

Changes in T-cell subsets identify responders to FcR-nonbinding anti-CD3 mAb (teplizumab) in patients with type 1 diabetes

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The mechanisms whereby immune therapies affect progression of type 1 diabetes (T1D) are not well understood. Teplizumab, an FcR nonbinding anti-CD3 mAb, has shown efficacy in multiple randomized clinical trials. We previously reported an increase in the frequency of circulating CD8⁺ central memory (CD8CM) T cells in clinical responders, but the generalizability of this finding and the molecular effects of teplizumab on these T cells have not been evaluated. We analyzed data from two randomized clinical studies of teplizumab in patients with new- and recent-onset T1D. At the conclusion of therapy, clinical responders showed a significant reduction in circulating CD4⁺ effector memory T cells. Afterward, there was an increase in the frequency and absolute number of CD8CM T cells. In vitro, teplizumab expanded CD8CM T cells by proliferation and conversion of non-CM T cells. Nanostring analysis of gene expression of CD8CM T cells from responders and nonresponders versus placebo-treated control subjects identified decreases in expression of genes associated with immune activation and increases in expression of genes associated with T-cell differentiation and regulation. We conclude that CD8CM T cells with decreased activation and regulatory gene expression are associated with clinical responses to teplizumab in patients with T1D.

Keywords: Anti-CD3 mAb · CD8⁺ T cells · Immune therapy · Tolerance · Type 1 diabetes



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Introduction

Type 1 diabetes (T1D) is a progressive autoimmune disease resulting from T-cell-mediated, targeted destruction of β cells

that leads to the loss of insulin production and dependence on exogenous insulin [1]. Metabolic control with insulin therapy does not achieve the same metabolic control as β cells in the islets of Langerhans. Studies of insulinitis in humans have highlighted the role of islet-infiltrating CD8⁺ T cells in the disease process, including those with specificities for known diabetes Ags [2].

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Over the past 15 years, clinical trials with FcR-nonbinding humanized anti-CD3 mAbs have shown slowed progression of disease [3–10]. Even the Protégé trial, which did not meet its clinical endpoint, showed improvement in C-peptide-treated subjects.

However, not all patients respond to treatment. Those who do respond may have a remarkably robust preservation of insulin production: In the AbATE trial the responders had <10% loss of C-peptide responses 2 years from diagnosis [5]. Studies to date have not identified the immunologic basis for responses in these subjects. Identifying biomarkers of responsiveness are important objectives for understanding the mechanisms of the treatment and disease, maximizing efficacy, and avoiding treatment of those who are not likely to respond to teplizumab.

Several metabolic and immunologic features have been found to distinguish responders to teplizumab. In the AbATE trial of teplizumab, we found that differences in glycemic control, insulin use, and changes in subsets of both CD4⁺ and CD8⁺ T cells at baseline predicted response; in other trials, younger age was a predictor [5]. However, these markers are not related to the actions of the anti-CD3 mAb and may identify differences in β cells, insulin sensitivity, or other parameters unrelated to the pathogenesis or drug response. Previously, we tracked the frequency of diabetes and other Ag-specific CD8⁺ T cells, and found that treatment with teplizumab did not eliminate Ag-specific or other effector T cells [11]. We also reported that responders to teplizumab could be distinguished from nonresponders, surprisingly, by an increase in the number of circulating CD8⁺ central memory (CD8CM) T cells in the former [12]. This is surprising because other successful immune therapies have been associated with a decrease in CD4⁺ and CD8⁺ memory T cells [13, 14].

A number of mechanisms of anti-CD3 mAbs have been suggested. Previous studies from our group and others showed induction of subpopulations of regulatory T cells [15–18]. Recent work has suggested that adaptive regulatory T cells that produce IL-10 and/or TGF- β may be induced following migration of T cells to the gut following treatment with teplizumab [19, 20]. Belghith et al. found that anti-CD3 mAb induced TGF- β -dependent CD4⁺ Treg cells in the pancreatic draining LNs in NOD mice [17]. Finally, CD8⁺ T cells isolated directly from drug-treated patients have regulatory function in ex vivo assays [15, 16, 18]. These cells were distinguished by low levels of expression of NKG2A (KLRC1). Collectively, the findings suggest that regulatory mechanisms are involved, either by direct induction of regulatory T cells or inactivation of subpopulations, such as memory T cells, that are involved in disease progression.

In this analysis, we determined the effects of teplizumab treatment on T-cell subsets in vitro and in vivo using cells and data from two randomized clinical trials of patients with T1D in order to identify cellular correlates of clinical responses [5, 12]. We identified changes in memory T cells immediately after drug treatment but clinical responses were associated with an increase in the frequency of CD8CM T cells. We analyzed gene expression in these cells and, in clinical responders, found reduced expression of genes associated with cell activation and changes in genes associated with differentiation and regulation.

Results

Teplizumab slows the rate of C-peptide loss in patients with T1D

Data and samples were collected from subjects with T1D enrolled in two randomized clinical trials of teplizumab [5, 7]. The AbATE trial enrolled subjects with new-onset disease and the Delay trial enrolled patients with T1D of 4–12 months' duration. The patient demographics have been published and were similar in the two trials. In both trials, patients with T1D, age range 8–35, were randomized to a control group (placebo in Delay, open label in AbATE) or teplizumab. The dosing regimen of teplizumab was the same in both trials and was daily four doses of 51, 103, 207, and 413 $\mu\text{g}/\text{m}^2$ on Study Days 0–3, respectively, and 826 $\mu\text{g}/\text{m}^2$ on each of Study Days 4–13. The total dose for a 14-day course was 9034 $\mu\text{g}/\text{m}^2$.

The primary clinical outcomes from these trials were reported [5, 7]. The C-peptide responses (area under curve (AUC)) to a 4-h mixed-meal tolerance test (MMTT) were measured at study entry and at 6 and 12 months after treatment. The 12-month change in C-peptide was significantly improved in drug-treated individuals in both studies (AbATE: -0.104 ± 0.037 nmol/L vs. -0.274 ± 0.056 nmol/L, $p = 0.002$, and Delay: -0.110 ± 0.035 nmol/L vs. -0.207 ± 0.039 nmol/L, $p = 0.03$; Fig. 1A).

Changes in T-cell subsets distinguish clinical responders to treatment

Not all patients receiving teplizumab therapy showed the same response. To identify the changes in T cells that distinguished responders and nonresponders and to allow direct comparison between these two and previous trials [8], we designated drug-treated patients as responders or nonresponders, based on a previously used definition of responders as having $\leq 7.5\%$ loss of baseline levels of C-peptide after 12 months [6] (Table 1). The C-peptide responses at study entry were not significantly different in the responders and nonresponders in AbATE or Delay. The percentage of responders to therapy was similar in the two trials (AbATE: 38.8%, Delay: 41.9%, $p = 0.82$). Responders, on average, had an improvement in C-peptide response at 12 months compared to baseline (0.166 ± 0.044 and 0.048 ± 0.045 nmol/L in AbATE and Delay, respectively), while nonresponders showed losses that were similar to untreated or placebo-treated control subjects (-0.24 ± 0.035 and -0.207 ± 0.038 nmol/L, AbATE and Delay, respectively; Fig. 1B).

