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METHODS. A randomized, single-blinded, placebo-controlled trial was performed on 25 subjects: 17 subjects received ATG (2.5 mg/kg intravenously) followed by pegylated G-CSF (6 mg subcutaneously every 2 weeks for 6 doses) and 8 subjects received placebo. The primary outcome was the 1-year change in AUC C-peptide following a 2-hour mixed-meal tolerance test (MMTT). At baseline, the age (mean ± SD) was 24.6 ± 10 years; mean BMI was 25.4 ± 5.2 kg/m^2; mean A1c was 6.5% ± 1.1%; insulin use was 0.31 ± 0.22 units/kg/d; and length of diagnosis was 1 ± 0.5 years.

RESULTS. Combination ATG/G-CSF treatment tended to preserve β cell function in patients with established T1D. The mean difference in MMTT-stimulated AUC C-peptide between treated and placebo subjects was 0.28 nmol/l/min (95% CI 0.001–0.552, $P = 0.050$). A1c was lower in ATG/G-CSF–treated subjects at the 6-month study visit. ATG/G-CSF therapy was associated with relative preservation of Tregs.

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Anti-thymocyte globulin/G-CSF treatment preserves β cell function in patients with established type 1 diabetes

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CONCLUSIONS. Patients with established T1D may benefit from combination immunotherapy approaches to preserve β cell function. Further studies are needed to determine whether such approaches may prevent or delay the onset of the disease.

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Introduction

Type 1 diabetes (T1D) is the end result of autoimmune-mediated destruction of insulin-producing β cells (1). To date, most efforts seeking to ameliorate the autoimmune process and reverse hyperglycemia in recent-onset cases have used single immunosuppressive or immunomodulatory drugs (2–6). While several agents have shown promise, no single agent has demonstrated long-term success in reversing T1D as a means of standard medical practice (7, 8).

▶ Related Commentary: p. 94

Conflict of interest: Mark A. Atkinson is listed as a coinventor on a patent for the use of ATG/G-CSF for T1D.
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We and others have questioned whether combination therapy using drugs that have already received regulatory approval might produce a synergistic response and provide for clinically meaningful preservation of β cell function (9–11). Perhaps the most aggressive and effective combination approach in T1D to date used autologous nonmyeloablative stem cell transplantation. With a regimen of cyclophosphamide, autologous stem cell harvest, anti-thymocyte globulin (ATG), stem cell infusion, granulocyte CSF (G-CSF), and intensive inpatient and outpatient support, this approach demonstrated the capacity to achieve short-term insulin independence, while simultaneously raising questions regarding equipoise (12–16). As such, we sought to deconstruct the autologous nonmyeloablative approach, eliminate the use of cyclophosphamide, and develop a lower risk, effective combinatorial approach to preserve β cell function in T1D.

Previously, we reported that low-dose ATG and G-CSF treatment leads to durable reversal of diabetes in nonobese diabetic mice (17). The combination appeared to impart its beneficial

