Rapamycin/IL-2 Combination Therapy in Patients With Type 1 Diabetes Augments Tregs yet Transiently Impairs β-Cell Function

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Rapamycin/interleukin-2 (IL-2) combination treatment of NOD mice effectively treats autoimmune diabetes. We performed a phase 1 clinical trial to test the safety and immunologic effects of rapamycin/IL-2 combination therapy in type 1 diabetic (T1D) patients. Nine T1D subjects were treated with 2–4 mg/day rapamycin orally for 3 months and 4.5 × 10^6 IU IL-2 s.c. three times per week for 1 month. β-Cell function was monitored by measuring C-peptide. Immunologic changes were monitored using flow cytometry and serum analyses. Regulatory T cells (Tregs) increased within the first month of therapy, yet clinical and metabolic data demonstrated a transient worsening in all subjects. The increase in Tregs was transient, paralleling IL-2 treatment, whereas the response of Tregs to IL-2, as measured by STAT5 phosphorylation, increased and persisted after treatment. No differences were observed in effector T-cell subset frequencies, but an increase in natural killer cells and eosinophils occurred with IL-2 therapy. Rapamycin/IL-2 therapy, as given in this phase 1 study, resulted in transient β-cell dysfunction despite an increase in Tregs. Such results highlight the difficulties in translating therapies to the clinic and emphasize the importance of broadly interrogating the immune system to evaluate the effects of therapy. Diabetes 61:2340-2348, 2012

Type 1 diabetes (T1D) results from immune-mediated destruction of pancreatic β-cells, leading to hyperglycemia and life-long dependence on exogenous insulin. Although targeting T cells, B cells, or costimulation have all induced transient maintenance of β-cell function in patients with recently diagnosed T1D, treatment resulting in lasting remission of disease remains elusive (1–4). Thus, alternative approaches, such as combination therapy, may be required to treat and prevent T1D.

An imbalance of autoreactive regulatory and effector cells is important in T1D pathogenesis. One predominant family of CD4+ regulatory T cells (Tregs) express high levels of CD25 (the high-affinity interleukin-2 [IL-2] receptor), and the forkhead box P3 (FOXP3) transcription factor. Impaired Treg function and decreased Treg persistence in response to IL-2 are present in T1D patients (5) and occur along with apparent increased activity of pathogenic T-effector cells (Teffs) (6).

Rapamycin is used in transplantation due to its ability to block activation of the mammalian target of rapamycin complex 1 (mTORC1), which results in growth factor–driven cell-cycle progression (7). Rapamycin preferentially inhibits proliferation of T helper (Th)1 and Th17 Teffs, with a weaker effect on Tregs, which do not require IL-2 dependent (13). In NOD mice and T1D patients, defects in IL-2 production and IL-2R signaling appear to both prevent and reverse hyperglycemia through selective activation and expansion of FOXP3+ Tregs (15–17). Moreover, in recently reported clinical trials of low-dose IL-2 in graft-versus-host disease (GVHD) (18) and autoimmune vasculitis (19), IL-2 therapy resulted in increased Treg numbers and beneficial clinical outcomes in some patients.

Treatment of NOD mice with rapamycin and IL-2 resulted in prevention of both spontaneous diabetes and recurrent diabetes after islet transplant (20). We performed an open-label phase 1 clinical trial in adult T1D subjects with rapamycin (Rapamune) plus IL-2 (Proleukin). β-Cell function, based on C-peptide values, transiently declined,
and significant changes were observed in Tregs, Teffs, and natural killer (NK) cells. Thus, rapamycin/IL-2 therapy boosted Tregs, but also promoted a proinflammatory environment that may have resulted in transient β-cell dysfunction. Clinical and mechanistic data from this study will help guide future uses of IL-2 and combination therapy.

