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References

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Increased T Cell Proliferative Responses to Islet Antigens Identify Clinical Responders to Anti-CD20 Monoclonal Antibody (Rituximab) Therapy in Type 1 Diabetes

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Type 1 diabetes mellitus is believed to be due to the autoimmune destruction of β-cells by T lymphocytes, but a single course of rituximab, a monoclonal anti-CD20 B lymphocyte Ab, can attenuate C-peptide loss over the first year of disease. The effects of B cell depletion on disease-associated T cell responses have not been studied. We compare changes in lymphocyte subsets, T cell proliferative responses to disease-associated target Ags, and C-peptide levels of participants who did (responders) or did not (nonresponders) show signs of β-cell preservation 1 y after rituximab therapy in a placebo-controlled TrialNet trial. Rituximab decreased B lymphocyte levels after four weekly doses of mAb. T cell proliferative responses to diabetes-associated Ags were present at baseline in 75% of anti-CD20- and 82% of placebo-treated subjects and were not different over time. However, in rituximab-treated subjects with significant C-peptide preservation at 6 mo (58%), the proliferative responses to diabetes-associated total (p = 0.032), islet-specific (p = 0.048), and neuronal autoantigens (p = 0.005) increased over the 12-mo observation period. This relationship was not seen in placebo-treated patients. We conclude that in patients with type 1 diabetes mellitus, anti-B cell mAb causes increased proliferative responses to diabetes Ags and attenuated β-cell loss. The way in which these responses affect the disease course remains unknown. The Journal of Immunology, 2011, 187: 1998–2005.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AUC, area under the curve; CI, confidence interval; CV, coefficient of variation; GFAP, glial fibrillary acidic protein; ITT, intention-to-treat; MMTT, mixed-meal tolerance test; PI, proinsulin; SI, stimulation index; TCP, T cell proliferation; T1D, type 1 diabetes; T1DM, type 1 diabetes mellitus; Treg, regulatory T cell.

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therapy, and by 12 mo, the circulating B lymphocytes had recovered to 69% of the baseline levels. Serum levels of IgG were not significantly different in the control and drug-treated subjects at that time, but the levels of serum IgM were still reduced 1 y after rituximab therapy.

The mechanisms of anti-CD20 effects are not clear because ultimately disease and disease progression are T cell dependent (17). To determine how B cell depletion with anti-CD20 mAb impacted disease-associated cellular immune responses, we compared T cell subset distributions and autoreactivity profiles in rituximab recipients who had positive C-peptide responses with those unresponsive to treatment.

Materials and Methods

Subjects and clinical samples

A detailed description of the TrialNet Rituximab trial (NCT00279305) and the baseline characteristics of the study subjects have been published (17). Briefly, 87 subjects were randomized in a 2:1 ratio to rituximab or placebo treatment, of which 81 formed the intention-to-treat (ITT) population, and 78 of whom completed the 1-y mixed-meal tolerance test (MMTT; the basis for the primary outcome, the area under the curve [AUC]). Based on the change in the AUC of the C-peptide response from baseline to 6 mo, each participant was designated as a C-peptide responder or nonresponder.

Of the ITT cohort, 75 (93%) were included in the flow analysis and 80 (99%) in the T cell proliferation (TCP) studies described in this article. Samples were collected for flow cytometry at baseline (prior to study drug dosing), 5 wk, and 3, 6, and 12 mo after the first dose of study drug. T cell assays were performed at baseline and 6 mo to determine an SI threshold to use in all samples.

Flow cytometric analysis

Whole blood was shipped overnight at ambient temperature to the Immune Tolerance Network Flow Cytometry Core (Roswell Park Cancer Institute, Buffalo, NY). Abs used in this study were purchased from BD Biosciences (San Jose, CA). Standard methods were used for staining of cells including blocking Fc receptors with mouse IgG. Cocktails of fluorochrome-labeled mAbs to CD3, CD4, CD8, CD62L, and CD25 were used, and the fluorometric analysis was performed using an FACSCanto (BD Biosciences) flow cytometer. The flow cytometry data were analyzed using WinList software.

