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# Combined Therapy With GABA and Proinsulin/Alum Acts Synergistically to Restore Long-term Normoglycemia by Modulating T-Cell Autoimmunity and Promoting β-Cell Replication in Newly Diabetic NOD Mice

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**Antigen-based therapies (ABTs) fail to restore normoglycemia in newly diabetic NOD mice, perhaps because too few β-cells remain by the time that ABT-induced regulatory responses arise and spread. We hypothesized that combining a fast-acting anti-inflammatory agent with an ABT could limit pathogenic responses while ABT-induced regulatory responses arose and spread. γ-Aminobutyric acid (GABA) administration can inhibit inflammation, enhance regulatory T-cell (Treg) responses, and promote β-cell replication in mice. We examined the effect of combining a prototypic ABT, proinsulin/alum, with GABA treatment in newly diabetic NOD mice. Proinsulin/alum monotherapy failed to correct hyperglycemia, while GABA monotherapy restored normoglycemia for a short period. Combined treatment restored normoglycemia in the long term with apparent permanent remission in some mice. Proinsulin/alum monotherapy induced interleukin (IL)-4– and IL-10–secreting T-cell responses that spread to other β-cell autoantigens. GABA monotherapy induced moderate IL-10 (but not IL-4) responses to β-cell autoantigens. Combined treatment synergistically reduced spontaneous type 1 T-helper cell responses to autoantigens, ABT-induced IL-4 and humoral responses, and insulitis, but enhanced IL-10 and Treg responses and promoted β-cell replication in the islets. Thus, combining ABT with GABA can inhibit pathogenic T-cell responses, induce Treg responses, promote β-cell replication, and effectively restore normoglycemia in newly diabetic NOD mice. Since these treatments appear safe for humans, they hold promise for type 1 diabetes intervention.**

The Immune Tolerance Network and Juvenile Diabetes Research Foundation Joint Taskforce as well as recent commentaries, have opined that ideal immunotherapies for type 1 diabetes (T1D) should reduce proinflammatory autoimmune responses, promote regulatory responses, and enhance β-cell survival and replication (1–4). Theoretically, antigen-based therapies (ABTs) are appealing because ABTs can induce antigen-specific regulatory responses with little interference with systemic immunity. Previous studies (5,6) have shown that ABT can induce strong regulatory T-cell (Treg) responses after T1D onset and prolong the survival of syngeneic islet grafts in preconditioned diabetic NOD mice. However, ABTs have little or no ability to restore normoglycemia in newly diabetic NOD mice. The inability of ABT to protect residual β-cell mass in newly diabetic NOD mice may stem from

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the 10–14 days that it takes for ABT to induce maximal immune responses to the administered autoantigen and the time it takes for Treg responses to spread to other β-cell autoantigens (reviewed in 4). Consequently, by the time ABT-induced regulatory responses peak in newly diabetic NOD mice, insufficient β-cell mass remains and the treatments appear ineffective. We hypothesized that administering a fast-acting anti-inflammatory agent along with an ABT could limit pathogenic autoimmune responses while ABT-induced regulatory responses arise and spread, and that their combined effects could synergistically inhibit inflammation and promote restoration of normoglycemia.

T cells express γ-aminobutyric acid (GABA) receptors (GABA-Rs) (7–9). The activation of GABA-Rs can inhibit autoreactive type 1 T-helper (Th1) cell responses and antigen-presenting cell function directly ex vivo (7,10–15), but increase the number of Tregs in vivo (13,16). GABA-R activation inhibits inflammation and disease in mouse models of T1D (10,13,17), rheumatoid arthritis (11), multiple sclerosis (12), and type 2 diabetes (16). Notably, GABA administration also very effectively restored normoglycemia in wild-type mice that had been rendered diabetic by multiple low doses of streptozocin, which induces low-grade β-cell autoimmunity (13). However, when the same treatment was given to mildly hyperglycemic NOD mice (which have a more robust autoimmune response), 60% of the mice did not respond to treatment and 40% of the mice displayed a delayed disease progression for <6 weeks (13). Thus, GABA monotherapy has limited beneficial effects in newly diabetic NOD mice. Because of its rapid anti-inflammatory effects, GABA is an excellent candidate for therapeutic testing in combination with ABTs. Importantly, GABA also promotes mouse and human β-cell survival and replication (13,15,18,19). Long-term treatment with GABA neither induces leukopenia (10) nor desensitizes immune cells to GABA (10,11), and GABA appears to be safe for human consumption (20–22).