RESEARCH DESIGN AND METHODS

Trial design and patient characteristics. After institutional review board approval and informed consent at Benaroya Research Institute, Oregon Health Sciences University, or Columbia University, subjects were screened for trial entry with the aim to enroll 10 subjects (21). Otherwise-healthy, autoantibody-positive subjects between 4 and 48 months from T1D diagnosis with peak C-peptide of $0.4 \text{ pmol} / \text{mL}$ after mixed-meal tolerance test (MMTT) were eligible. IL-2 (Prollekun) was administered at $4.5 \times 10^6 \text{ IU} \text{ s.c.}$, three times per week for a total of 12 doses. Rapamycin (Rapamune or Sirolimus) was administered without a loading dose at $2 \text{ mg} / \text{day}$, with adjustments to maintain trough blood levels of $5–10 \text{ ng} / \text{mL}$ for 3 months (Supplementary Fig. 1). Subjects also received prophylactic trimethoprim and sulfamethoxazole (Septa). All subjects met regularly with diabetes educators with the aim of achieving intensive diabetes control, targeting fasting values between 80 and 120 mg/dL and postprandial values $<180 \text{ mg} / \text{dL}$.

Autoantibodies. GAD-65 and islet-associated protein 2 were measured with radioimmunobinding assays. If other autoantibodies were negative, islet-cell autoantibodies were measured with indirect immunofluorescence (22).

C-peptide. Plasma C-peptide levels were measured (21) at the Northwest Lipid Pathologies at the University of Washington (Seattle, WA) using a two-site immunoenzymometric assay performed on a Tosoh 600 II autoanalyzer (21). The lower limit of quantification was $0.013 \text{ pmol} / \text{mL}$ and intra-assay and inter-assay coefficients of variation were $<10\%$. Results reported as less than a standard curve (run in triplicate) using a four-parameter fit equation. All assays passed the manufacturer’s quality control criteria.

Flow cytometry. For phenotypic studies, peripheral blood mononuclear cells (PBMCs) were thawed and stained for two surface panels and an intracellular panel. Cells were stained with CD4 AmCyan, CD45RO phycoerythrin (PE)-H7, CD25 phycoerythrin (PE), CD127 V450, IL-17 PerCP-Cy5.5, FOXP3 Alexa647, helios fluorescein isothiocyanate (FITC), and HLA DR eFluor605NC. For phosphostains, during the last 4 h, and cells were then fixed and stained with CD4 PE-Cy5, interferon-γ (IFN-γ) V500, IL-17 PerCP-Cy5.5, FOXP3 Alexa647, helios fluorescein isothiocyanate (FITC), and HLA DR eFluor605NC. For phosphostains, PBMCs were thawed, rested, and stained as described previously (23). After stimulation with IL-2 (100 IU/mL) or anti-CD3 (5 µg/mL) and anti-CD28 (1 µg/mL) antibody plus Fab x-linker (10 mg/mL), cells were fixed and permeabilized at designated times. IL-2-stimulated cells were stained with CD56 FITC, CD25 PE, pSTAT5(pY694) Alexa647, and CD4 PerCP. CD3/28-stimulated cells were stained with pAkt(pS473) Alexa488 or p65(pS253) Alexa647 for 5 min at $37\, ^\circ\text{C}$, followed by washing, adding a cocktail of biotinylated detection antibodies to each well. After incubation for 30 min at room temperature with shaking, PBMCs were fixed and permeabilized at $−20\, ^\circ\text{C}$ using Cytofix/Cytoperm (BD Biosciences). Then cells were stained with CD56 FITC, CD25 PE, and CD4 PerCP. Data were acquired on an LSR II or FACSCalibur and analyzed using FlowJo or Winlist. Samples from different draw dates for the same subject were assayed on the same day whenever possible.

Serum cytokine analysis. Serum cytokine levels were measured using Searchlight chemiluminescent platform in custom-designed multiplex arrays provided by Aushon Biosystems (Billerica, MA). A Clinical Laboratory Improvement Amendments–certified contract laboratory, The serum analytes tested included IFN-γ, IL-4, IL-5, IL-6, IL-8, IL-10, IL-1β, IL-2, IL-2p70, tumor necrosis factor-α, etoxin, IL-12p40, macrophage inflammatory protein (MIP)-1α, MIP-1β, E-selectin, IL-2RA, insulin-like growth factor–binding protein 3 (IGFBP3), IL-17, IL-13, transforming growth factor–β1 (TGF–β1), and IL-23. Samples (in duplicate) were incubated on array plates prespotted with capture antibodies specific for each cytokine. Plates were washed before adding a cocktail of biotinylated detection antibodies to each well. After incubating with detection antibodies, plates were washed and incubated with streptavidin–horseradish peroxidase conjugate followed by a chemiluminescent substrate and immediately imaged using the Aushon CCD imaging system. All incubations were performed at room temperature with shaking. Data were analyzed using Aushon Array Analyst software, and concentrations were interpolated from a standard curve (run in triplicate) using a four-parameter fit equation. All assays passed the manufacturer’s quality control criteria.