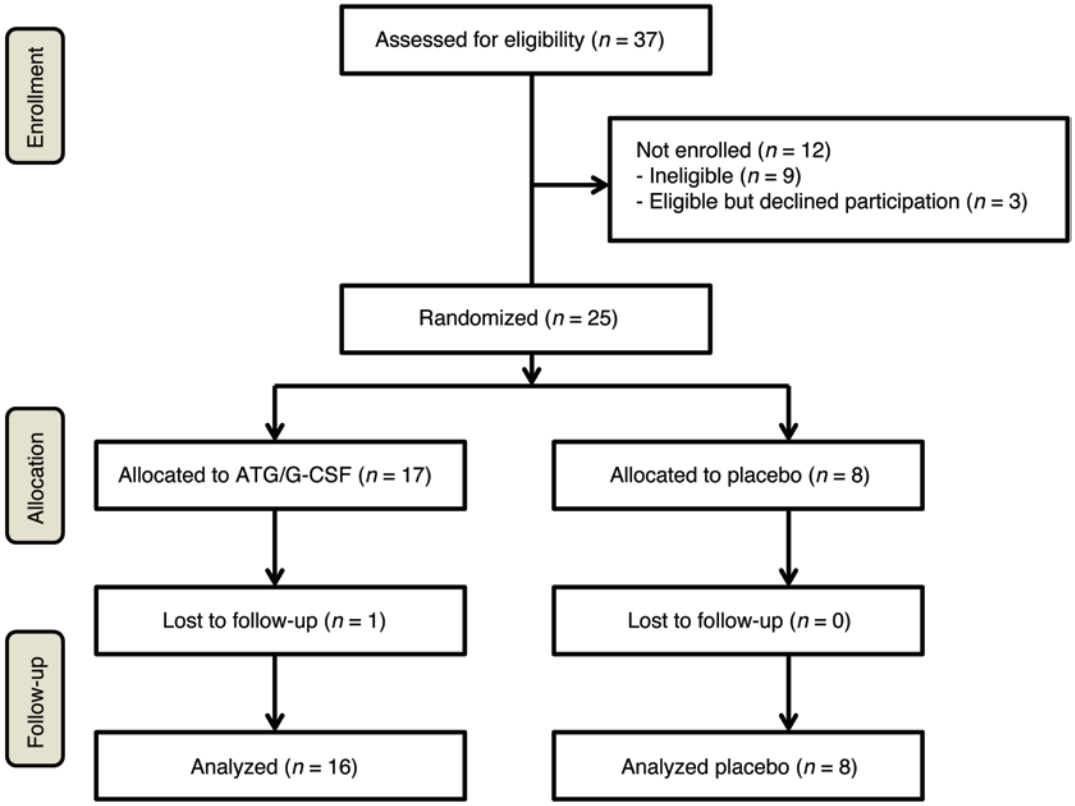

Figure 1. ATG/G-CSF combination therapy consort diagram.

effects synergistically via hematopoietic mobilization alongside a relative preservation of Tregs (17). Notably, efforts to use either G-CSF or ATG (6.5 mg/kg) as monotherapy in patients with recent-onset T1D failed to demonstrate benefit (18, 19).

To explore the potential of low-dose ATG and G-CSF to preserve β cell function in T1D and to determine whether the therapeutic window for immune-based therapies could be expanded beyond the first 100 days after diagnosis, we conducted a randomized, single-blinded, placebo-controlled trial in persons with "established" T1D (i.e., duration of disease of 4 months to 2 years), heretofore considered to be beyond the therapeutic window.

Results

Patients. From August 2010 through November 2012, we screened 37 individuals; 9 were ineligible for the study, 3 were eligible but declined participation, and 25 subjects were eligible and enrolled (Figure 1). Using a 2:1 randomization, 17 subjects were randomly allocated to receive ATG/G-CSF and 8 subjects were randomly allocated to receive placebo. Thirteen subjects had duration of diabetes greater than 1 year. Baseline characteristics were similar between groups (Table 1). The last subject completed the 12-month follow-up visit in November 2013. One subject, randomized to ATG and G-CSF, dropped out after the 6-month follow-up visit.

β Cell function. At baseline, no difference was observed in the mean 2-hour AUC C-peptide between the drug-treated and placebo-treated groups (Table 2). As study follow-up progressed, differences in AUC C-peptide between drug-treated and placebo-treated groups approached significance, as reflected by the *P* values decreasing sharply from 0.234 at 3 months to 0.076 at 6 months and 0.050 at 12 months (Table 2 and Figure 2). The mean difference in 2-hour AUC C-peptide between study groups, the study's a priori designated primary end point, was 0.28 nmol/l/min (95% CI 0.001–0.552, $P = 0.050$) after 1 year. The mean 2-hour AUC C-peptide in treated subjects was 0.74 nmol/l/min (SD 0.47), while the mean 2-hour AUC C-peptide in placebo-treated subjects was 0.43 nmol/l/min (SD 0.32). The mean 4-hour AUC C-peptide in treated subjects was 0.74 nmol/l/min (SD 0.46), while the mean 4-hour AUC C-peptide in placebo-treated subjects was 0.48 nmol/l/min (SD 0.35). The mean difference in 4-hour AUC C-peptide between study groups after 1 year was 0.22 nmol/l/min (95% CI –0.062–0.517, $P = 0.12$).

Data for individual subjects, showing the 2-hour AUC at baseline and 1 year after therapy, are shown in Figure 3. One year following therapy, 9 of 16 evaluable subjects had AUC C-peptide at or above baseline values and 11 of 16 had AUC C-peptide at or above 75% of the baseline value. In contrast, there was ongoing decline in β cell function in subjects who received placebo, with a nearly 40% decline in mean AUC C-peptide during 1 year of follow-up. Preservation of β cell function was not different when comparing subjects with T1D duration of 4 to 12 months with those of 12 to 24 months (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI78492DS1).

Glycemic control and insulin use. Subjects in both the ATG/G-CSF and placebo groups entered the study with excellent glycemic control and maintained it through the first year of follow-up (Table 3). A1c was lower, though not significantly, in treated subjects at the

