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Low-Dose ATG/GCSF in Established Type 1 Diabetes: A Five-Year Follow-up Report

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Previously, we demonstrated low-dose antithymocyte globulin (ATG) and granulocyte colony-stimulating factor (GCSF) immunotherapy preserved C-peptide for 2 years in a pilot study of patients with established type 1 diabetes ($n = 25$). Here, we evaluated the long-term outcomes of ATG/GCSF in study participants with 5 years of available follow-up data ($n = 15$). The primary end point was area under the curve (AUC) C-peptide during a 2-h mixed-meal tolerance test. After 5 years, there were no statistically significant differences in AUC C-peptide when comparing those who received ATG/GCSF versus placebo ($P = 0.41$). A modeling framework based on mean trajectories in C-peptide AUC over 5 years, accounting for differing trends between groups, was applied to recategorize responders ($n = 9$) and nonresponders ($n = 7$). ATG/GCSF reponders demonstrated nearly unchanged HbA_{1c} over 5 years (mean [95% CI] adjusted change 0.29% [–0.69%, 1.27%]), but the study was not powered for comparisons against nonresponders 1.75% (–0.57%, 4.06%) or placebo recipients 1.44% (0.21%, 2.66%). These data underscore the importance of long-term follow-up in previous and ongoing phase 2 trials of low-dose ATG in recent-onset type 1 diabetes.

Type 1 diabetes is characterized by T cell–mediated β-cell destruction, ultimately leading to lifelong dependence on exogenous insulin (1,2). Several immunotherapy trials in type 1 diabetes have resulted in transient preservation of C-peptide (3,4). However, few trials have reported results beyond 2 years, making long-term safety and efficacy data limited.

Persistence of endogenous insulin secretion, as measured by C-peptide, and glycemic control, as indicated by glycosylated hemoglobin (HbA_{1c}), are associated with lower risk of acute and chronic complications of type 1 diabetes (5,6). Therefore, long-term analyses of immunotherapy trials demonstrating short-term success remain critical. Previously, we showed that a combination of low-dose antithymocyte globulin (ATG) and granulocyte colony-stimulating factor (GCSF) in individuals with established type 1 diabetes provided for C-peptide preservation at both 1 and 2 years after therapy (7,8). Herein, we report 5-year clinical trial outcomes. Specifically, we tested the hypothesis that low-dose ATG/GCSF could preserve β-cell function up to 5 years after therapy. To better define responders and nonresponders, models were developed to predict C-peptide change from baseline to 5 years post-treatment, and modeled C-peptide trajectories were compared against measured C-peptide values. In addition, we assessed the effect of ATG/GCSF on peripheral blood gene expression and T-cell depletion shortly after treatment to determine if these changes could predict long-term outcomes.

RESEARCH DESIGN AND METHODS

Study Participants

Twenty-five participants (aged 12–45 years) with established type 1 diabetes (duration 4 months to 2 years)

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were enrolled in a single-blinded, randomized, placebo-controlled study as previously described (7,8). Of the 25 enrolled participants, long-term follow-up data collected at 30, 36, 42, 48, 54, and 60 months were available for 15 individuals (n = 5 placebo, n = 10 ATG/GCSF). Eligible participants had type 1 diabetes and minimum peak C-peptide of 0.1 nmol/mL during a 4-h mixed-meal tolerance test (MMTT) at time of enrollment. Participants were randomly assigned 2:1 to receive intravenous low-dose ATG (2.5 mg/kg total dose over 2 days) and subcutaneous pegylated GCSF (6 mg every 2 weeks for six doses) or placebo. At baseline and weeks 1, 2, 4, 6, 8, and 10, as well as months 3, 6, 9, 12, 18, 24, 30, 36, 42, 48, 54, and 60, metabolic and immunologic studies were performed.

Safety Monitoring

Adverse events (AEs), serious AEs, medical events of interest, and complete blood counts were evaluated during each visit (7,8). Safety monitoring procedures continued until 5 years after ATG/GCSF administration.