Epigenetic analysis. Cell pellets were snap frozen, and Epiontis performed DNA isolation and real-time based quantification of Treg-specific demethylation region (TSDR) (24) and CD3 demethylation (25).

Statistics. Change from baseline (time 0) was determined using a one-way ANOVA using Prism. For analysis of two time points, a paired Student’s t test was used. No adjustments were made for multiple comparisons. Unless otherwise noted, P values $<0.05$ were considered significant.

RESULTS

Rapamycin/IL-2 combination therapy results in a transient decrease in C-peptide levels. Nine adults were enrolled (Table 1). Subject 5 received only 10 of 12 IL-2 doses due to abdominal pain likely related to therapy. Review of 3-month MMTT data in the first seven subjects demonstrated that all had a marked decrease of β-cell function (Fig. 1). Further enrollment was halted, and subjects 8 and 9, who had already completed their IL-2 course, were instructed to stop ongoing rapamycin therapy at days 78 and 28, respectively (Table 1 and Supplementary Fig. 1). Follow-up of subjects through 3 years continues as per protocol. All subjects experienced transient eosinophilia and IL-2 injection site reactions, including firm nodules of

| Patient | Age (years) | Sex | Time since diagnosis (months) | Dosing‡ | Comments† | Symbol in figures |
|---------|-------------|-----|-------------------------------|---------|-----------|------------------|
| 1       | 20          | M   | 21                            | Complete| None      | ▪                |
| 2       | 22          | M   | 25                            | Complete| None      | ○                |
| 3       | 36          | F   | 7                             | Complete| Noncompliant with diabetes management | ■                |
| 4       | 28          | M   | 24                            | Complete| None      | ▲                |
| 5       | 22          | M   | 5                             | 10 of 12 doses of IL-2 treatment stopped due to abdominal pain | ▲                |
| 6       | 26          | M   | 42                            | Complete| None      | △                |
| 7       | 20          | F   | 19                            | Complete‡ | Anorexia, unrelated to therapy | ▲                |
| 8       | 34          | F   | 19                            | Stopped rapamycin on day 28 | None      | △                |
| 9       | 27          | F   | 42                            | Stopped rapamycin on day 28 | None      | △                |

F, female; M, male. §Patients received $4.5 \times 10^6 \text{ IU} \text{ IL-2 s.c.}$ three times per week for 1 month and rapamycin 2–4 mg/day orally for 3 months except where noted. †Characteristics of patients that may contribute to clinical and mechanistic phenotypes. ‡Subject 7 inadvertently took an extra five doses of rapamycin at 3 months.
1–3 cm and erythema in some individuals as much as 9 cm in diameter. Additional adverse effects were fatigue and malaise during the first month of treatment.

All subjects had a decrease in C-peptide at the first assessment at 3 months, including subjects 8 and 9, who were not receiving rapamycin therapy at the time of testing (day 0 mean = 8.926 pmol/mL and day 84 mean = 4.693 pmol/mL; $P < 0.0001$) (Fig. 1). This was associated with a stable HbA1c achieved with increasing doses of insulin (Supplementary Fig. 2). The mean rate of AUC C-peptide fall was 0.104 pmol/mL/month or 43% over 3 months. This is markedly greater than the expected rate of decline postdiagnosis in an adult with T1D (3,4,26–28). However, in almost all subjects, the subsequent C-peptide values increased by day 168 (day 84 mean = 4.693 pmol/mL and day 168 mean = 6.321 pmol/mL; $P < 0.0474$). Based on these data, combination therapy resulted in a transient decrease in $\beta$-cell function.

**Rapamycin/IL-2 combination therapy in T1D patients transiently increases FOXP3$^+$ natural Tregs.** To determine whether Tregs were augmented by combination therapy, we analyzed $CD4^+CD25^+CD127^{lo}$ cells and FOXP3 expression in $CD4^+$ T cells of peripheral blood (Fig. 2A and B). The frequency of $CD4^+CD25^+CD127^{lo}$ cells increased in all subjects, declining to baseline levels after completion of therapy. The absolute number of FOXP3$^+$ cells increased from baseline to day 28, falling back to baseline by day 56 when rapamycin therapy was still ongoing.