Table 1. Baseline characteristics

	ATG/G-CSF Mean (SD)	Placebo Mean (SD)
Gender	12 M/5 F	5 M/3 F
Age at diagnosis (yr)	23.64 (10.0)	23.55 (10.6)
Age at screening (yr)	24.67 (9.87)	24.48 (11.0)
Time from diagnosis (yr)	1.04 (0.55)	0.93 (0.50)
AUC C-peptide 2-hour MMTT (nmol/l/min)	0.71 (0.48)	0.71 (0.64)
A1c (%)	6.69 (1.09)	6.03 (0.97)
Daily insulin usage (units/kg/d)	0.44 (0.49)	0.45 (0.32)
GAD autoantibodies (units/ml)	292.12 (307.1)	357.13 (312.4)
ZnT8 autoantibodies (units/ml)	0.16 (0.32)	0.25 (0.31)
IA-2 autoantibodies (units/ml)	75.18 (125.0)	158.0 (162.7)
Total CD3 (cells/mm^3)	72.49 (7.24)	71.80 (4.01)
Total CD4 (cells/mm^3)	66.96 (7.05)	66.83 (7.35)
Total CD8 (cells/mm^3)	26.64 (5.83)	27.88 (6.37)
CD4/CD8	2.66 (1.07)	2.52 (0.66)
FOXP3$^+$Helios$^+$ Tregs (%)	5.80 (1.81)	5.81 (1.77)
Naive/memory T cells	3.49 (1.39)	3.69 (1.74)
TSDR Treg/CD3 (%)	3.86 (1.2)	3.47 (1.01)

M, male; F, female.

6-month visit (mean difference compared with baseline 0.88%, $P = 0.06$). While no significant differences in A1c were noted, mean A1c in ATG/G-CSF–treated subjects increased 0.5% (from 6.7 to 7.2) over the first year of study, while in placebo-treated subjects, it increased 1.0% (from 6.0 to 7.0). There were no significant differences in insulin use at 3, 6, 9, or 12 months (Table 4).

Autoantibody titers. There were no differences in autoantibody titers between study groups over time (Supplemental Table 2).

Flow cytometry and Treg/CD3 by FOXP3-TSDR analysis. Flow cytometry studies revealed lower total total CD3/CD8, CD19/CD8, and CD4/CD8 ratios, higher levels of FOXP3$^+$Helios$^+$ Tregs, and no differences in naive/memory ratios when comparing ATG/G-CSF– and placebo-treated subjects after baseline. Using epigenetic analysis of the Treg-specific demethylated region (TSDR) within the FOXP3 locus to determine Treg frequency, no significant differences in the Treg/CD3 ratio were noted at 2 weeks, 6 months, or 12 months when comparing study groups. Percentage change in cell counts and ratios from baseline to 2 weeks and 12 months after therapy are shown in Table 5. Detailed flow cytometry data and statistical results are provided in Supplemental Tables 3–10. There were no differences in CD4/CD45RO, CD8/CD45RO, CD45RO Treg, or CD45RA Treg frequencies (data not shown).

Complete blood count and complete metabolic profiles changes. When comparing subjects who received ATG/G-CSF to those receiving placebo, there were significant increases in white blood cell and neutrophil counts and decreases in lymphocyte count at the 2-week visit ($P = 0.02$, $P = 0.008$, and $P = 0.002$, respectively) (Supplemental Tables 11–13). Lymphocyte counts remained lower in treated subjects through 6 months, while neutrophil counts remained higher in treated subjects though 3 months of follow-up. There were no clinically relevant changes in hematocrit, hemoglobin, red blood cell, platelet, basophil count, or complete metabolic profile parameters between groups (data not shown).

Responders versus nonresponders. Responders were defined as subjects who maintained AUC C-peptide at or above the baseline value at 12 months. By this definition, the ATG/G-CSF group had 9 (of 16) responders, while the placebo group had only 1 (of 8) responder. Among subjects who received ATG/G-CSF, older age at diagnosis was a significant predictor of response. The mean age of responders at diagnosis of T1D was 27.5 years, with a mean difference in diagnosis age from nonresponders of 9.9 years ($P = 0.047$). In addition, baseline insulin dose was predictive of response, with responders using less insulin than nonresponders (mean difference 0.48 units/kg/d, $P = 0.049$). No differences were observed with respect to gender; baseline A1c; AUC C-peptide; total amounts of CD3, CD4, and CD8 cells; and the ratio of CD4/CD8 when comparing responders to nonresponders. Changes in complete blood count (CBC) cell subsets or T cell subsets during the 6 months following treatment did not differentiate responders from nonresponders.

Adverse events. There were 520 adverse events (AEs) reported through 12 months of study follow-up. No episodes of severe hypoglycemia requiring glucagon therapy or assistance by another person were observed, but numerous episodes of hypoglycemia were reported. Of the 17 subjects who received ATG, cytokine release syndrome (CRS) occurred during drug infusion in 14, whereas serum sickness developed in 13. There were no reports of grade 4 CRS or serum sickness. Among subjects

Table 2. AUC C-peptide following ATG/G-CSF versus placebo

	ATG/G-CSF AUC C-peptide, nmol/l/min (SD)	Placebo AUC C-peptide, nmol/l/min (SD)	Mean 2-hour AUC change from baseline ATG/G-CSF vs. placebo (SD)	95% Confidence interval	*P* value by *t* test (*P* value by 100,000 permutation test)
Baseline (17,8)	0.71 (0.48)	0.71 (0.64)	0.002 (0.46)	–	–
3 months (17,8)	0.69 (0.47)	0.56 (0.43)	0.121 (0.23)	−0.065–0.307	0.078 (0.007)
6 months (17,8)	0.79 (0.54)	0.49 (0.44)	0.029 (0.37)	−0.033–0.621	0.025 (0.025)
12 months (16,8)	0.74 (0.47)	0.43 (0.32)	0.277 (0.31)	0.001–0.552	0.017 (0.018)
12 months (16,8) 4-hour MMTT[A]	0.74 (0.46)	0.48 (0.35)	0.223 (0.32)	−0.062–0.517	0.039 (0.039)