The percentages and absolute numbers of T-cell subsets in the peripheral blood were evaluated in both trials in real time with freshly isolated PBMCs before and during the year following treatment with anti-CD3 mAb by flow cytometry. The laboratories used for analysis were different for the two trials but the same laboratories were used for all participants within each trial. The first analysis of T cells after treatment was performed on day 14

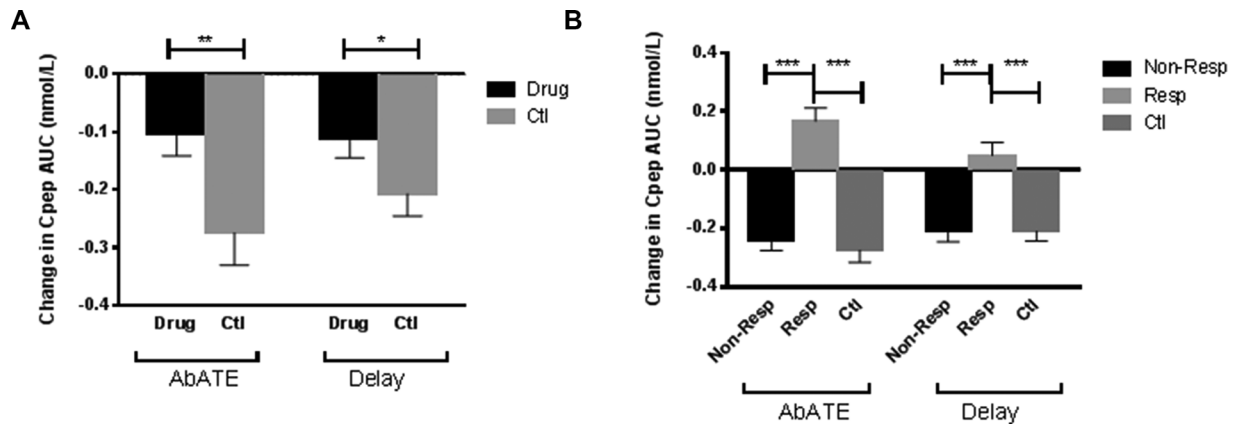


Figure 1. C-peptide changes in patients treated with teplizumab. (A) Comparison of 12-month changes in C-peptide AUC during a mixed-meal tolerance test between drug-treated ($n = 31$ and 49) and control subjects ($n = 26$ and 22) in Delay and AbATE trials, respectively [5, 12]. $**p = 0.002$, AbATE; $*p = 0.03$, Delay; Student's *t*-test. (B) Comparison of 12-month changes in C-peptide of responders, nonresponders, and control subjects in Delay and AbATE trials. Responders were defined as having lost $<7.5\%$ of C-peptide in the first year after treatment [8]. In both studies, responders had a positive increase in C-peptide in the first year of treatment and C-peptide change in nonresponders was indistinguishable from controls. Comparison of 12-month changes in C-peptide of responders, nonresponders, and control subjects in Delay and AbATE trials was made from the mixed linear model using a linear contrast (approximate *t*-test) at each time point. $p < 0.05$, $***p < 0.001$. Changes in C-peptide in the Delay study were corrected for imbalance in the baseline HbA1c levels in the mixed linear model [12].

(2 weeks: after the completion of treatment) in the AbATE trial, but at month 2 in the Delay trial. In the AbATE trial, the relative proportions of CD4⁺ effector memory (EM) and CD8⁺ EM T cells were significantly lower in the responders versus nonresponders at that time ($p = 0.006$ and $p = 0.03$, respectively) but these cells recovered by the next analysis at 1 month (Fig. 2A).

The relative proportions of CD8CM T cells were significantly increased in responders compared with the nonresponders following drug treatment in both trials (Fig. 2). In the AbATE trial, there was a trend for an increase in the frequency of CD8CM T cells by 2 months after the last dose of teplizumab (resp: $14.6 \pm 2.16\%$ vs. nonresp: $10.6 \pm 1.5\%$, $p = 0.13$) and a significant increase at 3 months (resp: $15.6 \pm 2.03\%$ vs. nonresp: $10.1 \pm 1.5\%$, $p = 0.029$; Fig. 2A). The differences in the proportions of CD8CM T cells were due to an increase in the absolute number of cells (Fig. 2B, $p = 0.05$) after 2 months (16.1 ± 2.10 vs. 10.1 ± 1.6 cells/ μ L, $p = 0.032$) and 3 months (17.1 ± 2.00 vs. 9.56 ± 1.59 cells/ μ L, $p = 0.004$). In the Delay trial, we found a significant increase in the proportion of CD8CM T cells at 2 months (Fig. 2C, 10.0 ± 1.41 vs. $6.25 \pm 1.23\%$, $p = 0.046$). The findings from the AbATE

trial show that both responders and nonresponders had an initial increase in CD8CM T cells with treatment, but the subpopulation of CD8CM T cells remained elevated in the responders for a longer period of time.

Source of CD8CM T cells

We cultured cells in vitro with teplizumab to determine how it affected CD8CM T cells. There was an increase in the proportion of CD4CM and CD8CM T cells after culture with teplizumab (Chi-squared $p < 0.0001$ for both, Fig. 3A). The changes in the proportions could reflect proliferation of CD8CM or killing of the non-CM populations, but the CD8CM T-cell subset showed increased proliferation (Fig. 3B and C).

To determine the source of the CD8CM T cells, we sorted CD8CM T cells, labeled them with CellTrace dye, and seeded them into CFSE-labeled PBMCs from which they had been sorted. The cells were then cultured for 2–3 days with teplizumab and anti-CD28 mAb. The proportion of CellTrace dye+ CD8CM T cells

Table 1. Demographics at entry of responders and nonresponders in AbATE and Delay

Trial	Group	N	Age	Duration of diabetes (days)	Baseline C-peptide (nmol/L)
AbATE	Responders	19	13.5 ± 1.33	40.7 ± 1.97	0.76 ± 0.071
AbATE	Nonresponders	30	12.2 ± 0.84	41.0 ± 1.43	0.70 ± 0.053
AbATE	Controls	25	12.3 ± 0.82	37.6 ± 7.58	0.67 ± 0.056
Delay	Responders	13	12.15 ± 1.09	210 ± 20.6	0.588 ± 0.07
Delay	Nonresponders	18	13 ± 1.06	219 ± 18.7	0.639 ± 0.08
Delay	Placebo	28	12.0 ± 0.76	216 ± 13.9	0.6 ± 0.08

Responders were defined as having lost $<7.5\%$ of their baseline C-peptide response at month 12 [6].