TCP assay

PBMCs were isolated by Ficoll-Hypaque gradient centrifugation from fresh blood samples shipped to the T Cell Core Laboratory at the Hospital For Sick Children (Toronto, ON, Canada). The washed cells were seeded (10^5/ml well) into flat-bottom microplates containing test or control Abs in 200 μl in serum- and protein-free Ex-Cell Hybri-Max medium (Sigma-Aldrich, St. Louis, MO). Test Abs were used at 1–4 concentrations (0.1–10 μg/ml), depending on cell yields. Ten units recombinant human IL-2 were added to all test wells (8, 9, 18). Depending on the blood volume sent and viable cell yields, not all Abs could be used in all tests. Cultures were incubated (37˚C, 5% CO2) for 5 d, receiving a [3H]thymidine pulse (1 μCi) for the last 18 h. Cultures were then harvested and counted with a β-counter. To compare results from different donors, results were transformed into stimulation indices (SIs; cpm test Ag/cpm Ag-free control cultures). An SI ≥1.5 was considered a positive response (10). Positive and negative controls included PHA, tetanus toxoid, and anti-CD3 mAb and actin, Cy3, HI, BSA193, and OVA, respectively. Test Abs were classified into three different groupings—isolet: Tep99, GAD, GAD55, and Pf; neuronal: S-100, GFAP, MBP, and EX2; and milk: casein, BLG, BSA, and ABB85 (19).

Because of the concern that the in vivo B lymphocyte depletion present only in the rituximab group could have a confounding impact on the in vitro TCP lymphocyte responses, in preliminary studies, we compared the SIs of samples in which B cells from all samples were depleted ex vivo with magnetic beads prior to culture. Depletion of B cells was confirmed by flow cytometry in a subset of samples. We found that the SIs of samples in which B cells were depleted prior to culture to the undepleted sample were largely unaffected by B cell depletion (Supplemental Table I). Therefore, we have reported results from the cultures that were studied without further manipulation of the cells. For each sample, a T cell reactivity score was generated (sum of all positive responses to test Abs). An overall T cell score of ≥4 was considered evidence for the presence of autoimmunity in a given sample.

Statistical analyses

Study investigators, flow cytometry, and T cell laboratories were masked to treatment assignment of each subject. We compared the groups who were treated with rituximab versus placebo and those who were classified as a C-peptide responder versus nonresponder to the drug treatment. The AUC of the C-peptide values over the 2 h of the mixed-meal tolerance test (MMTT) was calculated using the trapezoidal rule including the time 0 and 2 h values and the AUC mean C-peptide (pmol/ml) was obtained as AUC/120. The within-subject coefficient of variation (CV) of the AUC mean C-peptide was 0.097 from two repeat MMTT assessments conducted within 3–10 d from the MMTT-Glucagon Stimulation Test Comparison Study (20). A subject was classified as a C-peptide responder if the AUC mean increased from baseline to 6 mo or decreased by less than the within-subject CV of 0.097. If the subject's AUC decreased at 6 mo and the CV was >0.097, the subject was classified as a nonresponder.

The data from flow cytometry were analyzed by separate ANCOVA models for each cell population at each time point adjusted for baseline flow, age, and sex. SI sums were calculated in groups of Abs that were thematically clustered and divided by the number of Abs in the group to determine an SI group mean. The T cell SI and positivity (reactivity) at 6 and 12 mo were examined using a separate regression model for each Ag or Ag grouping to estimate the change in SI response from baseline by treatment group and responder status. Logistic regression models were used to examine whether measures of T cell reactivity at each time point were predictive of responder status with an adjustment for baseline. The association between T cell reactivity and quantitative C-peptide over time was analyzed using a repeated-measures regression model. Least squared means with 95% confidence limits are presented except for baseline continuous variables in which the mean ± SD is shown. The percent change was calculated by dividing the values at 6 mo by the baseline. A Wilcoxon test was used to compare the number of lymphocytes in each group.