Laboratory Measurements

HbA_{1c} was measured using a DCA2000 or Vantage analyzer (Siemens Healthcare Diagnostics), and C-peptide was measured at Northwest Lipid Research Laboratories, University of Washington. A 4-h MMTT was conducted at baseline and 1 year; 2-h MMTT was performed at 3 and 6 months and biannually to 60 months. The primary end point of the study was 2-h MMTT-stimulated area under the curve (AUC) C-peptide. Two-hour AUC C-peptide values are represented as absolute values (measured as ng/mL divided by 120 min) and change from baseline. Change in HbA_{1c}, total daily insulin dose (units/kg/day), and AUC glucose effect size from baseline were calculated as the difference from each time point and baseline divided by the SD for each variable in the total sample (Supplementary Table 1).

Flow Cytometry

Cryopreserved peripheral blood mononuclear cells (PBMCs) from fresh\whole blood were batch processed, thawed at 37°C, and washed into complete RPMI. Cells were then labeled with LIVE/DEAD Yellow Viability Dye (Invitrogen, Carlsbad, CA), followed by surface labeling with T-cell panel (CD3, CD4, CD8, CD38, CD45RA, CD197, and HLA-DR) antibodies used at concentrations previously reported (8). Data were acquired on a BD LSRFortessa flow cytometer and analyzed with FlowJo software.

Gene Expression

Gene expression from whole PBMCs was compared at baseline and 2, 4, and 12 weeks posttreatment with a custom Bar Harbor Biotechnology PCR array that contained 64 genes associated with GCSF signaling and T-cell signaling (Supplementary Table 2). RNA was extracted from PBMCs using the RNeasy Plus Kit (Qiagen, Germantown, MD). cDNA was prepared using RT^2 First Strand Kit reagents (Qiagen). All samples were run in duplicate. The relative transcript level for each gene was determined using the $2^{-\Delta\Delta CT}$ method and three candidate reference genes (*G6PD*, *GAPDH*, and *PPIA*).

Statistical Analyses

All statistical analyses were performed using SAS software (version 9.4), and data are expressed as means ± SD or frequencies and percentages. We previously defined low-dose ATG/GCSF responders and superresponders based on absolute AUC C-peptide levels at 12 and 24 months, respectively (8). Here, an updated model estimation analysis was performed in which responders were redefined using linear mixed modeling for all participants (treatment and placebo) that accounted for the cubic effect of time between the two groups. This modeling framework estimated mean trajectories in the two groups and allowed for model-estimated individual trajectories accounting for mean changes within the group as well as individual-level deviations. Differences among groups, time points, and interaction between group and cubic effect of time on change in HbA_{1c}, glucose level, insulin use, and delta AUC C-peptide effect size were assessed using a cubic mixed regression model (PROC MIXED in SAS), assuming a first-order autoregressive covariance structure using Kenward-Roger approximation. Baseline characteristics for placebo, ATG/GCSF responder, and ATG/GCSF nonresponder groups were compared by one-way ANOVA and Fisher exact test (Table 1). To further model change over time, linear regression was performed on each participant. For the regression models, adjusted estimates, 95% t-type CIs (95% CIs), and P values were reported, with P values <0.05 considered statistically significant. Linear mixed models were used to assess differences between gene expression level and group by time (9). In the models, we assumed unstructured covariance using Kenward-Roger approximation. Given the small size of this pilot study, posttests for multiple comparisons were not applied. P values <0.05 were considered statistically significant.

Data and Resource Availability

The data sets generated and analyzed are available by request. No applicable resources were generated or analyzed during the current study.

RESULTS

Modeling Framework to Predict Response to Treatment

Predicted C-peptide trajectories based on each individual's baseline and 1-year AUC C-peptide values were graphed for the 24 individuals enrolled in the study, irrespective of data availability at the 60-month time point (Fig. 1). Among participants who received ATG/GCSF, those with a predicted

