every 4 hours for 20 hours. Chemistries and complete blood count with differential blood count were repeated the next day before discharge from the clinical research unit. Patients were seen for follow-up assessments on day 4 after infusion, then weekly for 4 weeks, then every 13 weeks for 1 year, and then every 26 weeks for 2 years. Telephone monitoring for adverse events continues every 6 months for 5 years after infusion followed by a final clinic visit.

**Phenotypic analysis of expanded Treg populations and peripheral blood samples**

Freshly expanded cells were evaluated for expression of CD4, CD25, CD127, CD8 (BD Biosciences), and FOXP3. Intracellular staining was...
performed with Alexa 488–conjugated anti-FOXP3 (clone 206D) and the FOX3 staining kit (BioLegend) according to the manufacturer’s instructions and modified as follows: 2 × 10^6 cells were washed and fixed for 30 min at room temperature using fixation/permeabilization buffer. Cells were washed, resuspended in perm buffer containing deoxyribonuclease I (100 U/ml; Sigma-Aldrich), and incubated for 30 min at room temperature, followed by two washes in perm buffer. Cells were subsequently blocked with human immunoglobulin G (1gG) (5 μg per test) for 5 min and stained for cell surface and intracellular markers along with anti-human FOXP3–Alexa 488 (5 μl per test) or isotype control. Flow cytometric data were collected on a FACSCalibur cytometer (BD Biosciences) and analyzed with FlowJo software (version 9; Tree Star). In experiments to determine phenotype of expanded Tregs and localization of the deuterium labeling, antibodies used for flow cytometric sorting on a BD FACSARia II cytometer included CD45RA-APC (H100) (BioLegend), CD4–Alexa 488 (RPA-T4) (Becton Dickinson), CD38–PerCP-Cy5.5 (HIT2) (BioLegend), CCR7–V450 (150-503) (Becton Dickinson), CD45RO–PE–Cy7 (UCHL1) (BioLegend), CD127–PE (hIL-7R-M21) (Becton Dickinson), and CD25–BV786 (M-A251) (Becton Dickinson). FACS files were analyzed with FlowJo software version 9 or greater.

Studies of clinical samples conducted at the Benaroya Research Institute (BRI) were performed as follows. PBMCs collected at baseline and throughout the study were frozen for batch analysis. These PBMC samples were subsequently thawed, labeled, and analyzed as previously described (25) for STAT5 signaling and the immunophenotyping of lymphocyte and T cell subsets. Multicolor flow cytometry was conducted on a BD LSRII flow cytometer and analyzed in FlowJo using standardized panels developed by the Immune Tolerance Network (www.immunetolerance.org).

For studies of nonclinical samples at the BRI, cells (nonexpanded Tregs or D14-expanded Tregs from the same donor) were suspended in FACS buffer [phosphate-buffered saline + 1% fetal bovine serum (FBS) + 0.1% NaN3]. To block Fc receptor–mediated binding of antibodies, cells were suspended in FACS buffer with 1% human serum bodies, cells were suspended in FACS buffer with 1% human serum

Suppression assays

Treg suppression was assessed by measuring proliferation based on either a [3H]thymidine incorporation or CFSE dilution assay. [3H]thymidine incorporation Treg suppression assays were performed after expansion for all 14 individuals enrolled in the clinical trial as previously described (20), with the addition of a standard expanded Treg population in each assay to ensure that the data were comparable and could be combined. For all control subjects, suppression was assessed on the basis of CFSE dilution as analyzed by flow cytometry as previously described (41). In brief, CFSE-labeled autologous CD4+ CD25+ T cells were cocultured in round bottom plates with or without expanded Tregs or CD4+CD25hiCD127lo– Tregs sorted from freshly isolated PBMCs and activated with Dynabeads human T activator CD3/CD28 (Invitrogen) for 4 days. Varying ratios of Treg/Teff were plated in duplicate or triplicate. For comparisons of clinically expanded Treg to nonexpanded Treg from the same subject, a small-scale version of the previously described CFSE suppression assay was used, where Teff were plated at 10,000 cells per well at a 1:10 bead:Tcell ratio. For the small-scale CFSE suppression assays, all Tregs were isolated from cryopreserved material and in vitro suppression was assessed on the basis of Treg capacity to suppress the proliferation of a standard allogeneic responder T cells generated from a healthy donor whose PBMCs were cryopreserved in multiple aliquots containing 10 × 10^6 cells per vial. Additionally, standard nonexpanded Treg and expanded Treg populations were included in each assay to ensure that the data were comparable and could be aggregated.