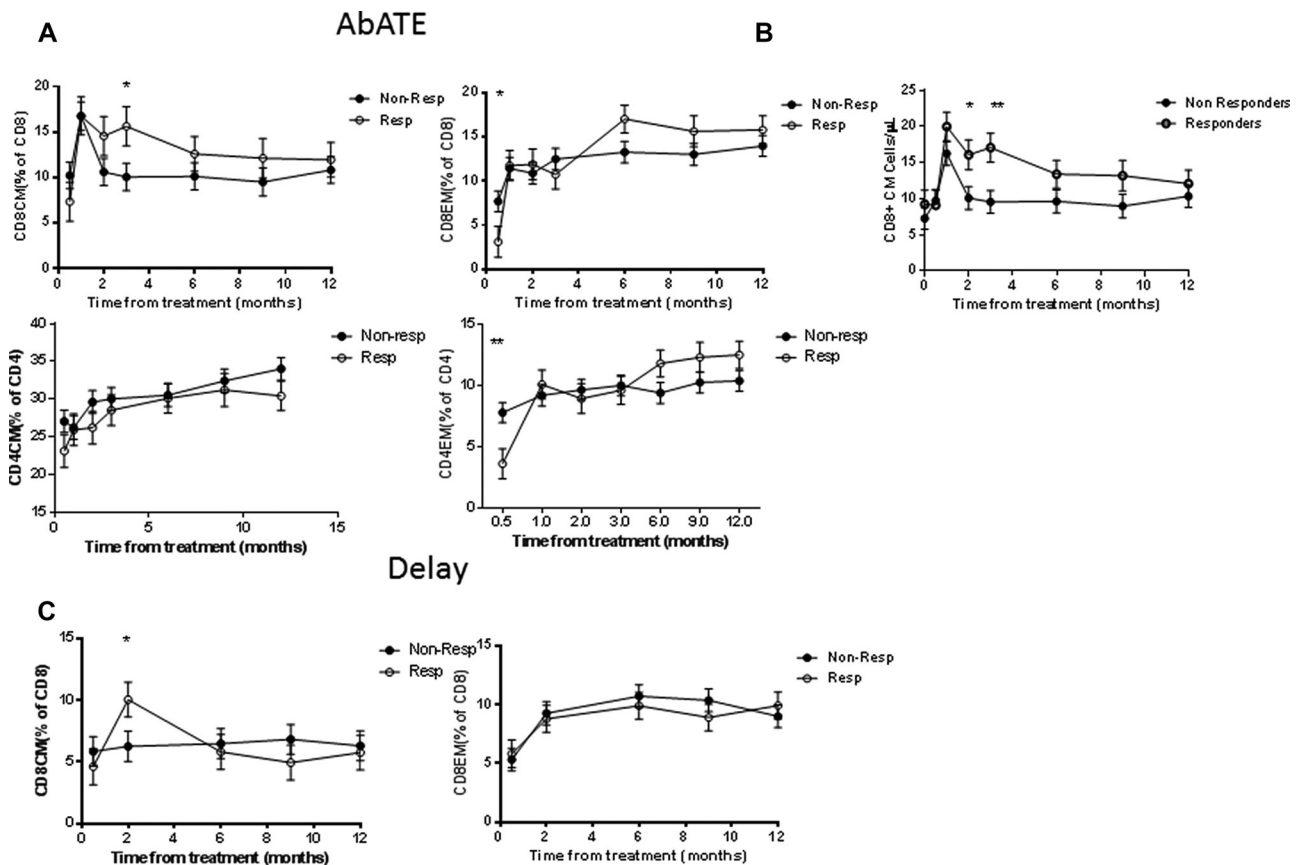


Figure 2. Changes in CD4⁺ and CD8⁺ T-cell subsets in patients with T1D following treatment with teplizumab. (A–C) CD4⁺ and CD8⁺ CM and EM T cells in responders and nonresponders in the first year after treatment in (A and B) AbATE and (C) Delay trials. (A and C) Percentage of total, (B) absolute cell counts. Data show the geometric mean \pm SEM from the repeated-measures mixed model corrected for the baseline values. Comparisons were made by linear contrasts (approximate t-test) at each time point. Data are shown as mean \pm SEM from the mixed model. Delay: responders $n = 14$, nonresponders $n = 18$; AbATE: responders $n = 19$, nonresponders $n = 30$; * $p < 0.05$, ** $p < 0.01$.

declined with time in culture while the frequency of CFSE+ CD8CM T cells increased with time (4.6% (median) CD8CM cells in control wells vs. 17.9% in teplizumab + anti-CD28 wells at 3 days) suggesting that some of the CD8CM T cells came from non-CD8CM T cells (Fig. 3D). These studies show that the CD8CM cells at the end of the cultures contained previously naïve cells that had differentiated into CM T cells. When we labeled naïve cells with CellTrace dye, between 3 and 9% of the CD8CM cells were positive for the dye at the end of the 3-day culture period (not shown).

Transcriptional analysis of CD8CM T cells from trial participants

We sorted CD8CM T cells from PBMCs collected at the 2-month visit in the Delay trial in responders and nonresponders, and from placebo-treated subjects, and measured gene expression by nanostring, using the human immunology codeset covering 594 genes (Fig. 4). Since drug-treated nonresponders and placebo-treated subjects showed a similar response, in our first analysis we pooled data from the placebo- and drug-treated nonresponders to identify

differences in gene signatures that were associated with preservation of C-peptide responses in the drug-treated responders. There were 53 genes that showed at least a 1.5-fold difference in expression between these groups with a p -value < 0.05 by Wilcoxon's test (Table 2, Fig. 4). Thirteen of the differentially expressed genes encode molecules associated with cell activation such as *LCK*, *EBI3*, *JAK1*, and *IKBKB* as well as decreased expression of two components of the CD3 complex. The expression of *STAT5B* was also modestly reduced in the responder group (1.4-fold, $p = 0.001$). There were differences in the expression of 12 genes differentially regulated that were associated with cell differentiation. These included decreased expression of *IL-17A*, and increased expression of two IL-10 family members (*IL-20* and *IL-26*). The latter may enhance IL-10 secretion [21]. There was decreased expression of *IL-22*, which is coproduced with IL-17 [22]. Expression of *IL-10* itself was also increased 2.1-fold ($p = 0.06$). There was also decreased expression of *IKZF1* (Ikaros family zinc-finger protein 1), which is a T1D susceptibility gene [23].

We compared the expression of these genes in the three groups (i.e., drug-treated responders and nonresponders and placebo-treated; Fig. 5). We found decreased expression of genes associated with T-cell activation: *LCK* ($p = 0.002$), *STAT5B* ($p = 0.002$),