Table 1—Baseline characteristics of study participants

Baseline characteristic	Total (*n* = 24)	Placebo (*n* = 8)	Nonresponders (*n* = 7)	Responders (*n* = 9)	*P*
Age at diagnosis, years	23.3 (10.1)	23.6 (10.6)	19.2 (5.1)	26.2 (12.2)	0.40
Age at screening, years	24.1 (10.0)	24.4 (10.8)	20.1 (4.9)	27.0 (11.9)	0.40
Duration of diabetes at screening, years	0.85 (0.52)	0.81 (0.37)	0.94 (0.57)	0.82 (0.65)	0.88
Male sex, *n* (%)	16 (66.7)	5 (62.5)	6 (85.7)	5 (55.6)	0.49*
Race, *n* (%)					
White	21 (87.5)	8 (100.0)	6 (85.7)	7 (77.8)	0.61*
Other	3 (12.5)	0 (0.0)	1 (14.3)	2 (22.2)	
Daily insulin use, units/kg/day	0.44 (0.43)	0.45 (0.30)	0.36 (0.27)	0.51 (0.62)	0.80
GADA, units/mL	303.7 (306.7)	357.1 (312.4)	254.3 (271.0)	294.6 (353.5)	0.82
ZnT8 A, units/mL	0.15 (0.27)	0.25 (0.31)	0.17 (0.34)	0.05 (0.07)	0.28
IA-2 A, units/mL	105.6 (142.0)	158.0 (163)	92.4 (146.0)	69.2 (120.1)	0.44
mIAA, units/mL	0.28 (0.50)	0.54 (0.79)	0.13 (0.10)	0.16 (0.24)	0.18
HbA_{1c}, %	6.5 (1.1)	6.0 (1.0)	6.1 (0.4)	7.3 (1.2)	**0.02**
HbA_{1c}, mmol/mol	48 (12.0)	42 (10.6)	43 (15.0)	56 (11.0)	
AUC C-peptide 2-h MMTT, ng/mL/min	1.13 (0.74)	0.90 (0.61)	1.90 (0.52)	0.76 (0.55)	**0.02**
AUC glucose 2-h MMTT, mg/dL/min	162.7 (53.5)	184.3 (54.5)	127.1 (53.1)	163.4 (46.4)	0.17
	(*n* = 20)	(*n* = 6)	(*n* = 5)	(*n* = 9)	
CD4 count, cells/mm^3	863.9 (353.3)	690.8 (169.3)	1,224.8 (332.8)	778.7 (333.4)	**0.02**
CD8 count, cells/mm^3	496.6 (150.7)	418.0 (54.5)	590.0 (188.6)	497.3 (155.6)	0.17
CD4/CD8 ratio	1.73 (0.56)	1.68 (0.44)	2.20 (0.65)	1.50 (0.45)	0.07
	(*n* = 23)	(*n* = 8)	(*n* = 7)	(*n* = 8)	
Hematocrit, %	41.3 (4.6)	40.1 (4.7)	43.3 (4.6)	40.7 (4.6)	0.40
Hemoglobin, g/L	14.1 (1.8)	13.6 (2.1)	14.6 (1.8)	14.0 (1.7)	0.61
Red blood cell count, cells/μL	4.86 (0.59)	4.84 (0.67)	5.02 (0.59)	4.74 (0.54)	0.67
Platelet count, cells/μL	239.2 (61.5)	243.6 (65.0)	269.4 (58.4)	208.4 (52.0)	0.15
White blood cell count, cells/μL	5.87 (1.64)	5.28 (1.83)	6.71 (1.64)	5.73 (1.28)	0.23
Lymphocytes, cells/μL	1.93 (0.64)	1.72 (0.33)	2.31 (0.68)	1.81 (0.73)	0.16
Neutrophils, cells/μL	3.26 (1.16)	2.92 (1.40)	3.60 (1.24)	3.31 (0.84)	0.54
Basophils, cells/μL	0.04 (0.04)	0.05 (0.05)	0.06 (0.03)	0.03 (0.03)	0.37
Eosinophils, cells/μL	0.17 (0.11)	0.17 (0.14)	0.20 (0.11)	0.14 (0.07)	0.51

Data are presented as mean (SD), except as otherwise noted. Individuals were categorized as receiving placebo or ATG/GCSF divided according to responder or nonresponder status. *P* values were calculated by one-way ANOVA, except as otherwise noted. Bold font denotes statistical significance at the 0.05 level. Sample size for cell counts differ because of loss of sample during processing. **P* value calculated by Fisher exact test.