In vitro suppression assays were performed in RPMI 1640 (Mediatech) supplemented with 5 mM Heps, 2 mM l-glutamine, penicillin/streptomycin (50 μg/ml each) (Invitrogen), 50 μM 2-mercaptoethanol (Sigma), 5 mM nonessential amino acids, 5 mM sodium pyruvate (Mediatech), and 10% FBS (Omega Scientific). Cultures were maintained in 200-μl volumes in U-bottom 96-well plates (Costar) incubated at 37°C and 5% CO2.

Cytokine analysis

Treg were assessed for cytokine production at day 14 of culture via intracellular staining. Intracellular cytokines were stained directly from cultures at day 14 or after 4 hours of reactivation with anti-CD3/anti-CD28–coated beads or PMA/ionomycin. Intracellular cytokine staining was conducted with FOXP3 staining using the manufacturer’s kit reagents from BioLegend as previously described along with the anti-human cytokine antibodies IFN-γ–PE and IL-10–APC (Becton Dickinson).

TCRβ repertoire analysis

Genomic DNA was extracted from 2.5 × 10^5 freshly isolated Tregs and 1 × 10^6 ex vivo–expanded polyTregs. The DNA was submitted to Adaptive Biotechnologies for deep-level TCRβ sequencing. TCR gene frequency analysis was performed using algorithms developed by Adaptive Biotechnologies.

TSDR methylation assay

Genomic DNA from 1 × 10^6 expanded Tregs was analyzed by Epiontis GmbH according to established protocol. Percentages of demethylated TSDR were calculated as follows: [mean copy numbers of unmethylated DNA/(mean copy numbers of unmethylated DNA + copy numbers of methylated DNA)] × 100. For female Tregs the percentages calculated above were multiplied by 2 to correct for X-chromosome inactivation.

Treg deuterium tracking

During the 14-day clinical expansion period, the 2H2 label from [6,6-2H2]glucose in the X-VIVO 15 culture medium was incorporated into the DNA of replicating polyclonal CD4+CD127lo/hi–CD25+ Tregs as previously described (42). Initial qualifying experiments showed no differences in fold expansions, phenotype, or percent TSDR demethylation between Tregs grown in X-VIVO 15 and Tregs grown in [6,6-2H2]glucose-containing X-VIVO 15. Functional suppression assay results showed similar inhibition between Treg expanded in either type of medium, and cultures were free from bacteria, fungi, mycoplasma, or endotoxin contaminants. MS analyses showed that Tregs expanded in X-VIVO 15 with [6,6-2H2]glucose in the medium at 100% enrichment were ~60% enriched for 2H2 in the deoxyribose moiety of purine deoxyribonucleotides isolated from DNA, which is the
theoretical maximum deuterium enrichment observed in deoxyribose in fully replaced cells that divided in the presence of [6,6-2H2]glucose ((42, 43). This 60% enrichment level was consistently observed in all seven preparations in this clinical study.

After infusion of the labeled Treg peripheral blood was collected from the study participants, purified for Tregs, and analyzed for stable isotope enrichment. In some experiments, the cells were subdivided into Tregs versus Teffs as well as subsets of Treg to determine the stability of the Treg surface markers.