[A]All data shown are for 2-hour MMTT, with the exception of the final row, which shows data for 4-hour MMTT. The number of subjects evaluated is shown in parenthesis for each time point (ATG-GCSF, placebo). AUC C-peptide are shown as total AUC divided by 120 minutes (2-hour data) or 240 minutes (4-hour data). The primary end point for the study was chosen a priori as the 2-hour MMTT.

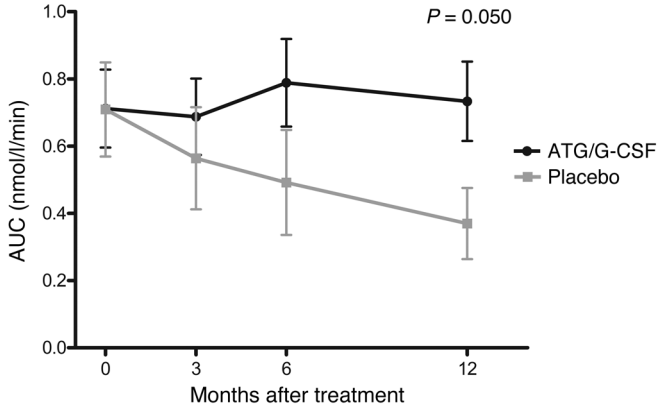

Figure 2. Preservation of C-peptide following combination therapy with low-dose ATG and G-CSF. The combination of ATG and G-CSF resulted in a significant preservation of AUC C-peptide when compared with placebo therapy. On average, subjects who received placebo experienced a 39% reduction in AUC C-peptide over 1 year, while subjects who received ATG and G-CSF experienced a 4.3% increase in AUC C-peptide over the same time period. AUC C-peptide is shown as the AUC divided by 120 minutes. Data were analyzed via 2-sample 2-sided *t* test and are presented as mean ± SD.

receiving ATG/G-CSF, the most common worst grade AEs were decreased CD4 (n = 15), lymphopenia (n = 15), serum sickness (n = 13), hypoglycemia (n = 13), and CRS (n = 11). In placebo-treated subjects (n = 8), the most common worst grade AEs were hypoglycemia (n = 6), headache (n = 4), fatigue (n = 3), anemia (n = 3), nausea (n = 3), sore throat (n = 3), and upper respiratory infection (n = 3) (Table 6). There were no differences in reported frequency of infections in either group. There were no cases of primary CMV or EBV infection, and no cases of clinical CMV reactivation. One subject who received ATG/G-CSF and who was CMV-seropositive at screening had a single-positive CMV PCR of 2,310 copies/ml that subsequently normalized. A second patient who received ATG/G-CSF and who was IgG positive, IgM negative, and PCR negative at enrollment had a mononucleosis-like illness (grade 2) 22 months after receiving ATG. This illness coincided with an elevation in EBV PCR to 11,002 copies/ml that subsequently returned to undetectable levels (<200 copies/ml).

Discussion

In this phase IIa clinical trial, treatment of established T1D (duration of diagnosis >4 months and <2 years) with a combination of low-dose ATG (2.5 mg/kg) and pegylated G-CSF (6 mg subcutaneously every 2 weeks for 6 doses) tended to preserve β cell function 12 months following initiation of therapy. While only the 2-hour AUC C-peptide data approached statistical significance, both the 2-hour and 4-hour AUC C-peptide values were higher in ATG/G-CSF–treated subjects than in placebo-treated subjects at 1 year (0.74 vs. 0.43 nmol/l/min and 0.74 vs. 0.48 nmol/l/min, respectively).

AUC C-peptide was 0.71 nmol/l/min in both the ATG/G-CSF– and placebo-treated groups at baseline. However, in an effort to determine if duration of diabetes affected response, we established subgroups with duration of T1D less than and greater than 1 year and analyzed their responses to therapy separately (Supplemental Table 1). While these analyses suggested no difference in response to therapy based on T1D duration, randomization and the relatively small sample size resulted in unevenly distributed baseline values that preclude definitive statements regarding efficacy in subjects with less than 1 year or greater than 1 year duration. Subjects with duration of T1D greater than 1 year had baseline mean AUC C-peptide of 0.80 nmol/l/min for those randomized to ATG/G-CSF but only 0.69 nmol/l/min for those randomized to placebo. Conversely, subjects with duration of T1D less than 1 year had baseline mean AUC C-peptide of only 0.59 nmol/l/min for those randomized to ATG/G-CSF but 0.92 nmol/l/min for those receiving placebo. As insulin secretory reserve may be more stable in subjects with higher baseline AUC C-peptide, we cannot exclude the possibility that differences in baseline C-peptide may have favored demonstration of efficacy of ATG/G-CSF in those with duration of T1D greater than 1 year or worked against demonstrating efficacy of ATG/G-CSF in those with duration of diabetes of less than 1 year. These observations underscore the urgency for larger studies to validate the apparent efficacy of ATG/G-CSF in patients with established T1D.