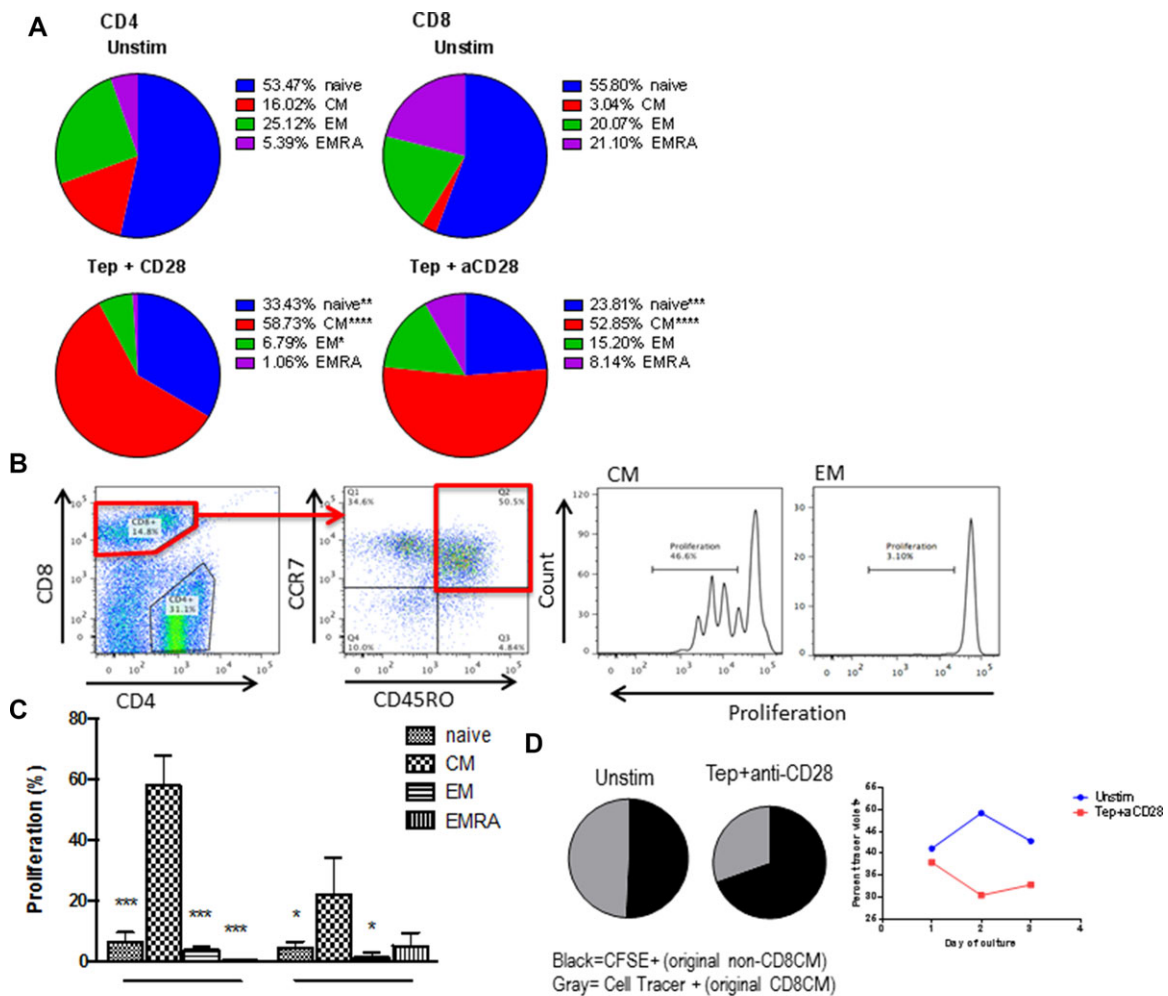


Figure 3. Proliferative responses of CD8⁺ T-cell subpopulations to teplizumab. (A) Changes in the subpopulations of cells after culture with teplizumab and anti-CD28 mAb. PBMCs from three healthy control subjects were cultured for 3 days in the presence or absence of teplizumab and anti-CD28 mAbs as described in the Materials and Methods section. The changes in the proportion of cells were determined by flow cytometry at the conclusion of the cultures. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; ANOVA. (B) To determine whether the changes in proportions of cells reflected proliferation of the CM subsets, we studied the proliferation of cell subgroups by dilution of CellTrace dye. An example of the gating strategy used to analyze proliferation of T-cell subsets in vitro is shown on the left. (C) Proliferation was assessed as percentage of cells and was greatest in the CD8CM T cells (* $p < 0.05$, *** $p < 0.001$). Data in (B) are from a single individual representative of three individuals studied independently and shown in (C). (D) To determine the source of the CD8CM T cells in the cultures, we sorted CD8CM T cells from PBMCs and labeled them with CellTrace dye. These labeled cells were placed into culture with PBMCs that had been stained with CFSE and we measured the proportion of CellTrace dye+ CD8CM T cells after 1, 2, and 3 days in culture with or without teplizumab+ anti-CD28 mAb. The pie charts show the proportion of CellTrace dye+ and CFSE+ cells after 2 days in culture. (Gray = CD8CM T cells labeled at the start of culture, black = CD8CM T cells that had differentiated from other subsets.) The graph shows the changes in the proportion of the CellTrace dye+ cells during cultures. The data are from a single experiment representative of three independent experiments.

IKBKB ($p = 0.01$), *JAK2* ($p = 0.007$), *IL-7R* ($p = 0.03$), *EBI3* ($p = 0.007$), and *CD3d* ($p = 0.0003$; all by Kruskal–Wallis test, Fig. 5A). We confirmed the differential expression of IL-7R by FACS on CD8CM cells from the patients ($p = 0.02$, Kruskal–Wallis test; Supporting Information Fig. 1), but did not find a difference in the expression of CD3 on the CD8CM T-cell surfaces (not shown). We likewise found differences among these subgroups in genes associated with cell differentiation including the IL-10 family members (*IL-10* ($p = 0.009$), *IL-20* ($p = 0.006$), *IL-22* ($p = 0.02$), *IL-26* ($p = 0.01$)), *IL-17A* ($p = 0.03$), and *IKZF1* ($p = 0.0004$, Fig. 5B).

We performed a principal components analysis (PCA, Fig. 6) of all genes in the three groups. We identified two dimensions that define the subgroups. The first dimension explained 24.8% of the overall variance and was correlated to placebo status ($p = 0.009$); 241 of 579 individual genes were in this dimension ($p < 0.05$ by Spearman's correlation). The second dimension explained 16.1% of the overall variance and was correlated to nonresponder and responder status ($p = 0.008$ and 0.011 , respectively), with 160 of 579 individual genes loading to this dimension ($p < 0.05$, Table 3). Thirty-three genes overlapped between the two dimensions (Table 3). In addition to the genes identified above, this

Table 2. Comparison of gene expression in CD8CM T cells from responders versus nonresponders+placebo

Activation			Differentiation			Regulation			Others		
Name	Fold R/NR + P	<i>p</i>	Name	Fold R/NR + P	<i>p</i>	Name	Fold R/NR + P	<i>p</i>	Name	Fold R/NR + P	<i>p</i>
IRF3	0.281	0.048	IKZF1	0.293	0.000	KLRC1	0.424	0.037	ATG10	0.104	0.008
CTLA4-TM	0.305	0.037	IL-15	0.339	0.044	IDO1	0.641	0.012	KLRC2	0.183	0.001
IKBKB	0.402	0.005	GZMA	0.409	0.048	LIF	1.820	0.024	ITGA6	0.372	0.001
IL-7R	0.475	0.014	ETS1	0.557	0.008				CFH	0.377	0.031
LCK	0.486	0.002	IL-17A	0.565	0.017				GBP1	0.425	0.037
CD3EAP	0.531	0.037	IL-22	0.580	0.009				CD3D	0.432	0.000
CD3D	0.432	0.000	IL-20	2.507	0.009				BCL2	0.458	0.048
KIT	0.562	0.023	IL-26	3.535	0.015				CCND3	0.464	0.005
EBI3	0.564	0.005	IL-9	0.605	0.015				CD8A	0.483	0.021
JAK1	0.641	0.018	IL-7	0.608	0.017				KLRD1	0.489	0.037
CXCR2	0.653	0.050	BATF3	2.122	0.024				NOD1	0.513	0.027
IL-32	1.855	0.028	CXCL13	0.641	0.039				CD34	0.561	0.012
									CEACAM6	0.607	0.015
									IRF5	3.140	0.003
									RAG1	0.610	0.010
									BAX	0.614	0.007
									ADA	0.618	0.027
									ARG1	0.646	0.034
									HLA-B	0.665	0.005
									SELPLG	1.566	0.015
									ATG10	0.104	0.008
									KLRC2	0.183	0.001
									ITGA6	0.372	0.001
									CFH	0.377	0.031
									GBP1	0.425	0.037
									CD3D	0.432	0.000
									BCL2	0.458	0.048
									CCND3	0.464	0.005

analysis also showed a correlation with the expression of *FOXP3* ($r = -0.75$, $p = 0.0005$), *GATA3* ($r = -0.725$, $p = 0.001$), and *CTLA4_all* ($r = -0.83$, $p = 0.382 \times 10^{-5}$).