In humans, FOXP3 is expressed constitutively in Tregs and transiently in activated Teffs. However, the FOXP3 locus remains methylated in Teffs, whereas similar analysis of natural Tregs (nTregs) displays demethylation at the TSDR, making TSDR methylation a more reliable marker of stable nTregs (29). An increased frequency of FOXP3 gene demethylation was observed on day 28 (Fig. 2C) and correlated with the frequency of CD4$^+$FOXP3$^+$ cells measured by flow cytometry, suggesting that the increase in CD4$^+$FOXP3$^+$ cells was primarily due to an increase in the nTreg subset. In comparison, IFN-γ secretion by FOXP3$^+$ cells is often indicative of activated Teffs. A limited amount of IFN-γ was secreted by CD4$^+$FOXP3$^+$ cells, and the frequency of CD4$^+$FOXP3$^+$ cells secreting IFN-γ did not increase with therapy (Fig. 2D). Thus, combination therapy promoted Treg expansion in the periphery.

**Enhanced response to IL-2 in CD25$^+$ cells of T1D patients treated with rapamycin/IL-2 combination therapy is maintained after therapy.** We have recently shown that IL-2 responses in CD4$^+$ T cells of T1D subjects are decreased as compared with controls (23). To determine whether treatment with rapamycin/IL-2 altered responses to IL-2, we assessed the phosphorylation of signal transducer and activator of transcription 5 (pSTAT5) in response to IL-2 (Fig. 3A). The T1D subjects in this trial had reduced IL-2 responsiveness prior to therapy (Fig. 3B). The basal level of pSTAT5 did not change with time (Supplementary Fig. 3), but the response to IL-2 stimulation was enhanced at day 28 (Fig. 3B and C). This increased responsiveness to IL-2 was maintained after IL-2 therapy, as seen by persistently higher responses on day 56, a time point by which the frequency and level of expression of CD25 had fallen (Figs. 3C and 2A). A significant increase in the frequency of CD25$^+$ cells responding to IL-2 was observed in the peripheral blood even as late as 1 year past initiation of therapy.
Rapamycin/IL-2 combination therapy in T1D subjects modulates pAkt, but not pS6. Rapamycin is an mTOR inhibitor, and both S6 and Akt are in the phosphatidylinositol 3-kinase pathway and are phosphorylated after T-cell receptor ligation. The levels of pS6 and pAkt were measured in the presence or absence of anti-CD3/anti-CD28 stimulation. T-cell activation was sufficient in these assays, as demonstrated by consistent phosphorylation of extracellular signal–related kinase (Erk) (Fig. 4A), a molecule that is activated by T-cell receptor engagement but is not targeted by rapamycin. Stimulated levels of pS6 in CD4+ T cells did not change with therapy (Fig. 4B). This lack of a reduction in pS6 after rapamycin therapy was not due to a compensatory increase in total S6 expression (data not shown). After combination therapy, CD4+ cells exhibited a markedly decreased induction of pAkt upon in vitro stimulation with anti-CD3/anti-CD28 (Fig. 4C). Similar responses were observed in both CD4+ and CD8+ T cells (data not shown). The decreased induction of pAkt, but not pS6, suggests that combination therapy resulted in modulation of proximal signaling molecules within the peripheral blood T cells of subjects in our study.

Rapamycin/IL-2 combination therapy in T1D patients does not alter CD4 or CD8 Teff differentiation. We found no difference in autoantibody titers, absolute white blood cell counts, the frequency of lymphocytes and monocyte populations, or the CD4:CD8 ratio with combination therapy (Supplementary Table 1 and data not shown). No changes were seen in the periphery in the frequency of naive and memory CD4+ or CD8+ T cells, effector and central memory T-cell subsets, or frequency of CRTH2+ or CXCR3+ cells, indicative of Th2 and Th1 cells, respectively (Fig. 5A and B and Supplementary Fig. 4). Likewise, no change in IFN-γ secretion, a cytokine made by Th1 cells, was observed over time (Fig. 5C). Despite some variance, no significant difference was observed over time in IL-17 secretion (Fig. 5C and Supplementary Fig. 5). Activation of CD4+ and CD8+ T cells was measured by expression of CD38 and Fas, and no differences were observed over time (data not shown). Together, these data demonstrate that T-cell activation and memory composition along with CD4+ helper subset differentiation may not have been altered in the peripheral blood during combination therapy in T1D patients.