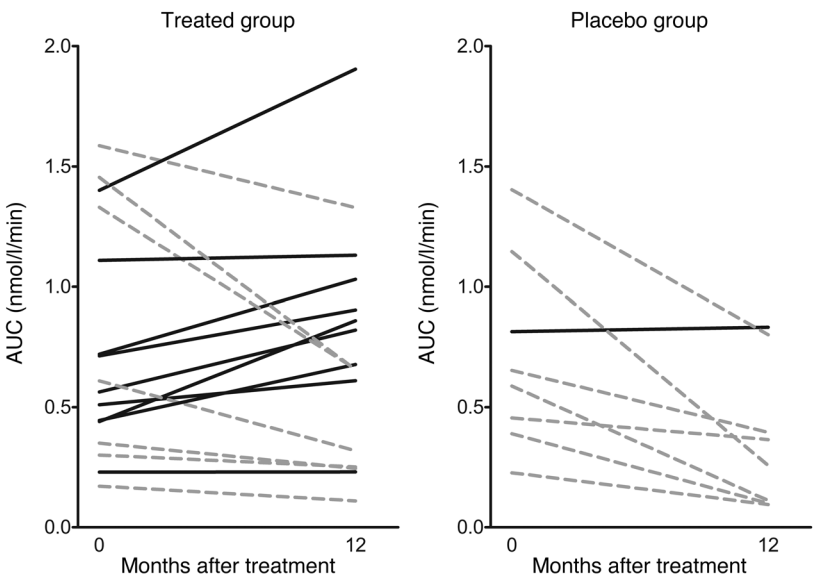

Figure 3. AUC C-peptide at baseline and 1 year following ATG/G-CSF compared with placebo. Data from each subject is shown. Subjects are separated by study drug assignment. Subjects depicted by solid black lines had sustained or increased AUC C-peptide over 1 year. Subjects depicted by dashed gray lines had a reduction in AUC C-peptide over 1 year. AUC C-peptide is shown as the AUC divided by 120 minutes.

Table 3. A1c following ATG/G-CSF versus placebo

	ATG/G-CSF A1c, % (SD)	Placebo A1c, % (SD)	Mean 2-hour AUC change from baseline ATG/G-CSF vs. placebo (SD)	95% Confidence interval	*P* value by *t* test
Baseline (17,8)	6.69 (1.09)	6.03 (0.97)	0.66 (1.06)	–	–
3 months (17,8)	6.58 (1.38)	6.41 (0.99)	−0.50 (0.77)	−1.18–0.18	0.14
6 months (17,8)	6.84 (1.64)	7.05 (1.35)	−0.88 (1.01)	−1.78–0.02	0.06
9 months (15,8)	7.14 (2.10)	6.63 (0.97)	−0.25 (1.35)	−1.20–0.70	0.58
12 months (16,8)	7.21 (2.06)	7.00 (0.98)	−0.51 (1.62)	−1.64–0.63	0.37

The number of subjects evaluated is shown in parenthesis for each time point (ATG-GCSF, placebo).

Additional efforts to determine whether baseline characteristics predicted response suggested that older subjects using less insulin at the time of enrollment were more likely to be responders. However, changes in the CBC, flow cytometry subsets, and Treg frequency during the first 3 months following therapy did not predict response.

Importantly, glycemic control was excellent throughout the study in both groups, with the mean A1c in placebo-treated subjects rising from 6.0% at baseline to 7.0% at 12 months, while ATG/G-CSF subjects had an initial mean A1c of 6.7% and A1c of 7.2% at 12 months. Similarly, insulin requirements remained relatively low in both groups, with average insulin requirements of 0.48 and 0.54 units/kg/d 12 months after randomization in the ATG/G-CSF–treated and placebo-treated groups, respectively. Though not statistically significant, the ATG/G-CSF group had higher absolute insulin requirements at the 3- and 6-month visits and higher absolute A1c at the 3-month visit when compared with the placebo group, despite achieving significant preservation of β cell function over the 1-year follow-up period.