Analysis of DNA enrichment by MS

Measurement of deuterium in newly synthesized DNA was performed by GC-MS as described in detail previously (42). Briefly, DNA from proteinase K digests was isolated using DNeasy microcolumns (Qiagen) and hydrolyzed using S-1 nuclease and acid phosphatase. Deoxyribose moieties from purine D4-nucleotides were converted to pentfluorobenzylhydroxylamine triacetate derivatives. Enrichment analysis was performed on an Agilent 6890/5973 GC/MS equipped with a 30-m DB-17MS column (inside diameter, 250 μm; film thickness, 25 μm; Agilent) using methane negative chemical ionization and collecting ions in SIM mode at mass/charge ratios (m/z) 435, 436, and 437 (M0, M+1, and M+2, respectively). Enrichment of the [5,5-2H2]deoxyribose derivative was determined from measured ratios of the peak abundances of the M+2 ion to the sum of the M+0 to M+2 ions, after subtracting the theoretical (unenriched) natural abundance ratios, which are validated with standard curves of % enrichment.

Laboratory tests

Biochemical autoantibody titers were assayed at the Barbara Davis Center using radioimmunobinding assays, and ICA was measured at the University of Florida. C-peptide and HbA1c were measured at the Northwest Lipid Research Laboratory. Viral loads for EBV and CMV were performed by ViraCor Laboratories. Chemistries and hematology were performed at local clinical laboratories at UCSF and Yale.

Statistics and methods of analysis

Data analyses were performed using GraphPad Prism 6.0 software, and values at P < 0.05 were deemed significant. Cytokine concentrations were determined using SoftMax Pro software (Molecular Devices) with four-parameter data analysis.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/7/315/315ra189/DC1

Table S7. Two-phase decay model parameters.

Table S1. Tabulation of cumulative number of adverse events for all cohorts categorized by system organ class and severity.

Table S2. Tabulation of cumulative number of adverse events categorized by cohort and severity.

Table S3. Listing of cumulative adverse events reported for cohort 1 by CTCAE term and severity.

Table S4. Listing of cumulative adverse events reported for cohort 2 by CTCAE term and severity.

Table S5. Listing of cumulative adverse events reported for cohort 3 by CTCAE term and severity.

Table S6. Listing of cumulative adverse events reported for cohort 4 by CTCAE term and severity.

REFERENCES AND NOTES


RESEARCH ARTICLE


Acknowledgments: We thank the whole team involved in developing and implementing this clinical trial, including F. Dekovic, T. Ghazi, G. Giunti, P. Preston-Hurlburt, H. Javier, L. Rink, C. Torok, M. Tatum, and the BRI Diabetes Clinical Research Unit for samples; J. Kricsher, E. Whalen, and M. Mason for statistical consultations; N. Warner and BD Biosciences for access to the cGMIP (Current Good Manufacturing Processes) antibodies; and the Immune Tolerance Network (UM1AI109565) for the mechanistic study support. Funding: This work was supported by the Juvenile Diabetes Research Foundation International (Collaborative Center for Cell Therapy; 2-SRA-2014-150 and the clinical trial; 17111-661), the Brehm Coalition, The Immune Tolerance Network, BD Biosciences, and Caladrius Biosciences. Author contributions: J.A.B. designed and analyzed experiments and wrote the manuscript; K.C.H., P.H.S., and S.E.G. were the clinicians conducting the study. Other authors played various roles in performing, analyzing, and overall contributions in the interpretation and editing of the manuscript. Competing interests: J.A.B., A.L.P., W.L., and Q.T. are co-inventors on patents (US 20080131445 A1 and US 7722862 B2) filed from Caladrius Biosciences and other in-kind contributions from BD Biosciences. The remaining authors declare that they have no competing interests.

Submitted 12 September 2015 Accepted 23 October 2015 Published 25 November 2015 10.1126/scitranslmed.aad4134

Type 1 diabetes immunotherapy using polyclonal regulatory T cells

Editor's Summary

Regulating type 1 diabetes

In patients with type 1 diabetes (T1D), immune cells attack the insulin-producing β cells of the pancreas. The resulting prolonged increase in blood sugar levels can lead to serious complications including heart disease and kidney failure. Regulatory T cells (Tregs) have been shown to be defective in autoimmune diseases. Now, Bluestone et al. report a phase 1 trial of adoptive Treg immunotherapy to repair or replace Tregs in type 1 diabetics. The ex vivo-expanded polyclonal Tregs were long-lived after transfer and retained a broad Treg phenotype long-term. Moreover, the therapy was safe, supporting efficacy testing in further trials.

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