Immune activation occurred upon rapamycin/IL-2 combination therapy. We observed significant eosinophilia in all subjects as early as day 14 (Fig. 6A), which coincided with IL-2 but not rapamycin therapy. In parallel with the increase in eosinophils was a decrease in the frequency of neutrophils in the peripheral blood (data not shown). Serum cytokine levels were assessed for all patients before and after combination therapy (day 0 vs. 84) and for a subset (n = 6) of patients at earlier time points. Significant levels of soluble IL-2Rα (sIL-2RA) were detected on days 14, 21, and 28, with levels declining back...
to pretreatment levels by day 35, a week after IL-2 therapy (Fig. 6B). We also observed a subtle decrease in TGF-β secretion. Notably, we did not detect significant amounts of other serum cytokines tested. Thus, a systemic excess of cytokines at and beyond day 14 is unlikely to have contributed to the decreases observed in C-peptide levels. Whether earlier production of cytokines contributed to initiation of inflammation is not known.

We observed a transient increase in the number of total peripheral blood CD56+ NK cells in all patients on day 28 (Fig. 7A), which declined to pretreatment levels by day 56. Within the CD56+ NK population, we observed a significant increase in CD56hi NK subsets (Fig. 7B) but no change in the CD56loCD16+ subset. CD3+CD56+ NK T-cell frequencies did not change with therapy (Supplementary Fig. 6).

Using sIL-2RA and rapamycin trough levels as a measure of the impact of IL-2 and rapamycin, respectively, we found a strong correlation between sIL-2RA levels and the frequency of multiple cell types (NK, FOXP3, and eosinophils), whereas rapamycin trough levels did not correlate with the frequency of any of these cell populations (Supplementary Fig. 7). These data suggest a prominent role for IL-2 in the induction of phenotypes observed in this clinical trial.

**DISCUSSION**

Coordinated analysis of both clinical and mechanistic data during trials may provide biomarkers of safety and efficacy. Using this approach, we found that rapamycin/IL-2 combination therapy had the unwanted effect of transiently decreasing β-cell function. This occurred despite the beneficial effects of this therapy in the NOD mouse model of diabetes (20), the beneficial effects of low-dose IL-2 in GvHD (18) and vasculitis (19), and a wealth of in vitro and in vivo data supporting the hypothesis that this therapy would result in coordinated enrichment of Treg responses and inhibition of Teff responses. Treg numbers and IL-2 responsiveness in peripheral blood were boosted in patients in this trial. Yet, the number of NK cells and eosinophils also increased, suggesting that combination therapy promoted an environment that may have been responsible for the transient decrease in β-cell function. These studies test, for the first time, IL-2 and rapamycin therapy in human T1D subjects and highlight the challenges in translating studies from preclinical models. Moreover, this work suggests specific tests that may be informative in future trials to assess safety and efficacy.
creased at later time points. This transient decrease in overall amount of IL-2 given in those studies over months was similar to the amount we used (54). Although administered in different dosing schedules, the first month of therapy concordant with IL-2. In addition, in long-standing T1D patients waiting for islet transplant, rapamycin monotherapy at doses similar to those used in our trial did not result in a drop in C-peptide levels (11,32). Nevertheless, in other clinical settings higher doses of rapamycin have resulted in impaired regulation of insulin levels (33). Thus, although we cannot rule out an impact of rapamycin on β-cell function, our evidence points to a detrimental effect of IL-2 or the combination of rapamycin and IL-2 in this patient population.

Rapamycin/IL-2 combination therapy resulted in a significant increase in FOXP3+ Tregs. Whether the function of FOXP3+ cells in our trial was altered by therapy was not addressed. Isolated CD25hi T cells from patients in the low-dose IL-2 trial in GVHD were functional Tregs. Yet, it is interesting that not all GVHD patients showed beneficial effects of therapy even though Tregs were significantly elevated in all study subjects. In addition, the level of Treg enhancement was highly variable in the vasculitis study (19) while all patients improved clinically, suggesting that other cells beyond Tregs may play a role.