delta AUC C-peptide effect size at 60 months > -0.5 were defined as responders (n = 9), and those with a predicted delta AUC C-peptide effect size < -0.5 were defined as nonresponders (n = 7). There were significant differences in baseline HbA_{1c} (P = 0.0247), 2-h AUC C-peptide (P = 0.0002), and $CD4^+$ T cell counts (P = 0.0177) between placebo recipients, responders, and nonresponders (Table 1). Specifically, responders had higher HbA_{1c} at enrollment (7.3% [56 mmol/mol]) compared with nonresponders (6.1% [43 mmol/mol]) and placebo recipients (6.0% [42 mmol/mol]). At baseline, the C-peptide AUC mean ± SD was higher in nonresponders (1.90 ± 0.52 ng/mL/min) versus responders (0.76 ± 0.55 ng/mL/min) and placebo recipients (0.90 ± 0.61 ng/mL/min). Nonresponders had a higher baseline $CD4^+$ T-cell count (1,224.8 ± 332.8 cells/mm^3) compared with responders (778.7 ± 333.4 cells/mm^3), although all were within the clinical reference range. All other baseline characteristics were similar across groups. There do not seem to be specific baseline characteristics differentiating the three placebo and five ATG/GCSF recipients who were not available at year 5 from those who remained in the study (Supplementary Table 3).

Effectiveness

When comparing low-dose ATG/GCSF with placebo in the individuals who completed follow-up at 60 months (n = 15), there were no statistically significant differences in delta AUC C-peptide effect size from baseline to 5 years

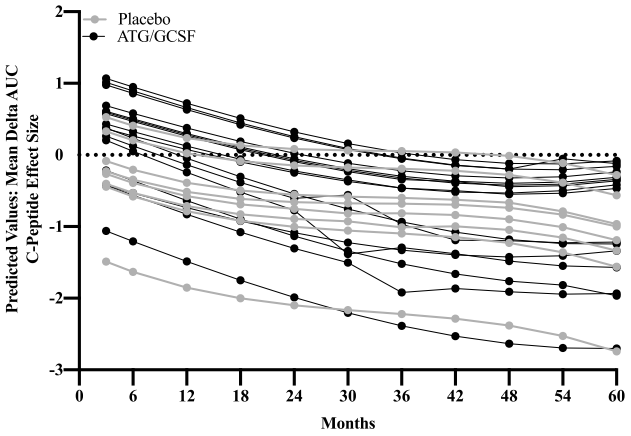

Figure 1—Projected model-based trajectories for mean delta AUC C-peptide effect size in study participants receiving ATG/GCSF treated and placebo. A linear mixed model to predict all participants (ATG/GCSF [*n* = 16] and placebo [*n* = 8]) shows cubic trends that differ from individual to individual.

(*P* = 0.41) (Table 2). At the 5-year visit, ATG/GCSF (*n* = 10) and placebo (*n* = 5) recipients had mean (95% CI) delta C-peptide AUC effect sizes of –0.79 (–1.23, –0.35) and –1.30 ng/mL (–1.86, –0.75), respectively (Fig. 2*A*), with apparent concordance between predicted C-peptide AUC trajectories (Fig. 2*A*) and absolute C-peptide AUC values (Supplementary Fig. 1). Although not statistically significant, when separated by response status, nonresponder effect size was –1.41 (–2.14, –0.68) and responder effect size was –0.37 (–0.94, 0.20) (Table 2 and Fig. 2*B*).

Secondary End Points

Change in HbA_{1c} from baseline was not significantly different between participants receiving treatment and placebo at 5 years (*P* = 0.9059) (Supplementary Table 1). However, in ATG/GCSF responders, the mean (95% CI) adjusted change in HbA_{1c} was 0.29% (–0.69%, 1.27%) vs 1.75% (–0.57%, 4.06%) in nonresponders and 1.44% (0.21%, 2.66%) in placebo recipients (Fig. 2*C*). Change in insulin requirements did not differ significantly between placebo recipients, nonresponders, and responders at any time point (*P* = 0.0546) (Fig. 2*D*).

As expected, total lymphocyte counts were lower in ATG/GCSF recipients in the first 2 weeks posttreatment (7,8). However, there were no differences in lymphocyte count when comparing ATG/GCSF versus placebo recipients between 1 month and 5 years of study follow-up (Supplementary Fig. 2*A*). $CD3^+$ T-cell percentages at 1 week posttreatment did not statistically differentiate nonresponders from responders (Supplementary Fig. 2*B*).