Discussion

In four randomized clinical trials of patients with new- and recent-onset T1D, teplizumab has caused improvement in C-peptide responses for a year after treatment, compared with untreated or placebo-treated patients. Previous clinical and preclinical studies have suggested that anti-CD3 mAb may affect immune responses by induction of regulatory cells, release of inhibitory cytokines, or even by depleting effector T cells but these studies did not distinguish the immunologic effects of the drug in general from those that were associated with clinical responses. In this analysis, we have focused on the immunologic differences that lead to preservation of insulin secretion. At the conclusion of 14-day drug treatment, clinical responders had a decline in CD8 and CD4 CM and EM subsets that was significantly greater in the CD8⁺ EM and CD4⁺ EM subsets. However, these acute changes alone are unlikely to account for the lasting effects of the drug because

these cell subpopulations recovered quickly and the changes in these subpopulations were not lasting. In both trials, an increase in CD8CM T cells identified the responders compared with drug-treated nonresponders and controls. The analysis of cell counts in the AbATE trial showed that there was a similar expansion of the CD8CM T cells in both responders and nonresponders initially but a decline in the proportion and absolute number of circulating CD8CM T cells by 2 months after treatment in the nonresponders. This observation may not have been appreciated in the Delay or other trials due to less-frequent sampling of PBMCs between the 0 and 2 months time-points.

The differences in the CD8CM T cells are not likely to be due to differences in the drug pharmacokinetics since drug is not detectable on the surfaces of T cells after 3–4 weeks ([6, 8] and personal observation), and the levels of coating and modulation of CD3 cannot identify clinical responders. Our studies in vitro showed that the anti-CD3 mAb preferentially induced proliferation of CD8CM T cells. The source of the CD8CM cells involved both conversion of naïve or other non-CD8CM cells that had been activated by the anti-CD3 mAb and proliferation of CD8CM T cells, because there were CD8⁺ cells that became CM T cells during the cultures. We are unable to perform similar lineage-tracing studies

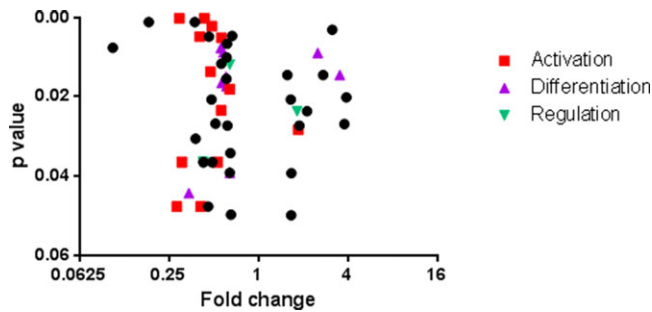


Figure 4. Effects of teplizumab on gene expression in CD8CM T cells, measured by nanostring, from teplizumab-treated responders, nonresponders, and placebo-treated subjects after treatment. Volcano plot of a comparison of gene expression in CD8CM T cells in drug-treated responders ($n = 6$) versus drug-treated nonresponders ($n = 3$) and placebo-treated ($n = 6$) subjects. The \log_2 of the fold change (X-axis) and p -value are plotted. Differences in gene expression of at least 1.5-fold with a $p < 0.05$ (Wilcoxon's rank-sum test) are shown. Genes that are associated with T-cell activation (red square), differentiation (purple triangle), and regulation (green inverted triangle) are highlighted in color. Other genes are shown with black dots and are listed in Table 2.

in vivo but the acute changes that were seen in the effector population would suggest that there has been differentiation of the CD8⁺ cell population following teplizumab treatment, and our previous studies also suggest changes in CD8 subpopulations [11]. The effects of the anti-CD3 mAb on the original CM T cells and the newly differentiated CM cells may not be the same. In this regard, the timing of the anti-CD3 mAb treatment has been shown to be an important determinant of responses—drug efficacy appears to be greater after initiation of an immune response [5, 24–26].

Since our objective was to identify gene signatures associated with clinical responses, we first combined samples from placebo- and drug-treated nonresponders and compared them to the drug-treated responders. We identified 53 genes that differentiated the responders from the other two groups. These genes suggest that modulation of cell signaling, induction of regulatory cells or cytokines, or other mechanisms may be involved. Several of the genes are involved in T-cell signaling (*LCK*, *EBI3*, *JAK1*, *IKBK*, *KIR*). These observations suggest that the anti-CD3 mAb may reduce signaling of CD8CM T cells. Since these cells may represent a source of diabetogenic effector T cells, this mechanism may account for the long-term action of the drug in preventing disease progression.

We also found differences in the expression of genes associated with T-cell differentiation including pathways involved in the development of pathogenic or regulatory T cells. The role of IL-17A in T1D has been controversial but diabetes Ag specific T cells have been shown to produce IL-17 and IL-22 in response to their Ags [27]. In other studies, IL-10 production in response to islet Ags was seen in healthy subjects [28]. We found lower expression of IL-17A and IL-22 in the responders compared with the nonresponders and increased expression of other IL-10 family cytokines such as IL-20 and IL-26. One of the differences we identified was in the expression of *IKZF1*, Ikaros family zinc-finger 1, which is also a T1D susceptibility gene. This gene is a regulator of lymphopoiesis and immune homeostasis. Its link to T1D was shown in genome-wide associated studies—the minor allele

(rs10272724) is protective for the disease but the susceptibility allele is not correlated with the levels of transcripts in peripheral blood cells [23].

The changes in the expression of genes associated with immune regulation were variable: The expression of *IDO1* was decreased but we found increased transcripts of *LIF*, which has been shown to induce Treg cells [29]. Interestingly, we also found lower levels of expression of *KLRC1* (NKG2A). In previous studies, we had shown that low expression of NKG2A identified CD8⁺ T cells with regulatory function from teplizumab-treated subjects [16].

When we separately compared gene expression in the three subgroups, we also found differential expression of genes associated with T-cell activation, differentiation, and regulation in the responders and nonresponders. Our PCA allowed us to identify a gene signature that could distinguish responders and nonresponders and also to identify genes that were associated with anti-CD3 mAb treatment but not necessarily with clinical response. For example, consistent with our previous findings in humanized mice we found increased expression of *CCL20* (MIP3A) by the CD8CM T cells that was correlated with the first principal component distinguishing placebo from treatment samples (Spearman's $\rho = -0.49$, $p = 0.047$), but this correlation is not found with the second principal component that distinguished responders and nonresponders. We also found increased intracellular CCL20 production by CD8CM T cells from responders + nonresponders from month 2 compared with CD8CM T cells from placebo-treated subjects when they were stimulated with PMA/ionomycin (1.02 ± 0.22 vs. $0.37 \pm 0.05\%$, $p = 0.008$, Mann–Whitney) but CCL20 production did not distinguish responders from nonresponders (not shown).