The aim of this study was to investigate the therapeutic potential of ABT in combination with GABA. As a prototypic ABT, we chose to study proinsulin, because it is a key β-cell autoantigen and contains more T-cell determinants than insulin or fragments thereof. We assessed the ability of each monotherapy and their combination to restore euglycemia in newly diabetic NOD mice, their impact on autoimmune and immunoregulatory responses, and their ability to promote β-cell replication.

## RESEARCH DESIGN AND METHODS

### Mice

NOD mice (Taconic Farms, Derwood, MD) were housed in a specific pathogen-free facility. Only female NOD mice were used. All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles.

### Treatment

We monitored the blood glucose levels of female NOD mice, and those with two blood glucose levels >250 mg/dL on consecutive days were considered to be newly diabetic. The mice were randomly assigned to groups that continually received water containing 0, 2, 6, or 20 mg/mL GABA, pH 7.2 (Sigma-Aldrich, St. Louis, MO). Each mouse consumed about 5 mL of water per day.

Some newly diabetic mice also received 100 μg of proinsulin (provided by Eli Lilly, Indianapolis, IN) complexed with alum (Pierce, Rockford, IL) intraperitoneally. The mice were boosted with the same dose of proinsulin/alum 10 days after the first vaccination. The mice were monitored for the recurrence of hyperglycemia.

### ELISPOT Assay

NOD mice at 15 weeks of age received proinsulin/alum, GABA (20 mg/mL), or combined therapy, as described above. Control groups included untreated and alum (alone)–injected mice. Ten days after the second vaccination, the frequency of antigen-specific interferon-γ (IFN-γ)–, interleukin (IL)-4–, and IL-10–secreting splenic T cells in the different groups of mice was determined by ELISPOT assays, as previously described (23), with the addition of using JES5–16E3 and JES5–2A5 (BioLegend, San Diego, CA) as capture and detection antibodies for IL-10. The tested antigens included control self-antigen mouse serum albumin (MSA), and β-cell autoantigens GAD65 (Diamyd Medical), HSPp277, and proinsulin (all at 100 μg/mL). The cells in medium alone were used as the negative controls, while cells challenged with 1 μg/mL anti-CD3 provided positive controls.

### Flow Cytometry Analysis of Tregs

The percentages of splenic $CD4^+Foxp3^+$ Tregs in individual mice were determined by flow cytometry, as per our previous study (16).

### ELISA for Proinsulin Autoantibodies

The concentrations of serum anti-proinsulin IgG, IgG1, and IgG2a in individual mice were determined by ELISA (5) using proinsulin as the antigen.

### Histological Examination

Insulitis scores were determined from at least 20 islets per pancreas of individual diabetic mice 10 days after initiating treatment, as previously described (24).

### Analysis of β-Cell Replication

β-Cell replication and $insulin^+$ cells in mouse islets were assessed 10 days after initiating treatment as previously described (19).

### Statistical Analysis

Data are expressed as the mean ± SEM. The difference between groups was analyzed by Student *t* test, and the periods of normoglycemia between groups were analyzed by the log-rank test. A *P* value of <0.05 was considered statistically significant.

## RESULTS

### GABA Monotherapy Dosing Studies

After NOD mice develop hyperglycemia (2 consecutive days of blood glucose levels >250 mg/dL), they generally progress to severe hyperglycemia within 1 week (Fig. 1*A*). We found that oral treatment with GABA at 2 mg/mL, a dose can inhibit the development and severity of rheumatoid arthritis in mice (11) as well as the development of insulin resistance in a high-fat diet mouse model (16), failed to correct hyperglycemia in newly diabetic mice (data not shown), suggesting that T1D intervention will require a higher dosage. Treatment with GABA at 6 mg/mL delayed disease progression for a very short period (Fig. 1*B*). Treatment with GABA at 20 mg/mL restored normoglycemia in all mice (Fig. 1*C*). Eight of 10 mice maintained euglycemia for 2–5 weeks, and two mice did so for 18–23 weeks (Fig. 1*C*). Thus, oral GABA monotherapy at an appropriate dose can quickly correct hyperglycemia and maintain normoglycemia for a short period in newly diabetic mice, extending previous findings (13). These observations underscore the need for systematic studies to identify an effective GABA dose and frequency of administration to inhibit inflammatory responses in humans. The therapeutic effects of GABA may be mediated by the ability of GABA to inhibit autoreactive Th1 responses (7,10,11), enhance Treg responses (13,16), and promote the functional recovery and replication of β-cells (13,18,19).