Gene Expression Studies

Baseline characteristics were enumerated to confirm no significant differences in PBMC gene expression between the groups (Supplementary Table 2). Low-dose ATG/GCSF was not associated with significant changes in gene expression in whole PBMCs at 2, 4, or 12 weeks posttreatment as compared with baseline for any of the 64 T-cell or granulocytic genes assayed (Supplementary Table 3). Moreover, gene expression was not significantly different for those receiving placebo versus ATG/GCSF or between responder, nonresponder, and placebo groups at any time point examined (Supplementary Table 4).

AEs

AEs from the 1- and 2-year time points were previously reported (7,8). Of note, in the 3rd, 4th, and 5th years after treatment, there were no significant differences in infections or other AEs reported between ATG/GCSF and placebo recipients.

DISCUSSION

The small sample size available at 5 years (15 [63%] of 24 of the original pilot study cohort) rendered findings of

Table 2—Delta AUC C-peptide effect size for ATG/GCSF responder, nonresponder, and placebo groups

Time, months	Placebo (*n* = 5)	Nonresponders (*n* = 4)	Responders (*n* = 6)	*P**
3	–0.36 (–0.85, 0.13)	0.58 (–0.05, 1.21)	0.19 (–0.31, 0.69)	0.41
6	–0.49 (–0.92, –0.06)	0.35 (–0.23, 0.93)	0.18 (–0.27, 0.63)	
12	–0.68 (–1.11, –0.26)	–0.09 (–0.66, 0.49)	0.12 (–0.32, 0.57)	
18	–0.80 (–1.24, –0.36)	–0.47 (–1.06, 0.12)	0.03 (–0.43, 0.48)	
24	–0.86 (–1.29, –0.43)	–0.80 (–1.38, –0.22)	–0.09 (–0.54, 0.36)	
30	–0.89 (–1.31, –0.46)	–1.07 (–1.65, –0.49)	–0.21 (–0.65, 0.22)	
36	–0.91 (–1.34, –0.47)	–1.28 (–1.87, –0.68)	–0.33 (–0.77, 0.12)	
42	–0.93 (–1.40, –0.47)	–1.42 (–2.04, –0.80)	–0.41 (–0.87, 0.04)	
48	–1.00 (–1.46, –0.53)	–1.49 (–2.11, –0.87)	–0.46 (–0.92, 0.00)	
54	–1.11 (–1.57, –0.65)	–1.49 (–2.11, –0.87)	–0.45 (–0.92, 0.02)	
60	–1.30 (–1.86, –0.75)	–1.41 (–2.14, –0.68)	–0.37 (–0.94, 0.20)	

Data presented as effect size (95% CI) in ng/mL/min, treating time as a cubic effect. **P* value group × time^3. The *P* value of 0.41 applies to all time points.