The results of these studies should be interpreted cautiously. First, the sample numbers that we have studied were relatively small. In addition, we did not correct for multiple comparisons in our statistical analysis of gene expression. Although our studies of T-cell proliferation in vitro suggest that the anti-CD3 mAb itself caused the expansion of the CD8CM T cells, it is possible that some cells within the expanded CD8CM population represent viral Ag reactive T cells. In a previous analysis of CD8⁺ T cells following treatment with oteelixumab, Keymeulen et al. reported that there was transient EBV reactivation that was associated with an EBV-specific T-cell response [30]. In participants in the AbATE trial, we found that, at month 3, the frequency of CD8CM T cells was higher in the six EBV sero⁺ responders ($294 \pm 106 \times 10^3/\text{mL}$) compared with the seven EBV sero⁻ responders ($86.2 \pm 24.4 \times 10^3/\text{mL}$, $p = 0.14$, Student's t -test with Welch's correction) but together, these values in the responders were greater than the CD8CM T cells in the 13 EBV sero⁻ nonresponders ($182 \pm 57 \times 10^3$ vs. $41 \pm 12 \times 10^3/\text{mL}$, $p = 0.003$, Mann–Whitney). We cannot exclude the possibility that EBV sero⁺ subjects are more likely to respond to the anti-CD3 mAb but the experience from that trial of oteelixumab, in which all subjects were EBV sero⁺ at entry would suggest that other factors determine whether individuals will respond to the treatment.

In summary, this study of participants in two clinical studies has identified a gene signature that is associated with

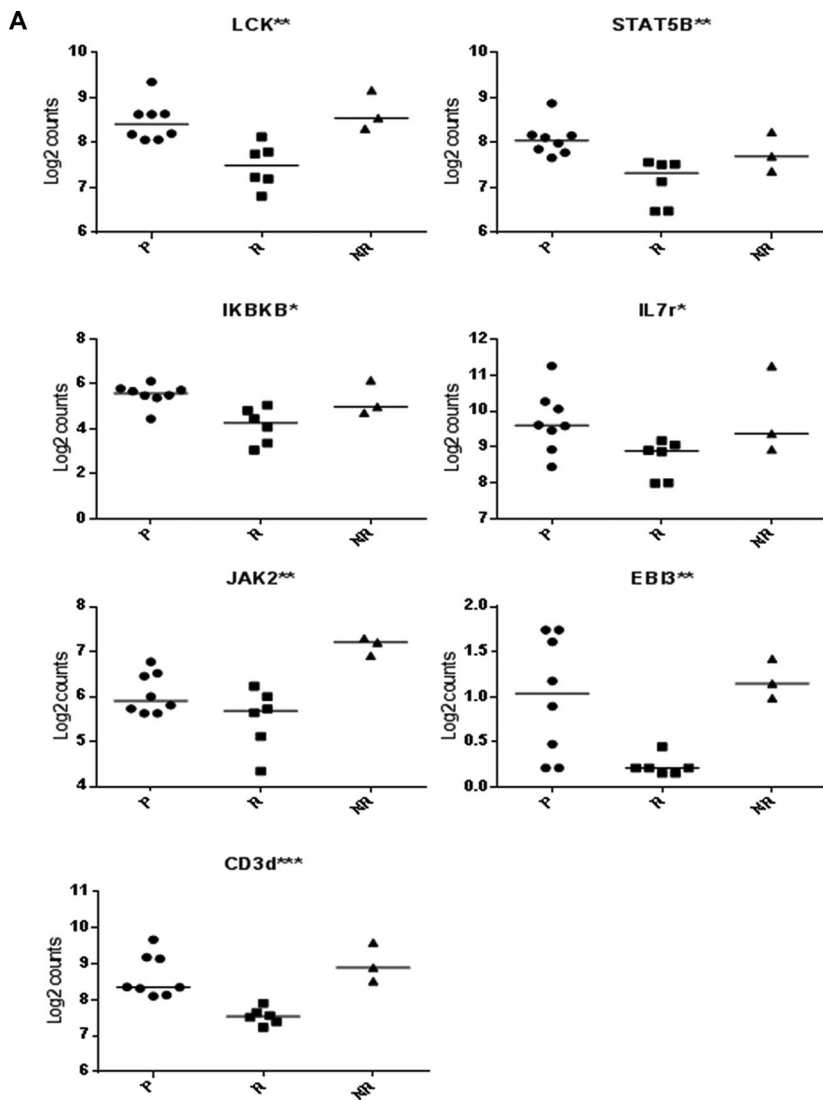


Figure 5. Gene expression among responder subsets. Gene expression was compared by nanostring in the drug-treated responders and nonresponders and placebo-treated subgroups. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Kruskal-Wallis ANOVA). (A) Genes associated with T-cell activation. (B) Genes associated with T-cell differentiation and regulation are represented. The lines represent the median values. $n = 8$ placebo, 3 drug-treated nonresponders; 6 drug-treated responders.

clinical responses and can differentiate drug-treated responders and nonresponders. Reduced expression of genes associated with activation of T cells was found in the CD8CM T-cell subpopulation that distinguishes the responders. In addition, there was enhanced expression of genes associated with differentiation of regulatory cells such as *IL-10* and *KLRC1*, and reduced transcripts of pathogenic cytokines such as *IL-17A*. These findings may be used to identify individual factors that can predict clinical responses to anti-CD3 mAb, which will help to improve the safety and efficacy of this form of immune modulation.

Materials and methods

Human subjects

We studied subjects with T1D between the ages of 8 and 35 enrolled in the AbATE and Delay studies (NCT00129259 and NCT00378508). The AbATE study was a randomized open-label

study of patients with new-onset (<3 months) diabetes, and the Delay study was a randomized placebo-controlled study that enrolled subjects with 4–12 months' duration of disease. The drug-treated subjects in both trials received the same dose of teplizumab for 14 days (total dose = 9.033 mg/m²). The descriptions of the study groups and results of the primary endpoint analysis for both trials have previously been published [5, 7]. The data presented herein are limited to the first year of each study to permit direct comparison of studies. The study protocols and use of samples for the mechanistic studies were approved at each study site. Written informed consent was obtained from the participants.

Analysis of C-peptide responses/designation of responders

The subjects in both trials underwent a 4-h mixed-meal tolerance test at the time of study entry and after 6 and 12 months. C-peptide levels were measured at Northwest Lipid Research Laboratory (Seattle, WA, USA), and the AUC was calculated using the