Results

Study population

The demographics of the study cohort within treatment groups and those designated as C-peptide responders and nonresponders are shown (Table I). As reported recently (17), the C-peptide responses increased at 3 mo in the rituximab-treated group, whereas placebo-treated subjects showed a decline of C-peptide responses (p = 0.038). After 6 mo, there was a parallel decline in both study groups, but a significant difference remained between the groups in average responses over 12 mo (p = 0.0013).

Based on the observed change in C-peptide responses relative to the coefficient of variation (CV) of repeated measurements, 58% of the subjects in the rituximab-treated group were responders. The age and sex distribution, prevalence of autoantibodies at baseline, HLA genotypes, and baseline MMTT C-peptide responses were similar between responders and nonresponder groups (Table I).

Analysis of lymphocyte subsets by flow cytometry

In rituximab recipients, the total lymphocyte counts had fallen to 87.6% of baseline by 3 mo (Fig. 1A, p < 0.001), but recovered later as measured at 6 and 12 mo. The decline was accounted for by depletion of CD19+ B cells. With some fluctuation, the placebo group remained at an average of 107.7% of the baseline lymphocyte count over the first year. Using ANCOVA to compare treatment groups, the number of CD3+, CD4+, and CD8+ cells, activated CD4+CD25+ cells, or regulatory T cells (Tregs) were not significantly different in the rituximab and placebo groups over the 1-y study period (Fig. 1B–F; p values >0.05).

T cell studies

We compared ex vivo T cell proliferative responses to an array of test and control Abs as described (8, 9, 21) in responders and nonresponders to rituximab treatment as well as placebo recipients. The baseline responses to disease-associated test Abs were...
similar in the two treatment groups and in the drug responders and nonresponders (Table II). Using the definition of a positive response in the assay overall as an SI of $1.5$ to at least four Ags, 75% of the rituximab- and 82% of the placebo-treated subjects showed positive T cell autoreactivity at baseline ($p = \text{NS}$), with similar incidence and response amplitudes of positive responses to each of the 12 individual disease-associated Ags (mean SI was $2.02 \pm 0.66$ in the rituximab-treated and $2.28 \pm 0.72$ in the placebo-treated group; $p = \text{NS}$). There were also no differences when test Ags were thematically clustered (Fig. 2, Table II).

At months 6 and 12, T cell autoreactivities in the two treatment groups were also similar (Fig. 3, Table II). Although the differences in the percentage of positive responses were not statistically significant between groups for any specific Ags or overall at either 6 or 12 mo, for all 12 Ags, the proportion positive was higher in the rituximab- versus placebo-treated subjects at 6 mo, but not at
Likewise, the SIs were not significantly different for any of the Ag groups or any of the individual Ags. However, sizable differences emerged when grouping rituximab recipients into C-peptide responders versus nonresponders (Fig. 3, Table III). In the rituximab group at 6 mo, 86.7% of subjects had a positive response overall, whereas 96.5% of responders versus 75.2% of nonresponders were positive ($p = 0.028$). There was a significant increase in the responses to 9 of the 12 Ags tested in the responders compared with the nonresponders. At month 12, these differences were reduced; only the proportion of responses to EX2 and MBP were significantly greater in C-peptide responders (Table III). There was also a significant ($p < 0.05$) rise in response amplitudes (SIs) over 12 mo to 4 out of 12 Ags including responses to the islet ($p = 0.037$) and neuronal ($0.043$) Ag groupings (Table III). Over 6 and 12 mo combined, the association was statistically significant for 8 out of the 12 test Ags (S-100, GAD, GAD555, PI, ABBOS, BLG, BSA, and casein), for each grouping (islet: $p = 0.01$; neuronal: $p = 0.048$; milk: $p = 0.006$), and overall ($p = 0.009$). These differences were the result of treatment with rituximab because we did not detect any significant differences in responders and nonresponders at baseline (Supplemental Table II).