### Combined Therapy Provides Synergistic Therapeutic Effects

Next, we tested whether combined therapies of proinsulin/alum vaccination with GABA (20 mg/mL) could correct hyperglycemia and better maintain normoglycemia in newly diabetic NOD mice. Monotherapy with proinsulin/alum vaccination failed to correct hyperglycemia (Fig. 1*D*).

In contrast, all mice treated with a combination of proinsulin/alum and GABA rapidly re-established normoglycemia (Fig. 1*E*). Five of nine mice developed hyperglycemia again between 13 and 24 weeks after initiating treatment. Four mice remained normoglycemic at 20, 30, 45, and 50 weeks post-treatment, suggesting permanent remission in some mice (Fig. 1*E*). Combined therapy maintained a significantly longer normoglycemic period than GABA (20 mg/mL) monotherapy ($P = 0.001$). These data demonstrate for the first time that the combination of ABT with an immunoregulator effectively corrects hyperglycemia and can maintain long-term normoglycemia in newly diabetic NOD mice.

### Combined Therapies Induce Antigen-Specific IL-10 Responses in NOD Mice

To understand the mechanisms underlying the therapeutic effects, female NOD mice at 15 weeks of age were treated with monotherapy or combined therapy, as described for newly diabetic mice. Ten days after the second vaccination, we characterized splenic antigen-specific T cells by ELISPOT (Fig. 2). There was no significant difference in the numbers of splenic mononuclear cells among the different groups of mice (data not shown), and no significant difference in the frequency of GAD65, HSPp277, and proinsulin-specific IFN-γ–secreting T cells between alum-vaccinated and unmanipulated control NOD mice (Fig. 2*A*). These control mice had few autoantigen-reactive IL-4 or IL-10 spot-forming cells (Fig. 2*B* and *C*), which is consistent with the notion that unipolar Th1 responses drive disease progression in NOD mice (24).

Monotherapy with proinsulin or GABA significantly reduced IFN-γ–secreting Th1 responses to all autoantigens tested (Fig. 2*A*). Proinsulin monotherapy induced IL-4– and IL-10–secreting T-cell responses to proinsulin that spread to autoantigens GAD65 and HSPp277, but not to control self-antigen MSA (Fig. 2*B* and *C*). GABA monotherapy elevated the levels of IL-10, but not IL-4, responses to proinsulin, GAD65, and HSPp277, but not to MSA (Fig. 2*C*). More interestingly, combined therapy further reduced the frequency of GAD65-, HSPp277-, and proinsulin-specific Th1 cells (Fig. 2*A*), and increased the frequency of IL-10 responses to β-cell autoantigens to twofold to threefold greater than that of either monotherapy (Fig. 2*C*). Notably, IL-4 responses to β-cell antigens induced by combined therapy were significantly lower than that of proinsulin monotherapy (Fig. 2*B*). Consistent with these findings, monotherapy with proinsulin or GABA significantly reduced the levels of proinsulin-specific IgG2a, and monotherapy with proinsulin, but not with GABA, enhanced IgG1 responses (Fig. 2*D*). In contrast, combined therapy significantly decreased the levels of Ig2a and IgG1 responses relative to proinsulin monotherapy. The reduced frequency of autoreactive IL-4–secreting T cells and IgG1 responses by combined therapy suggests that GABA may mitigate ABT-induced IL-4 responses. Moreover, histological characterization revealed that treatment with GABA or combined therapies (but not with proinsulin monotherapy) significantly reduced insulitis (insulitis scores 1.008 and 1.006, respectively) compared with those in untreated mice (1.82, $P < 0.01$ for both treatments). Collectively, combined therapy synergistically inhibited pathogenic T-cell autoimmunity and enhanced IL-10 responses, contributing to the restoration of normoglycemia in newly diabetic NOD mice.