On the basis of mouse models (15–17,20), we predicted that combination therapy would halt damage of β-cell cells. However, we observed a significant decline in 2-h AUC C-peptide values that occurred in all subjects at an accelerated rate, with a mean percent change of approximately four times that of comparable published control groups (3,4,26–28). Of importance, β-cell function increased at later time points. This transient decrease in β-cell function may have occurred due to IL-2 and rapamycin dosing/timing issues, combination of rapamycin with IL-2, and/or factors unique to T1D subjects. In two recently reported clinical trials, low doses of IL-2 were beneficial in promoting tolerance in GVHD and vasculitis (18,19). Although administered in different dosing schedules, the overall amount of IL-2 given in those studies over ~2 months was similar to the amount we used (54 × 10^6 IU over 1 month) with 52.5 × 10^6 IU over 9 weeks in the vasculitis trial and the tolerated dose of ~95 × 10^6 IU over 8 weeks in the GVHD trial. In our study, IL-2 was given for 1–2 weeks before rapamycin reached targeted blood levels (Supplementary Fig. 1). This is unlike the mouse models where rapamycin was initiated at full dose with IL-2 (21) and may explain some of the early proinflammatory responses, even though IL-2 doses were similar to other low-dose regimens for tolerance induction. Future studies will benefit from including early assessment of both immunological and clinical parameters as biomarkers of safety and mechanisms of action.

Although earlier observations in vitro and in transplant models have suggested that rapamycin may be β-cell toxic (30,31), evidence from this trial suggests that rapamycin may not be the primary or only cause of β-cell damage. C-peptide decline was seen even in two subjects who did not receive the full course of rapamycin, and the most significant changes in mechanistic data occurred within the first month of therapy concordant with IL-2. Whether NK cells from patients in this trial secrete pro- or anti-inflammatory cytokines is not known. In multiple sclerosis patients, there is an increase in CD56^bright NK cells that produce IL-10 and are associated with clinical benefit upon daclizumab therapy (36). Yet, a clinical trial of daclizumab plus mycophenolate mofetil did not alter the disease course in new-onset T1D subjects (26). As in our trial, a transient increase in eosinophils has been observed in HIV patients with IL-2 monotherapy (37) and low-dose IL-2 therapy for GVHD (18) but, interestingly, not in the low-dose IL-2 vasculitis study (19). Eosinophils constitutively express the low-affinity IL-2R. Upon activation, they express CD25 and gain the potential to secrete peroxidase and IL-6 (38), suggesting that eosinophilia could also contribute to the proinflammatory responses observed during combination therapy. Future studies should address the functional properties of NK cells and eosinophils that changed upon therapy.
Remarkably, combination therapy resulted in a durable rescue of IL-2R signaling even a full year after initiation of therapy, most dramatically in the CD25+ population. Increased response to IL-2 is most likely not solely due to increased expression of CD25, since CD25 MFI (mean fluorescence intensity) transiently increased with IL-2 therapy (data not shown) and the percentage of CD25+CD127lo Tregs increased during therapy whereas

FIG. 5. Rapamycin/IL-2 combination therapy did not alter CD4 helper subset differentiation or CD8 memory composition. Thawed PBMCs were stained for viability, CD4, CD8, CD45RO, CXCR3, and CRTH2. A: The frequency of memory CD45RO+ (solid square) and naive CD45RO− (open circles) in the CD4+ (left) and CD8+ (right) T-cell populations is shown. B: The frequency of CXCR3 and CRTH2 of CD4+CD45RO+ cells is shown as approximate markers of Th1 and Th2 cells, respectively. C: PBMCs were stimulated for 6 h with PMA/ionomycin in the presence of brefeldin A/myosin prior to staining for CD4, CD45RO, IL-17, and IFN-γ. Frequencies of IFN-γ+ (open squares, left axis) and IL-17+ (solid circles, right axis) cells of CD4+CD45RO+ gated cells are shown. Means ± SD are shown for all subjects assayed. Statistical significance was determined by a one-way ANOVA and is shown in each plot.