This observation is likely explained by a number of factors. First, the study's relatively small sample size lead to imperfect randomization as it related to A1c, with baseline mean A1c in the ATG/G-CSF group of 6.7% compared with 6.0% in the placebo group. Our analyses focused on the change in A1c and insulin requirement over time and showed no statistical differences between the treatment groups. However, baseline differences in A1c and insulin usage clearly contributed to the differences noted in the absolute values at the 3- and 6-month visits. Second, we believe that the cytokine release and serum sickness that frequently followed ATG/G-CSF therapy resulted in physiologic stress and insulin resistance that induced temporary β cell dysfunction. Third, we observed one marked outlier in the treatment group who maintained poor glycemic control and reported markedly higher insulin requirements than physiologically expected. The subject later acknowledged falsifying insulin usage reports. While the subject's data were included in the primary analysis, a post-hoc analysis (data not shown) excluding this subject's insulin dose and A1c data revealed nearly identical A1c and insulin usage in both treatment groups at the 3-month visit.

Another important consideration of the low-dose ATG/G-CSF approach relates to the AE profile of these agents. Despite selecting a low-dose of ATG in an effort to minimize AEs, a considerable number of subjects experienced cytokine release and serum sickness. In addition, the use of any agents associated with immunosuppression increases concern for infection and specifically primary infections of EBV. In this trial, there were no notable differences in infection rates between placebo- and ATG/G-CSF–treated subjects. No subjects developed primary EBV or CMV infections. One ATG/G-CSF–treated subject, who was EBV seropositive at study enrollment, developed a mononucleosis-type illness 22 months after completing study therapy. Given the time from exposure to ATG and the subject's EBV reactivation, a causal relationship seems highly unlikely. Considering issues of risk versus benefit and given the growing number of therapies which have failed to provide benefit to patients with T1D, we must continue to discuss equipoise as we rationally evaluate therapies that appear to preserve C-peptide, as we seek to ultimately prevent and reverse T1D.

Related to discussions of equipoise, our study differs importantly from the results of the Study of Thymoglobulin to Arrest Type 1 Diabetes (START) trial (19). The START trial used a 3-fold higher dose of ATG monotherapy (6.5 mg/kg) and treated new-onset T1D subjects, yet it failed to demonstrate preservation of β cell function, although subanalysis suggested benefit in adults.

Table 4. Insulin use following ATG/G-CSF versus placebo

	ATG/G-CSF Insulin, units/kg/d, (SD)	Placebo Insulin, units/kg/d, (SD)	Mean 2-hour AUC change from baseline ATG/G-CSF vs. placebo (SD)	95% Confidence Interval	*P* value by *t* test
Baseline (16,8)	0.44 (0.49)	0.45 (0.30)	−0.01 (0.44)	–	–
3 months (15,6)	0.67 (1.09)	0.34 (0.24)	0.33 (0.94)	−0.62–1.29	0.47
6 months (15,8)	0.69 (1.08)	0.55 (0.33)	0.14 (0.91)	−0.50–0.97	0.72
9 months (14,7)	0.54 (0.47)	0.58 (0.44)	−0.05 (0.46)	−0.50–0.40	0.83
12 months (15,8)	0.48 (0.40)	0.54 (0.29)	−0.64 (0.37)	−0.40–0.27	0.69

The number of subjects evaluated is shown in parenthesis for each time point (ATG-GCSF, placebo).

Table 5. Cell subsets following ATG/G-CSF versus placebo (percentage change from baseline)

	ATG/G-CSF		Placebo	
	% Change from baseline		% Change from baseline	
	2 weeks	12 months	2 weeks	12 months
CD3[A]	−30	−14	−10	−10
CD4[A]	−38	−26	−0.7	−3
CD8[B]	+56	+45	+11	−28
CD4/CD8[A]	−61	−51	−4	+4
Treg/CD3 by TSDR	+75	+4	−6	+10
FOXP3$^+$Helios$^+$ Treg[A]	+86	+33	+1.3	+0.3
CD4$^+$ CD45RA/CD45RO	−0.1	−0.2	−0.1	−0.2
CD19 (B cells)[A]	+129	+38	−29%	−24

[A]Significant differences at 2 weeks and 12 months in ATG/G-CSF vs. placebo groups. [B]Significant differences at 12 months in ATG/G-CSF vs. placebo groups. See Supplemental Tables for raw data and *P* values.

When comparing mechanistic data from the ATG/G-CSF combination trial and the START trial, it is apparent that low-dose ATG/G-CSF induces a less severe T cell depletion and allows for faster T cell recovery than high-dose ATG alone (19). Furthermore, TSDR analysis confirms that low-dose ATG/G-CSF combination therapy preserves Tregs (Supplemental Table 7), while higher dose ATG monotherapy results in marked reduction of Tregs (19). In addition, low-dose ATG/G-CSF may be associated with a slight reduction in AEs relative to high-dose ATG monotherapy. While both CRS and serum sickness occurred in 97% of START subjects, 82% of ATG/G-CSF–treated subjects experienced CRS and only 76% developed serum sickness. Future efforts should seek to determine whether alternate ATG/G-CSF dosing schedules could further minimize AEs while preserving efficacy.