### Combined Treatment With Proinsulin With GABA Synergistically Increases the Frequency of Splenic Tregs in NOD Mice

Next, we characterized the frequency of splenic Tregs in the different groups of NOD mice by flow cytometry. In comparison with that in unmanipulated NOD mice, vaccination with alum alone did not significantly alter the percentages of splenic Tregs (Fig. 2*E*). In contrast, vaccination with proinsulin/alum or treatment with GABA alone increased the frequency of splenic Tregs by ~18% and 50%, respectively. The combination of proinsulin and GABA treatments further elevated the percentages of splenic Tregs to ~200% over that in controls, and

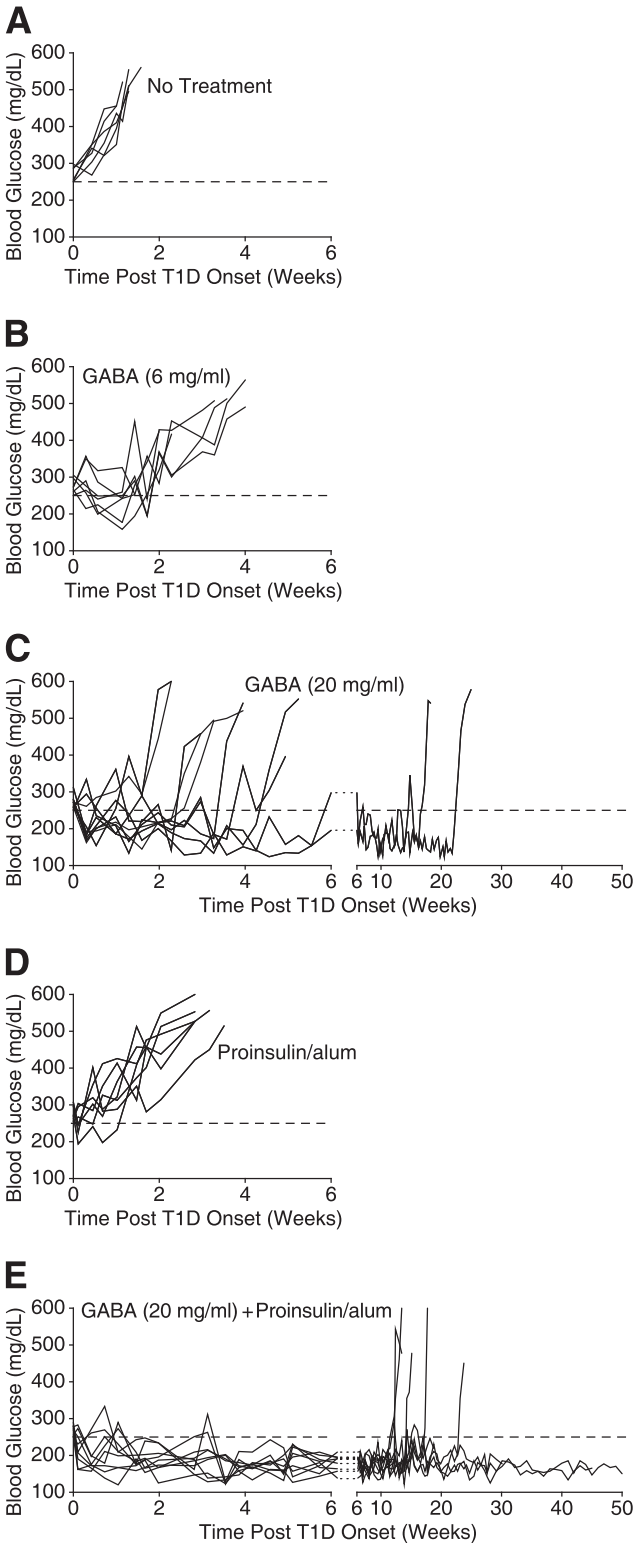


**Figure 1**—Longitudinal blood glucose levels in newly diabetic NOD given GABA or proinsulin/alum monotherapy or combined treatment. Newly diabetic NOD mice (with two blood glucose levels >250 mg/dL recorded on consecutive days) were untreated ($n$ = 6) (*A*) or were continually given GABA, 6 mg/mL ($n$ = 7) (*B*), or GABA, 20 mg/mL ($n$ = 10) (*C*), through their drinking water. Other groups of mice received proinsulin/alum monotherapy ($n$ = 7) (*D*) or combined proinsulin/alum + GABA, 20 mg/mL ($n$ = 9) (*E*). Four of the mice shown in *E* remained normoglycemic at 20, 30, 45, and 50 weeks after initiating treatment (at the time of manuscript proofing). Data shown are longitudinal blood glucose levels for individual mice. Note the change of scale in *C* and *E*. The dashed line indicates a blood glucose level of 250 mg/dL. Combined therapy significantly prolonged the period of normoglycemia compared with GABA (20 mg/mL) monotherapy ($P$ = 0.001 by the log-rank test).