FIG. 6. Rapamycin/IL-2 combination therapy increased eosinophils and sIL-2RA while decreasing serum TGF-β. A: The frequency of eosinophils in whole blood was determined in a standard hematologic assay and is shown for each subject over time. B: Serum analytes were measured using a multiplex Searchlight chemiluminescent assay. Averages of duplicates for all subjects are shown for day 0 and 84, and subjects 1, 2, 3, 5, and 7 are shown for time points between days 14 and 70. Of the 22 cytokines and cytokine receptors tested, data are shown where statistical differences were observed. Measures are shown for each subject over time, and statistical significance was determined by a one-way ANOVA, noted in the graph. Significance from time 0 to each time point was determined using a paired Student t test and is indicated with an asterisk. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.
was maintained, the increased frequency of FOXP3+ Tregs of IL-2 molecules and/or promote selective outgrowth or homing and IL-2 may stably enhance IL-2R complex signaling siveness in NK cells (40). Thus, treatment with rapamycin Wiskott-Aldrich syndrome rescues poor IL-2 respon-
siveness is not unprecedented. In some cancer response to IL-2 persisted beyond therapy. Rescue of IL-2 responsiveness is not unprecedented. In some cancer patients treated with high doses of IL-2, pSTAT5 in CD4+ and CD8+ T cells is enhanced (39), and IL-2 therapy for Wiskott-Aldrich syndrome rescues poor IL-2 responsive-
siveness in NK cells (40). Thus, treatment with rapamycin and IL-2 may stably enhance IL-2R complex signaling molecules and/or promote selective outgrowth or homing of IL-2–responsive cells. Even though IL-2 responsiveness was maintained, the increased frequency of FOXP3+ Tregs was transient in the peripheral blood. These FOXP3+ cells may have migrated from the periphery or may have undergone activation-induced cell death upon withdrawal of IL-2.

Rapamycin has been shown to increase memory CD8+ T-cell formation, increase the Th2:Th1 ratio, and decrease activation and proliferation of CD4+ Teffs (10), whereas IL-2, when given alone, leads to an expansion of the CD8+ and CD4+ Teffs (37). Yet, we did not observe such changes in peripheral blood samples. It remains possible that once activated, the relevant cells leave the circulation and move to the tissues or secondary lymphoid populations, or that combination of rapamycin with IL-2 negated the effect of rapamycin alone on T-cell differentiation. Alternatively, T cells of T1D subjects may be relatively resistant to rapamycin due to disease-specific defects in mTOR sig-
naling, although the reduction in pAkt after therapy sug-
gests that rapamycin did affect the Akt/phosphatidylinositol 3-kinase pathway. Thus, the functional composition of the memory T-cell pool may have been altered with combi-
nation therapy without causing measurable changes in the frequency of memory or helper T-cell subsets.

Recently, the immunostimulatory effects of rapamycin on innate cells have been recognized (41). Although we did not test monocyte function, we did find a significant decrease in TGF-β secreted by myeloid cells. We also noted increased serum sIL-2RA, a marker of immune activation. This in-
crease is most likely due to IL-2RA shedding upon activation (42). Whether sIL-2RA has any biological effects is unclear, although one report suggested that high sIL-2RA levels block Treg function (43). Future studies will benefit from a broader survey of immune cells, including array technol-
ogies, to determine all cell types impacted by therapy.

Overall, these data suggest that Tregs of T1D subjects can be enhanced by IL-2 therapy, providing a foundation upon which to build future treatments designed to augment Tregs. However, rapamycin/IL-2 combination therapy also promoted an environment that presumably resulted in transient β-cell dysfunction. In future studies, approaches need to be identified that dissociate Treg expansion from effector responses. For next-generation trials, markers of IL-2 therapy safety should include early assessment of β-cell function as well as FOXP3 expression, eosinophil and NK frequencies, and changes in sIL-2RA levels.

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S.A.L. wrote the manuscript and researched data. M.R., P.L.S., and S.A. researched data. S.S., J.B.B., R.G., and A.A. conducted the clinical trial. A.R., S.A., D.P., L.A.T., and J.A.B. reviewed and edited the manuscript and contributed to discussion. M.E. and P.J.B. assisted with trial design and conduct, reviewed and edited the manu-
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**FIG. 7.** Rapamycin/IL-2 combination therapy transiently augments NK cell frequency. Thawed PBMCs were stained for viability, CD56, CD3, and CD16. A: The number of CD56+CD3− NK cells of PBMCs are shown for individual subjects. B: The average ± SD frequencies for CD56+CD16− (open squares), CD56+CD16− (closed triangles), and CD56+CD16+ (closed circles) subsets of CD56+ NK cells within the live lymphocyte gate are shown. Statistical significance was determined by a one-way ANOVA, noted in the graph. Significance from time 0 to each time point was determined using a paired Student t test and is indicated with an asterisk. *P ≤ 0.05, **P ≤ 0.01.
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