This study also differs from previous combination therapy approaches in T1D. Notably, the effort to combine mycophenolate mofetil and daclizumab in patients with new-onset T1D failed to preserve β cell function (20). One observation used to explain the failure was the potential negative effect of daclizumab on Treg number (20). In contrast, another combination approach using rapamycin and IL-2 resulted in an increase in Tregs yet transiently impaired β cell function (21). As such, not all combination approaches will be effective in preserving β cells and, as demonstrated by ATG and G-CSF, not all ineffective monotherapies should be discounted when considering combination therapy approaches. Furthermore, Treg frequency alone cannot serve as surrogate for an effective immunotherapy. Therapies such as ATG/G-CSF, which appear to simultaneously reduce effector T cells and preserve Tregs, may be required to achieve β cell preservation through immunomodulation.

With the exception of the anti-CD3 mAb teplizumab, which demonstrated preservation of C-peptide in patients with T1D 4 to 12 months after diagnosis (22), low-dose ATG/G-CSF remains the only other approach known to preserve C-peptide in patients with established T1D and, importantly, it extends the observation to patients with durations of T1D of nearly 2 years. In addition, the effect of ATG/G-CSF was the same when comparing patients with disease duration of less than or greater than 1 year. This observation provides further support for the potential of immunotherapy to benefit patients beyond the days or months immediately following diagnosis. As both ATG and G-CSF are FDA-approved agents, there is a clear pathway for the translation of this approach if larger studies confirm the observed benefits.

In conclusion, these data provide robust support for two emerging concepts in the treatment of T1D. First, the use of combination approaches, specifically low-dose ATG and G-CSF, may provide therapeutic synergy in preserving β cell function in patients with T1D. Second, subjects with T1D can achieve benefit from immunomodulatory therapy well beyond the classic "new-onset" period immediately following diagnosis.

Methods

Study patients. Participants from 3 sites (UCSF, University of Colorado, and University of Florida) were screened and enrolled. Eligible subjects were 12 to 45 years old and had been diagnosed with T1D for more than 4 months but less than 2 years. All subjects were positive for at least one T1D-related autoantibody other than insulin (glutamic acid decarboxylase [GAD], insulinoma-associated-2 [IA-2], or zinc transporter 8 [ZnT8]). To evaluate the potential for treating subjects with longer-standing T1D, we chose a minimum stimulated C-peptide response of 0.3 ng/ml (0.1 nmol/ml) during a 4-hour mixed-meal tolerance test (MMTT) (Boost, 6 ml/kg, maximum 360 ml) as an entry criteria instead of the 0.6 ng/ml (0.2 nmol/ml) threshold more commonly used in T1D intervention trials (6, 8). Eligible subjects had normal values at screening for CBC and complete metabolic profiles. Patients with immunodeficiency, chronic infection, evidence of past or current TB, HIV, hepatitis B or C infection, known allergy to ATG or G-CSF, or prior treatment with ATG were excluded. Subjects were randomized within 8 weeks of the screening visit. All patients provided written informed consent and minors provided assent. All eligibility criteria are listed in the study protocol (See Supplemental Materials).

Study design. In this randomized, single-blinded, placebo-controlled trial, subjects received either ATG (Thymoglobulin, Sanofi; 2.5 mg/kg given intravenously as 0.5 mg per kilogram on day 1 and 2 mg/kg on day 2) followed by pegylated G-CSF (Neulasta, Amgen; 6 mg subcutaneously every 2 weeks for a total of 6 doses) or placebo. Subjects were randomly assigned in a 2:1 ratio to the treatment groups. All subjects underwent baseline metabolic and immunologic studies, were hospitalized for the 2-day masked ATG or placebo infusion and first G-CSF or placebo injection, and thereafter were seen in an outpatient research center. The first dose of G-CSF or placebo was given within 24 hours of completing the ATG or placebo infusion. Subjects randomized to active drug therapy received pretreatment for ATG infusions with methylprednisolone (0.25 mg/kg) before and 12 hours after the start of each infusion. Subjects randomized to placebo received placebo infusion in place of methylprednisolone. All subjects received infusion pretreatment with acetaminophen and diphenhydramine. Those receiving ATG were given antimicrobial prophylaxis with trimethoprim-sulfamethoxazole and acyclovir for a minimum of 3 months and until the CD4 count was above 200 cells/μl. Subjects randomized to placebo received placebo prophylaxis. Subjects received ongoing intensive diabetes management from their personal diabetes physicians during study