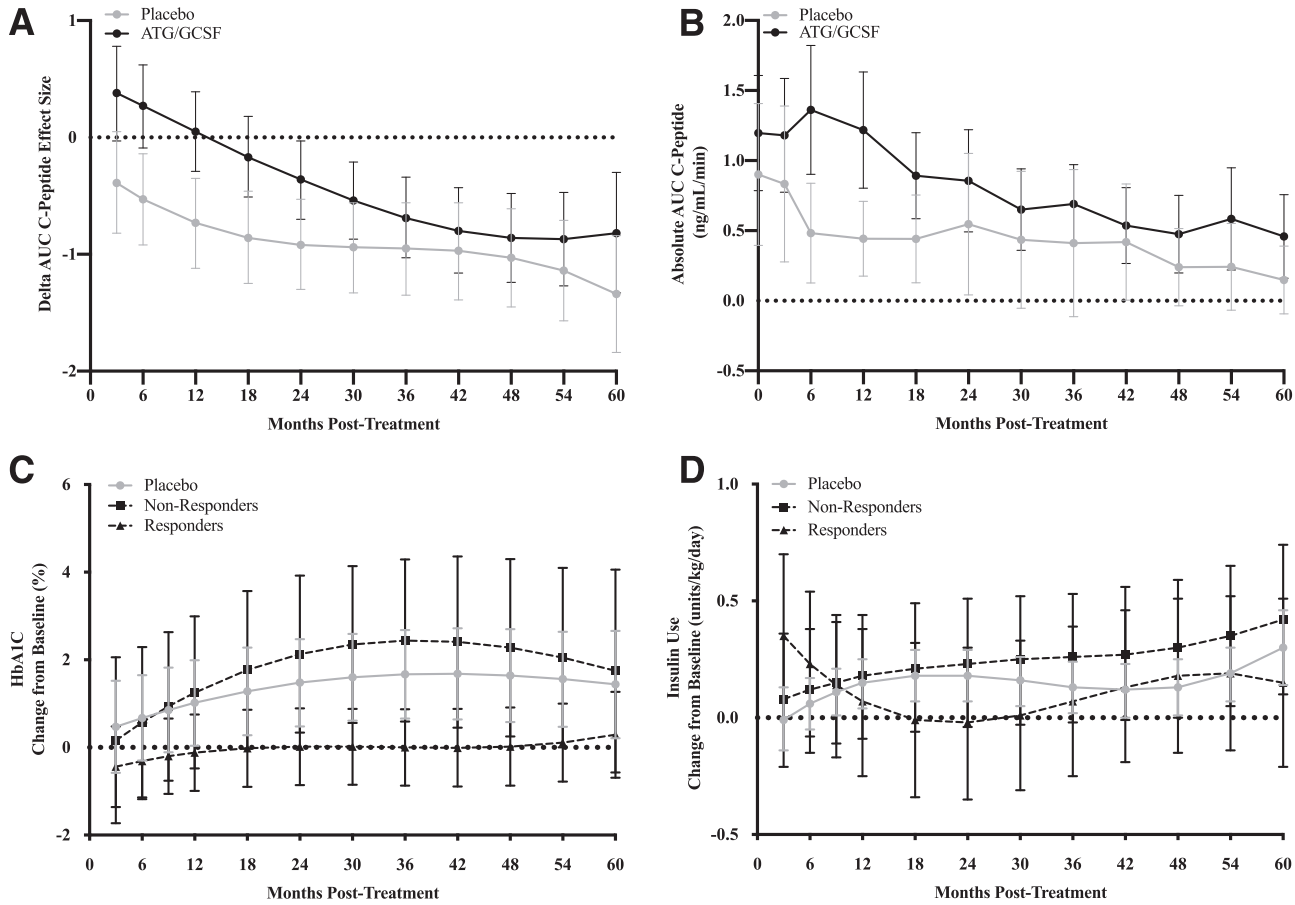

Figure 2—C-peptide, HbA_{1c}, and insulin use over time. *A*: Delta AUC C-peptide effect size over the 60-month period for ATG/GCSF (n = 10) and placebo (n = 5) groups. *B*: AUC C-peptide change from baseline by ATG/GCSF responder (n = 6), ATG/GCSF nonresponder (n = 4), and placebo (n = 5) groups. *C*: HbA_{1c} change from baseline by ATG/GCSF responder (n = 7), ATG/GCSF nonresponder (n = 4), and placebo (n = 4) groups. *D*: Insulin use change from baseline by ATG/GCSF responder (n = 8), ATG/GCSF nonresponder (n = 5), and placebo (n = 5) groups.

statistical significance in this analysis highly unlikely. Indeed, change in AUC C-peptide, HbA_{1c}, and insulin use between ATG/GCSF and placebo recipients did not reach significance at the 3-, 4-, or 5-year time points. Nevertheless, the point estimates for each of these parameters lend support to the notion that low-dose ATG/GCSF may provide long-term benefit in established type 1 diabetes, although data from fully powered trials are needed to formally assess long-term therapeutic efficacy. Importantly, there were no observations of long-term safety concerns in participants who received low-dose ATG/GCSF.