the percentages were significantly higher than that in mice that received either monotherapy (Fig. 2*E*). Thus, consistent with significantly increased IL-10 responses (Fig. 2*C*), combined proinsulin and GABA treatments synergistically enhanced splenic Treg responses in NOD mice.

### Combined Therapy More Effectively Reduces Insulitis and Increases β-Cell Replication and the Percentage of Insulin-Producing Cells in the Islets of Newly Diabetic NOD Mice

Since GABA and proinsulin/alum have immunoregulatory actions, and GABA can also promote β-cell survival and replication, we studied the effect of each therapy on insulitis and β-cell replication in newly diabetic NOD mice. Newly diabetic NOD mice were untreated, or were treated with a monotherapy or combined therapy, and were provided with water containing BrdU for 10 days. Histological characterization revealed that GABA, but not proinsulin, monotherapy significantly reduced the insulitis scores and that combined therapy further reduced the insulitis scores in newly diabetic NOD mice (Fig. 3*A*).

We focused on measuring β-cell replication by Ki67/insulin immunostaining rather than by determining β-cell mass because *1*) low levels of β-cell replication would be difficult to detect as a change in β-cell mass and *2*) GABA treatment can promote functional recovery of degranulated β-cells leading to an increase in insulin$^+$ islet cells and an apparent increase in β-cell mass (19). We observed almost no insulin$^+$ islet cells in pancreatic islets from untreated or proinsulin/alum (only)–treated mice (Fig. 3*B* and *C*), which is consistent with their rapid progression to severe hyperglycemia during this time period (Fig. 1*A* and *D*). In contrast, Ki67$^+$insulin$^+$ cells were detected in the islets from the GABA monotherapy and combined therapy mouse groups, and accounted for ~1.2% and 1.7% of insulin$^+$ cells, respectively (Fig. 3*B* and *C*). Similarly, there were ~80 insulin$^+$ cells per islet in the GABA monotherapy group, and a significantly greater number of insulin$^+$ cells (≈120 per islet) in the combined therapy group, suggesting that combined therapy more effectively promoted β-cell health and survival. These observations are consistent with the longitudinal blood glucose levels (Fig. 1), cellular immune responses (Fig. 2), and insulitis scores (Fig. 3*A*).

## DISCUSSION

In summary, proinsulin/alum monotherapy had no ability to correct hyperglycemia. GABA monotherapy had a limited