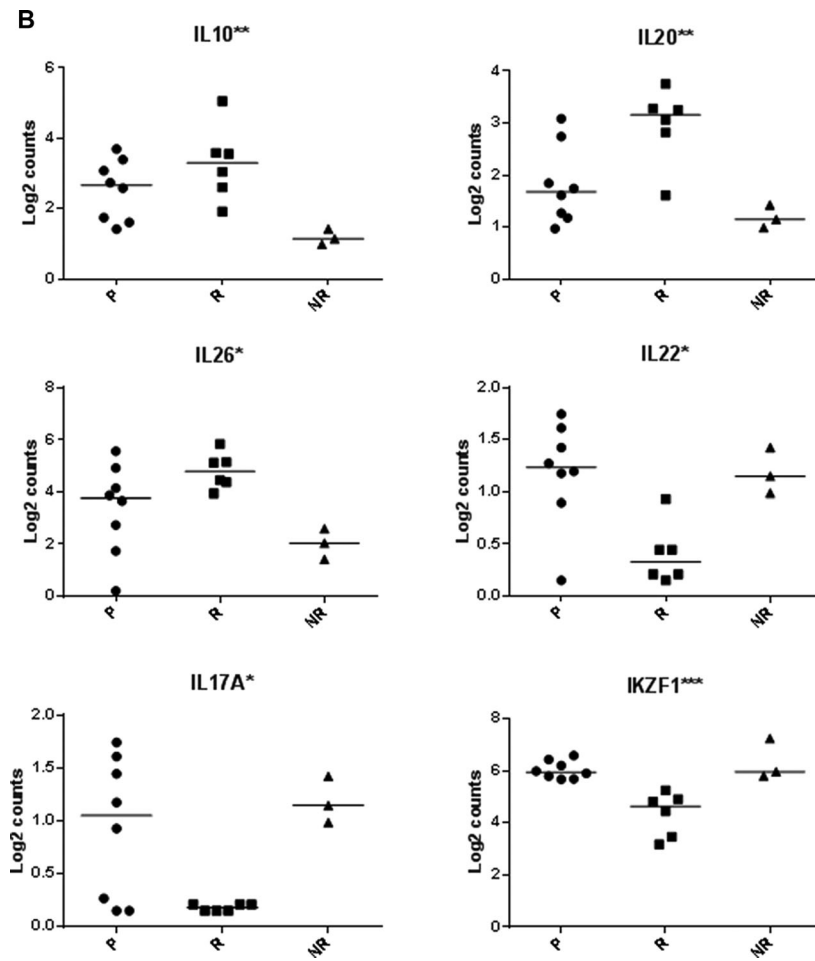


Figure 5. Continued.

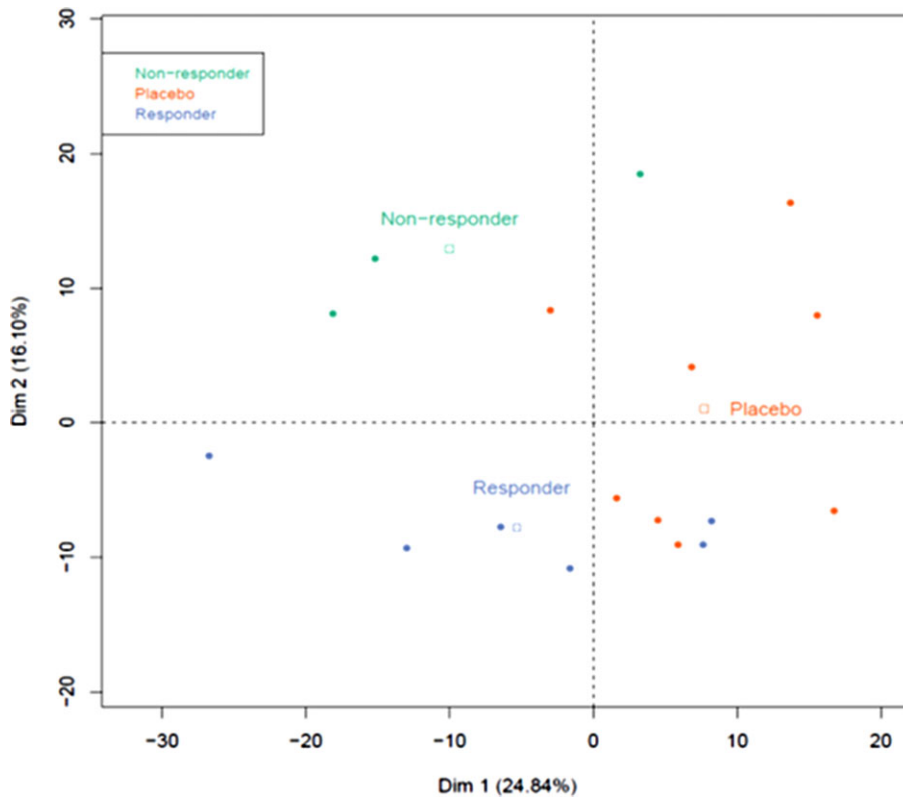


Figure 6. PCA of the three groups. We performed a PCA analysis with nanotraining data. Each circular symbol represents an individual subject who was a drug-treated responder (blue, $n = 6$), nonresponder (green, $n = 3$), or placebo-treated subject (orange, $n = 8$). The squares indicate the median values of the two dimensions of each group. The components of Dim2, which differentiated responders and nonresponders, are listed in Table 3.

Table 3. Listing of genes in Dim2 and correlation

Name	Correlation	<i>p</i>	Name	Correlation	<i>p</i>	Name	Correlation	<i>p</i>	Name	Correlation	<i>p</i>
AIRE	-0.546	0.023	CFH	0.588	0.013	IL-18RAP	0.681	0.003	NFATC1	-0.503	0.040
APP	-0.553	0.021	CFI	-0.891	0.000	IL-1R2	-0.592	0.012	NFKB1	-0.552	0.022
ARG1	0.529	0.029	CFP	-0.593	0.012	IL-1RL1	0.521	0.032	NFKB2	-0.635	0.006
ATG10	0.942	0.000	CIITA	0.775	0.000	IL-20	-0.723	0.001	NFKBIZ	-0.503	0.040
ATG5	-0.518	0.033	CMKLR1	0.685	0.002	IL-22	0.708	0.001	PDGFB	-0.626	0.007
B3GAT1	-0.574	0.016	CSF1	-0.773	0.000	IL-26	-0.592	0.012	PIGR	0.559	0.020
BATF3	-0.497	0.042	CSF2	0.580	0.015	IL-28A	-0.711	0.001	PRF1	0.849	0.000
BAX	0.629	0.007	CTLA4_all	-0.830	0.000	IL-28A/B	-0.568	0.017	PSMB7	-0.534	0.027
BCAP31	0.533	0.028	CTLA4-TM	0.602	0.011	IL-29	0.679	0.003	PSMB8	0.747	0.001
BCL10	-0.570	0.017	CTNNA1	-0.751	0.001	IL-32	-0.646	0.005	PSMC2	-0.651	0.005
BCL3	-0.605	0.010	CTSC	0.529	0.029	IL-4	0.765	0.000	PSMD7	-0.551	0.022
C14orf166	0.571	0.017	CTSG	0.526	0.030	IL-5	0.491	0.046	PTGER4	-0.680	0.003
C1QB	0.508	0.037	CXCR1	0.636	0.006	IL-6ST	-0.672	0.003	RAG1	0.577	0.015
C4BPA	0.849	0.000	CXCR6	0.519	0.033	IL-7	0.596	0.012	RAG2	-0.523	0.031
C6	-0.716	0.001	DEFB103B	0.697	0.002	IL-7R	0.507	0.038	SELPLG	0.534	0.027
C7	0.483	0.049	DEFB4A	0.611	0.009	IL-9	0.803	0.000	SIGIRR	-0.510	0.036
C9	0.678	0.003	EBI3	0.848	0.000	ILF3	-0.547	0.023	SLAMF1	0.630	0.007
CASP1	0.523	0.031	ETS1	0.538	0.026	IRF4	-0.658	0.004	SLAMF6	0.633	0.006
CCBP2	-0.536	0.027	FCAR	-0.497	0.042	IRGM	-0.514	0.035	SMAD3	0.519	0.033
CCL18	0.643	0.005	FCGR1A/B	0.662	0.004	ITGB1	0.711	0.001	SRC	-0.573	0.016
CCL19	0.523	0.031	FN1	-0.522	0.032	JAK1	0.557	0.020	STAT2	0.482	0.050
CCL23	-0.681	0.003	FOXP3	-0.751	0.001	JAK2	0.849	0.000	STAT3	-0.530	0.029
CCL4	0.493	0.044	GATA3	-0.725	0.001	KIR3DL1	0.511	0.036	STAT5A	-0.734	0.001
CCL8	0.722	0.001	GBP1	0.494	0.044	KIR3DL2	-0.639	0.006	STAT5B	0.523	0.031
CCND3	0.567	0.018	GZMA	0.812	0.000	KLRB1	0.662	0.004	TBX21	0.483	0.050
CCR5	0.498	0.042	HFE	0.748	0.001	KLRC2	0.590	0.013	TCF7	-0.546	0.023
CCR7	-0.561	0.019	HLA-B	0.554	0.021	KLRD1	0.690	0.002	TFRC	-0.759	0.000
CCRL1	-0.652	0.005	HLA-DOB	-0.528	0.029	KLRG1	0.598	0.011	TGFBR2	-0.615	0.009
CD160	0.553	0.021	ICOS	-0.520	0.032	LCK	0.643	0.005	TIRAP	-0.675	0.003
CD164	-0.699	0.002	IDO1	0.707	0.002	LIF	-0.502	0.040	TLR1	-0.586	0.013
CD1A	0.683	0.003	IFNA2	0.572	0.016	LITAF	-0.613	0.009	TLR3	-0.509	0.037
CD247	-0.639	0.006	IFNAR2	0.693	0.002	LY96	-0.539	0.026	TLR7	-0.696	0.002
CD34	0.662	0.004	IFNB1	0.746	0.001	MAF	0.599	0.011	TLR9	-0.788	0.000
CD3D	0.850	0.000	IKBKB	0.582	0.014	MALT1	-0.800	0.000	TNF	0.482	0.050
CD45RO	0.535	0.027	IKZF1	0.723	0.001	MAP4K4	-0.780	0.000	TNFAIP6	-0.571	0.017
CD5	-0.572	0.016	IL-10	-0.731	0.001	MAPK11	-0.571	0.017	TRAF5	0.668	0.003
CD81	0.823	0.000	IL-11RA	-0.506	0.038	MAPK14	-0.499	0.042	VCAM1	0.667	0.003
CD8A	0.540	0.025	IL-15	0.864	0.000	MBL2	0.553	0.021	VTN	0.846	0.000
CEACAM6	0.542	0.025	IL-17A	0.731	0.001	MUC1	-0.564	0.018	XCR1	-0.495	0.043
CEACAM8	-0.490	0.046	IL-17F	-0.548	0.023	NCAM1	-0.912	0.000	ZBTB16	0.669	0.003