Importantly, the positive and negative proliferative control responses were similar in C-peptide responders and nonresponders ($p = 0.519$ and 0.425, respectively). The responses to tetanus toxoid were 12.95 (confidence interval [CI] 11.24–14.65) in the responders and 12.04 (CI 9.9–14.19) in the nonresponders. The extent of in vivo B cell depletion by rituximab, which was not significantly different in responders and nonresponders, did not appear to account for the differences in the T cell responses. When corrected for the number of CD19 cells or the same B cell subsets, there was minimal effect on the $p$ values that describe the differences in the frequency of responses at 6 mo (data not shown).

### Table II. Percent positive T cell Ag reactivity by treatment group at months 6 and 12

<table>
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<tr>
<th>Group</th>
<th>Analyte</th>
<th>Baseline</th>
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<th>12 mo</th>
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<td></td>
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<td>Control ($n = 28$)</td>
<td>Rituximab ($n = 49$)</td>
<td>Control ($n = 27$)</td>
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<td>Ex2</td>
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<td>84.0</td>
</tr>
<tr>
<td></td>
<td>S-100</td>
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<td>84.0</td>
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<td>82.1</td>
<td>86.7</td>
<td>73.9</td>
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</tbody>
</table>

Estimates of percent positive as a function of treatment group for each Ag, and overall, from a separate logistic model at 6 or 12 mo adjusted for baseline positivity comparing treatment group. None of the differences between the rituximab and control groups are nominally statistically significant at $p \leq 0.05$.  

**FIGURE 2.** Average SIs in the TCP assay for each Ag group using cells from rituximab- or placebo-treated patients. The average SI (sum of [cpm for each analyte/cpm for media alone]/number of analytes) and 95% CIs for the mean for the positive control analytes, negative control analytes, total diabetes Ags, and thematically grouped Ags: islet, neuronal, and milk are shown for the rituximab-treated (●) and placebo (■) groups at the three study time points. None of the differences between the two groups are of statistical significance. The numbers of subjects per group at each time point were the following (rituximab, control): baseline (49, 29), month 6 (49, 27), and month 12 (46, 28).
Relationship of cellular and metabolic responses

We regressed the log (C-peptide + 1) values on the T cell proliferative responses (SI) at 6 and 12 mo, separately and jointly, adjusting for age, sex, and the SI and C-peptide responses at baseline. Within the rituximab group, Fig. 4 shows that the C-peptide increased as the SI increased (a positive relationship) at 6 mo for all of the Ags and groups of Ags, six of which were statistically significant: S-100 (p = 0.022) and PI (p = 0.033), as well as milk Ags overall (p = 0.021), ABBOS (p = 0.029), BLG (p = 0.0171), and BSA (p = 0.029). Conversely, within the placebo group, there was in inverse association (C-peptide decreased as the SI increased) for all Ags and Ag groupings, though not significantly so for any one in particular. Neither the negative nor positive controls were associated with C-peptide responses.

In like analyses within the placebo group, there was no significant relationship between 6 and 12 mo C-peptide levels and any Ag responses (Fig. 4 and data not shown). Further analyses of the two groups combined showed that the relationship between the change in C-peptide/change in SI was significantly different between the rituximab versus placebo groups (i.e., the test of the group*SI interaction) for all 16 of the Ags and Ag groupings. In a similar comparison between responders and nonresponders to rituximab, we found a significant difference between responders and nonresponders for each analyte for the slope of percent change in C-peptide/unit change in SI (Fig. 4B). However, within the nonresponders, none of these slopes were significantly different from zero. Within the responders, there were three analytes with a slope significantly different from zero: PI, ABBOS, and BLG (p < 0.05).