Our analyses of responders and nonresponders represented a critical opportunity to predict response to ATG/GCSF therapy independent of the data points obtained at month 60. We previously defined responders and nonresponders on the basis of absolute change in C-peptide 1 and 2 years after treatment (8). In this longer-term analysis, we developed a linear model based on each individual's baseline and 1-year AUC C-peptide values to predict AUC C-peptide from month 36 to 60. Our modeling projected a potential long-term benefit of ATG/GCSF among the nine individuals identified as responders who were predicted to still have AUC C-peptide values near baseline, even at 60 months after treatment. As validation for the model, when compared with actual AUC C-peptide at month 60, responders (n = 6) did, in fact, seem to have a smaller change in AUC C-peptide than nonresponders (n = 4), although the difference was not significant. An alternate measure of success used in many type 1 diabetes intervention studies has been the percentage of participants with peak C-peptide >0.2 pmol/mL over time (10). Notably, 50% of ATG/GCSF-treated individuals had >0.2 pmol/mL at the 5-year time point, compared with only 20% of those receiving placebo, but the small sample size precluded statistical analysis of this finding. Unfortunately, our T-cell depletion and gene expression studies failed to improve our capacity to predict or explain response and nonresponse.

In addition to the effects of low-dose ATG/GCSF treatment in those with established type 1 diabetes reported here and previously (7,8), a subsequent TrialNet study of low-dose ATG with and without GCSF in recent-onset type 1 diabetes demonstrated the ability of a single 2-day

course of low-dose ATG monotherapy to preserve C-peptide and reduce HbA_{1c} for up to 2 years (11,12). Because the TrialNet study indicated GCSF was not a significant contributor to C-peptide preservation, future efforts should focus on ATG monotherapy and combination approaches with alternative agents likely to provide synergy. Notably, the European INNODIA network is currently enrolling participants in the MELD-ATG phase 2 study, which seeks to compare ATG at 2.5, 1.5, 0.5, and 0.1 mg/kg and placebo in new-onset type 1 diabetes (clinical trial reg. no. NCT04509791, ClinicalTrials.gov).

As we continue to consider the practical application of therapies to preserve C-peptide in those with type 1 diabetes, we must consider issues of efficacy, logistics, risk/benefit, and cost. In a cross-trial comparison of studies in patients with recent-onset disease, low-dose ATG clearly outperformed high-dose ATG, alefacept, rituximab, and abatacept, and it was equivalent to teplizumab in terms of C-peptide preservation (13). ATG also has considerable practical advantages. Namely, ATG requires a 2-day outpatient infusion compared with teplizumab, which reqires a 12–14 day infusion. In addition, the retail cost of low-dose ATG is ∼$7,500, and although pricing information is not available for teplizumab, existing U.S. Food and Drug Administration–approved monoclonal antibody therapies for other autoimmune diseases have a median annual cost of ∼$54,000 (14). Given that low-dose ATG and teplizumab differ only in terms of short-term AE profiles (relating to serum sickness), with no differences in observed long-term AEs, low-dose ATG should be considered a reasonable option for clinical translation in the type 1 diabetes immunotherapy space.

In closing, the data reported herein represent one of only a few reports seeking to elucidate long-term outcomes after immunotherapy in participants with type 1 diabetes (15,16). Although statistically limited by the sample size, these 5-year data provide an important point of reference for long-term follow-up of participants in both the TrialNet study of low-dose ATG and ATG/GCSF in new-onset disease (11,12) and the INNODIA MELD-ATG study. Finally, the TrialNet study (clinical trial reg. no. NCT04291703, ClinicalTrials.gov) evaluating the ability of low-dose ATG to delay progression from stage 2 (autoantibody positive with dysglycemia) to stage 3 disease (clinical type 1 diabetes) (17) is well positioned to determine if low-dose ATG can prevent or delay type 1 diabetes.

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Duality of Interest. M.A.A. holds patent US 8758761 B2, Combination therapies for treating type 1 diabetes, which includes the use of ATG in combination with GCSF for the treatment of type 1 diabetes. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. A.L., J.A.M., and M.J.G. analyzed the data and wrote the manuscript. B.B., L.M.J., A.L.P., C.H.W., T.M.B., M.A.A., S.E.G., P.A.G., and C.E.M. contributed to discussion and reviewed and edited the manuscript. D.A.S. conceived of the study and reviewed and edited the manuscript. M.J.H. conceived of the study, researched the data, and wrote, reviewed, and edited the manuscript. M.J.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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