Table 6. AEs in ATG/G-CSF and placebo-treated subjects by maximum grade

	ATG/G-CSF					Placebo				
Event	1	2	3	4	Total	1	2	3	4	Total
Alkaline phosphatase increased	7	0	0	0	7	0	0	0	0	0
Allergic reaction	1	0	0	0	1	0	0	0	0	0
Anemia	6	1	0	0	7	2	1	0	0	3
Arthralgia	6	2	1	0	9	2	0	0	0	2
AST increased	1	0	0	0	1	1	1	0	0	2
Back pain	6	0	0	0	6	1	0	0	0	1
CD4 lymphocytes decreased	0	2	11	2	15	0	0	0	0	0
Chills	2	3	0	0	5	1	0	0	0	1
CRS	1	10	0	0	11	0	0	0	0	0
Dizziness	4	0	0	0	4	0	0	0	0	0
Fatigue	4	1	0	0	5	3	0	0	0	3
Fever	1	1	0	0	2	0	0	0	0	0
Headache	4	4	0	0	8	3	1	0	0	4
Hypoglycemia	5	5	3	0	13	1	4	1	0	6
Hyponatremia	3	0	0	0	3	2	0	0	0	2
Hypotension	0	0	0	0	0	0	1	0	0	1
Infusion related reaction	2	1	0	0	3	0	0	0	0	0
Lymphocyte count decreased	0	1	2	12	15	1	1	0	0	2
Myalgia	6	1	1	0	8	1	0	0	0	1
Nausea	4	0	0	0	4	1	2	0	0	3
Neutrophil count decreased	2	0	0	0	2	1	0	0	0	1
Others	5	7	1	1	14	3	4	0	0	7
Platelet count decreased	5	1	0	0	6	0	0	0	0	0
Serum sickness	0	2	11	0	13	0	0	0	0	0
Sore throat	6	0	0	0	6	3	0	0	0	3
Upper respiratory infection	5	2	0	0	7	3	0	0	0	3
Vomiting	3	2	0	0	5	1	0	0	0	1
White blood cell decreased	2	0	0	0	2	2	0	0	0	2

AST, aspartate aminotransferase.

participation and were advised not to discontinue insulin therapy. In addition to a safety visit 1 week after initiation of study drug, subjects were seen every 2 weeks for 10 weeks to have metabolic and immunologic samples obtained, physical examination performed, and G-CSF or placebo given. Subjects returned for immunologic and metabolic testing at 3, 6, 9, and 12 months. The 3- and 6-month study visits included a 2-hour MMTT and the 12-month study visit included a 4-hour MMTT.

Study assessments. In addition to standard laboratory and clinical tests performed locally, A1c was determined using a DCA2000 or Vantage (Siemens Healthcare Diagnostics), C-peptide was measured at the Northwest Lipid Research Laboratories, and T1D autoantibodies were evaluated at the Barbara Davis Center. Whole blood T cells (CD3, CD4, CD8, CD45RA, CD45RO, CD25, FOXP3, Helios), B cells (CD19), and APCs (CD11c, CD14) and G-CSFR (CD114) were determined by flow cytometry. Frozen aliquots of whole blood were processed for DNA isolation and quantification of the TSDR of FOXP3 in proportion to T cells demethylated at the CD3 locus (Epiontis) (23).

End points. The primary outcome was the change from baseline to 1 year in the 2-hour AUC for C-peptide. Specifically, change in AUC C-peptide from the baseline to the 1-year visit was calculated for each subject. Mean change from baseline to 1 year was then calculated for the group of subjects randomized to either ATG/G-CSF or placebo. The mean of the changes from baseline to 1 year were then compared by treatment assignment using a 2-sample 2-sided *t* test, as described below. AUC C-peptide values were divided by either 120 minutes or 240 minutes for the 2-hour and 4-hour tests, respectively. Secondary outcome measures included change in insulin use, A1c, Treg/CD3 ratio by FOXP3-TSDR analysis, and frequency and severity of AEs. In addition, we determined whether baseline characteristics or laboratory measures obtained within the first 3 months following therapy predicted response to therapy. Responders were defined as those who maintained AUC C-peptide at or above the baseline value at 1 year.

Statistics. The treatment effect (i.e., the 2-hour AUC C-peptide change from baseline) at each time point was evaluated with the efficient parametric 2-sample 2-tailed *t* test. Results were secondarily compared via a permutation t test (100,000 replications), with corresponding *P* values reported within the parenthesis (24). Specifically, the analysis compared changes in 2-hour AUC C-peptide (i.e., Y_{ijk} for subject *j* in treatment arm *i*, where $i = 1$ for drug and $i = 0$ for placebo, at measurement time point *k* [e.g., 3, 6, 12 months] from baseline measurement [i.e., Y_{ij0}]) between the 2 treatment groups, with 12 months as the primary time point. This notation

applied to secondary outcomes as well. Analysis of AEs included tabulation of all reported events and reporting of the maximum AE grade and maximum AE attribution per subject. Significance was defined as $P < 0.05$.

Study approval. This study was conducted according to the Declaration of Helsinki and in accordance with good clinical practice guidelines, performed under an investigational new drug exemption (IND 107185), and approved by institutional review boards at the University of Florida, UCSF, and the University of Colorado. Written informed consent and assent, when appropriate, was received from each participate prior to inclusion in the study. A data safety monitoring board and an independent medical monitor provided additional study oversight. AEs were recorded and reported according to Common Terminology Criteria for Adverse Events classifications (version 4.0; http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_8.5x11.pdf).

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