Lymphocyte subsets in subgroups of rituximab recipients

We compared the relative and absolute number of T cell subsets in C-peptide responders and nonresponders within the rituximab-treated group (Fig. 5). By an ANOVA for repeated measures,
the absolute number of CD19+ cells over 12 mo was not significantly different (-41%) in responders and nonresponders (p = 0.632), but the number of CD3+ cells was higher in the responders (1512, CI: 1422, 1608 versus 1346, CI: 1255, 1444; p = 0.016). The most significant differences between the groups were accounted for by an increase in the number of CD4+ cells (937, CI: 878, 999 versus 764, 884; p = 0.01) because the number of CD8+ cells was only slightly different (423, CI: 393, 454 versus 383, CI: 353, 417; p = 0.08). However, to assess this hypothesized effect of CD4 cells among responders and nonresponders, we conducted additional analyses adjusting for the CD4 values at baseline, week 5, month 3, and month 6 of the positivity/negativity of each analyte. There was very little change in the p values, and 9 out of the 13 (versus 10 out of 13 without adjustment) were still significant at the 0.05 level. The number of activated, CD4+CD25+ cells was not significantly different in responders and nonresponders. The number of Tregs (CD4+CD25+ CD62L-) was higher at week 12 (48, CI: 41, 57 versus 34, CI: 28, 41; p = 0.005) in responders, but over the 12-mo period with four separate measurements, the differences were not statistically significant (p = 0.17).

Discussion

This trial discovered that enhanced, T cell proliferative responses to diabetes-associated target Ags are predictive of a positive C-peptide response to rituximab in newly diagnosed patients with T1D. Enhanced T cell reactivity in C-peptide responders was not explained by overall elevated T cell reactivity, because neither positive nor negative control responses were changed by therapy, nor could any control responses be associated with stimulated C-peptide levels. The enhanced T cell reactivity cannot be explained by an artifact or assay drift. Background counts were not significantly different between C-peptide responders and nonresponders at 6 mo (p = 0.632), and positive (p = 0.519) and negative (p = 0.425) controls were unchanged in the same assays. There was also no change in T cell reactivity of placebo recipients. We conclude that the observed elevation of Ag-specific responses to diabetes-associated environmental, islet, and neuronal Ags was caused by rituximab-driven attenuation of postonset β-cell loss over the first year posttherapy. These strictly blinded assays, conducted over a 27-mo period, delineated an almost absolute (95.6%) linkage between enhanced T cell reactivity and the presence of definitive signs of β-cell preservation. It was conceivable that increased proliferative responses were a function of being a clinical responder per se and not due to the effects of the rituximab treatment. Comparing the change in SIs with C-peptide AUC in both study arms, we found that the positive association between changes in C-peptide responses only occurred in rituximab recipients. In the placebo group, this relationship was more often negative. Thus, the observed changes in T cell responses were a function of the rituximab therapy received rather than sequelae of the improved metabolic response.

In addition, our findings cannot be explained by differences in the extent of B cell depletion. The number of circulating T cells overall were not significantly different in the rituximab- and placebo-treated groups. We found modest but statistically significant changes in CD3+ and CD4+ T cells, but the mechanistic relevance of these minimal changes in polyclonal populations to the Ag-specific responses is not clear. Even if subtle, a numeric expansion of the T cell compartment might reflect brief homeostatic proliferation into hemopoietic space, vacated by rituximab. The observation that this space was only 69% refilled by B lineage cells 1 y after the single treatment course as well as the growth of the T cell compartment both support the homeostatic expansion scenario. This expansion might include the Ag-specific T cell pools we measured ex vivo. There is precedence for this with rapid diabetes development in lymphocyte-free NOD.scid mice following transfer of small number of diabetesogenic wild-type NOD lymphocytes or following lymphotoxic therapy of wild-type NOD mice with cyclophosphamide (22, 23). However, homeostatic expansion alone does not appear to explain the differences in the TCP assay in responders and nonresponders because the responses to control Ags were not different between these two groups, yet the responses to the diabetes-related Ags were different. This suggests that there has been expansion of disease Ag-specific T cells and/or function.

However, in the present trial, loss of β-cells/insulin secretory reserve was not accelerated but significantly slowed. Thus, therapeutic B cell depletion relieved a somewhat counterintuitive, disease progression-associated restraint of T cell expansion. These data demonstrate, for the first time in human T1D to our knowledge, a progression-promoting role of B lineage cells at diabetes onset. This B cell role is amenable to therapeutic intervention and adds a fundamental similarity to T1D pathogenesis in NOD mice and in humans. We conclude, therefore, that the statistically significantly different relationships between C-peptide and T cell
responses between the rituximab and placebo groups show that the T cell responses between responders and nonresponders suggest a biological effect of rituximab treatment and not a general response regardless of the treatment received.