Bold indicates genes that overlap between Dim1 and Dim2.

trapezoidal rule. We defined responders as subjects who lost <7.5% of their baseline C-peptide response at month 12 [6]. This criterion was more stringent than criteria used to designate responders in the AbATE trial based on year-2 C-peptide results but had been used in previous analyses of treatment responses [6].

Flow cytometry

Fresh whole blood samples from AbATE were analyzed at the Immune Tolerance Network Flow Cytometry Core and fresh whole blood samples from the Delay trial were analyzed at the Yale Flow Cytometry Core. Fresh samples from study participants were

stained with mAbs to CD8, CD4, CD45RA, CD45RO, and CD62L (BD Pharmingen, CA, USA) to identify the following subsets of CD4⁺ and CD8⁺ T cells: CM: CD45RA⁻CD45RO⁺CD62L⁺, EM: CD45RA⁻CD45RO⁺CD62L⁻, EMRA: CD45RA⁺CD45RO⁻CD62L⁻, naïve: CD45RA⁺CD45RO⁻CD62L⁺. Gating strategies were kept consistent throughout each study and percentages of cell types or absolute cell counts (using the number of circulating lymphocytes from the complete blood count (CBC)) were compared to identify changes with treatment. For gene expression analysis and cell culture studies, frozen cells were thawed prior to study. CD62L expression is affected by freezing and thawing [31], therefore, CCR7 was used to identify CD4⁺ or CD8⁺ T-cell subsets: CM: CD45RO⁺CCR7⁺, EM: CD45RO⁺CCR7⁻, EMRA: CD45RO⁻CCR7⁻,

naïve: CD45RO⁺CCR7⁺. Cells were acquired with an LSRII or a FACSAria cytometer and analyzed with FlowJo software. Samples were analyzed without knowledge of the treatment assignment.

Cell cultures

To analyze the effects of teplizumab on T-cell proliferation, PBMCs from three different healthy controls were intracellularly labeled with CellTrace Violet dye (Life Technologies, Grand Island, NY, USA). Thawed PBMCs were cultured with 0.5 µg/mL teplizumab and 1.0 µg/mL of anti-CD28 mAb for 3 days. Proliferation of different T-cell subsets was determined based on dilution of CellTrace Violet dye, staining with the above mAbs and flow cytometry. For some experiments, CD8CM or naïve cells were sorted from PBMCs and labeled with CellTrace Violet dye. The remaining cells were labeled with CFSE. The two populations were combined and cultured with teplizumab and anti-CD28 mAb as above. The frequency of CellViolet⁺ CD8CM T cells was determined after 1, 2, and 3 days by flow cytometry.

Gene expression analysis

To identify differences in gene expression among CD8CM T cells, PBMCs from the 2 months time-point from drug-treated responders ($n = 6$), nonresponders ($n = 4$), and placebo-treated control subjects ($n = 8$) from the 2 months time-point in the Delay trial were labeled with mAbs to CD2, CD8, CD45RO, and CCR7 for cell sorting. CD8CM T cells (CD2⁺CD8⁺CD45RO⁺CCR7⁺) were sorted and RNA was prepared. The nanostring nCounter GX Human Immunology gene expression panel was used for gene expression analysis of 594 immunology-related genes.

Pathway and network analyses

The Nanostring data were normalized by (Nalini transformation) followed by quantile normalization across all samples [32, 33] as implemented in the R BioConductor Project library *preprocessCore* (Bioconductor: open software development for computational biology and bioinformatics R. (<http://bioconductor.org/>)). For technical reasons, the results from one of the nonresponders could not be normalized to the other 17 samples and the results from this sample were not used in the analyses. PCA was performed as described [34].

Statistical analyses

We compared the change in C-peptide AUC over 1 year in the drug-treated responders and nonresponders versus control or placebo-treated subjects in each trial. Due to a baseline imbalance in HbA1c between placebo- and drug-treated subjects in the Delay trial [7], C-peptide analysis was corrected for HbA1c levels at study

entry in a mixed linear model. Changes in the proportion of T-cell subsets from flow studies were compared by repeated-measures ANOVA and corrected for the baseline levels. Absolute cell counts (Fig. 2B) were calculated from the proportion of cells multiplied by the lymphocyte counts at the same blood draw. The presented flow cytometry data were corrected for the baseline unless otherwise indicated. Unless otherwise indicated, the data shown in the figures represent the LSMEANS values from the statistical model performed with SAS. Grouped data were compared either by *t*-tests, Wilcoxon's test, chi-squared test, ANOVA, or Kruskal–Wallis test (for data without normal distribution) as indicated. Calculations were done with GraphPad Prism (v. 6.0) and SAS (v. 9.3).

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Abbreviations: AUC: area under curve · CD8CM: CD8⁺ central memory · EM: effector memory · PCA: principal components analysis · T1D: type 1 diabetes

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