Our findings cannot be explained by chance alone, but it is important to note that the statistical relationship that we identified with the T cell assay has an unclear biologic foundation. We do not have a confirmatory assay in which the number of Ag-specific cells or the magnitude of their responses to Ags can be confirmed, and the mechanism that accounts for the increase in the proliferative responsiveness to disease-associated Ag-specific T cells is not clear. The TCP assay is performed in the presence of IL-2, and therefore, it is also possible that the increased proliferative response that we found was due to an increased proportion of activated or anergic CD4+ or CD8+ T cells (21). Indeed, our flow cytometric analysis of peripheral blood cells did show a modest increase in the proportion of CD4+ and CD8+ T cells that expressed CD25 but the frequency of CD4+ or CD8+ T cells (21). It is also possible that B cell depletion altered T cell trafficking rather than purely numeric change in the Ag-specific T cells. For example, Piccio et al. (24) recently reported that there was a decrease in the number of T cells in the CSF after rituximab treatment of patients with relapsing/remitting multiple sclerosis. They suggested that B cells may be critical for T cell trafficking into the CNS inflammatory lesions. An analogous reduction in T cell trafficking into the islet following B cell depletion with the mAb might account for an increase in the number of Ag-specific cells in the periphery. A similar mechanism might be operative to explain the finding of increased Tregs that we observed in the peripheral blood of rituximab-treated patients. An analysis of cytokine responses to the tested Ags might provide insights into the relationships between the increased proliferative responses and reduced β-cell destruction and determine, for example, whether production of cytokines such as IL-10 and/or TGF-β may have been increased in the Ag-specific cells. However, this type of analysis will require further studies with fresh cells from a separate cohort.

We found that the changes in T cell responses correlated with clinical responses in the rituximab-treated group and with responses to certain Ags when comparing the drug and placebo groups. One explanation that might account for this finding is that the Ag-specific cells that are assessed in the assay have regulatory function. This notion was suggested in preclinical studies of Hu et al. (16), who found that the proportion of CD4+CD25+Foxp3+ cells was increased during the recovery phase following depletion of human CD20-expressing NOD mice. Interestingly, we found a similar difference in the number of circulating Tregs in C-peptide responders compared with nonresponders at week 12. In addition to these conventional Tregs, it is also possible that the Ag-specific cells, which are increased in the patients, have regulatory function by virtue of production of cytokines such as IL-10 or TGF-β or through other mechanisms (25, 26). Further studies of the cytokines that are made by the T cells in response to Ag might be helpful in addressing this question. The mechanism that would lead to induction of T cells with this phenotype has not been identified, but it is of note that IL-10-secreting B lymphocytes have been identified, and the secretion of this cytokine might create an environment that induces differentiation of T cells that produce regulatory cytokines. Induction of Tregs might be postulated to occur through a bystander mechanism in the presence of IL-10 production by B cells that repopulate after CD20 depletion (i.e., regulatory B cells) (26, 27).

Collectively, we have found that proliferative responses to diabetes-associated target Ags are similar in patients who receive antirituximab compared with placebo. However, among those treated with rituximab, individuals who are clinical C-peptide responders show an increased proliferative response to islet, neuronal, and disease-relevant environmental Ags, and the changes in proliferative responses to islet Ags are associated with an increase in insulin secretory function. The perhaps simplest explanation of our data would be that rituximab allowed recovery and/or regeneration of β cells through transient interruption of pathogenic T cell recruitment via surface Ig-captured autoantigen but...
providing new β-cell targets during recovery of the CD19+ B cell compartment posttreatment (28).

Disclosures
M.D.P. has been a speaker and consultant to Roche/Genentech and has received research support for a clinical trial from Genentech. The other authors have no financial conflicts of interest.

References