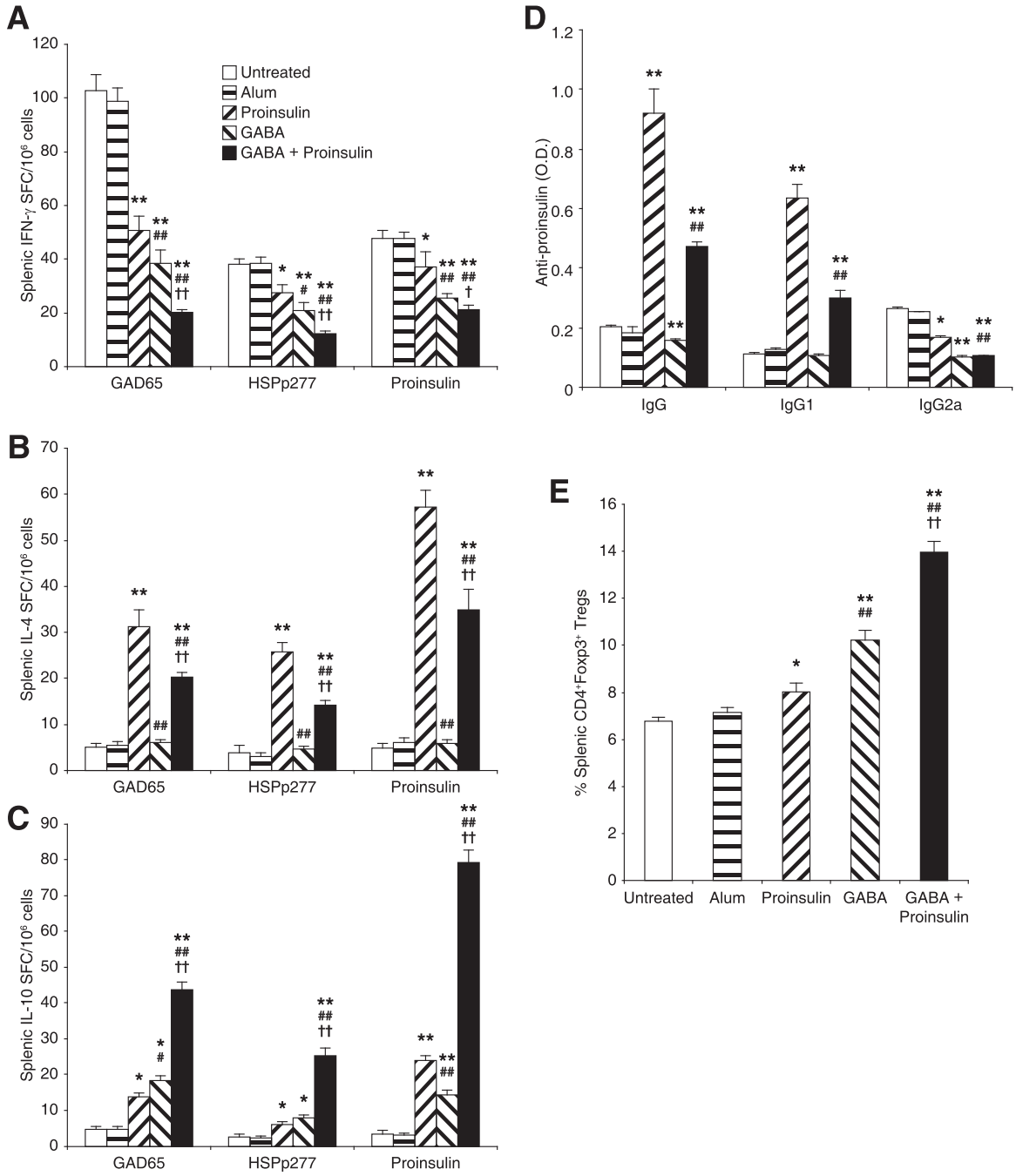


**Figure 2—**Effect of monotherapy and combined therapy on T-cell immunity and humoral responses. NOD mice at 15 weeks of age were treated with alum, proinsulin/alum, GABA, or proinsulin/alum + GABA, as described in RESEARCH DESIGN AND METHODS. A control group of mice received no treatment. Ten days after completing treatment, their splenic T cells were isolated, and the frequency of T cells secreting IFN-γ (*A*), IL-4 (*B*), or IL-10 (*C*) in response to MSA, proinsulin, GAD65, or HSPp277 was determined by ELISPOT. Responses to control MSA were at background levels in all mice (data not shown). Data are expressed as the mean number of spot-forming cells (SFCs) ±SEM per million splenic mononuclear cells. *D*: The levels of proinsulin-specific IgG, IgG1, and IgG2a antibodies, as determined by ELISA. Data are expressed as the mean optical density (O.D.) value ± SEM for each group. *E*: Effect of monotherapy and combined therapies on Treg responses, as determined by flow cytometry. Data are expressed as the mean percentage of Tregs ± SEM. The experimental and control groups of mice ($n$ = 5 per group) were tested simultaneously in two separate experiments. *$P$ < 0.05, **$P$ < 0.01 vs. the control. #$P$ < 0.05, ##$P$ < 0.01 vs. the proinsulin/alum group. †$P$ < 0.05, ††$P$ < 0.01 vs. the GABA group.

ability to restore normoglycemia in newly diabetic NOD mice. The combination of these therapies not only rapidly corrected hyperglycemia, but also restored long-term normoglycemia in newly diabetic NOD mice. We found that combined therapies induced antigen-specific IL-10 responses that spread to other β-cell antigens, increased the frequency of splenic Tregs, and inhibited the severity of inflammation in the pancreatic islets of NOD mice.

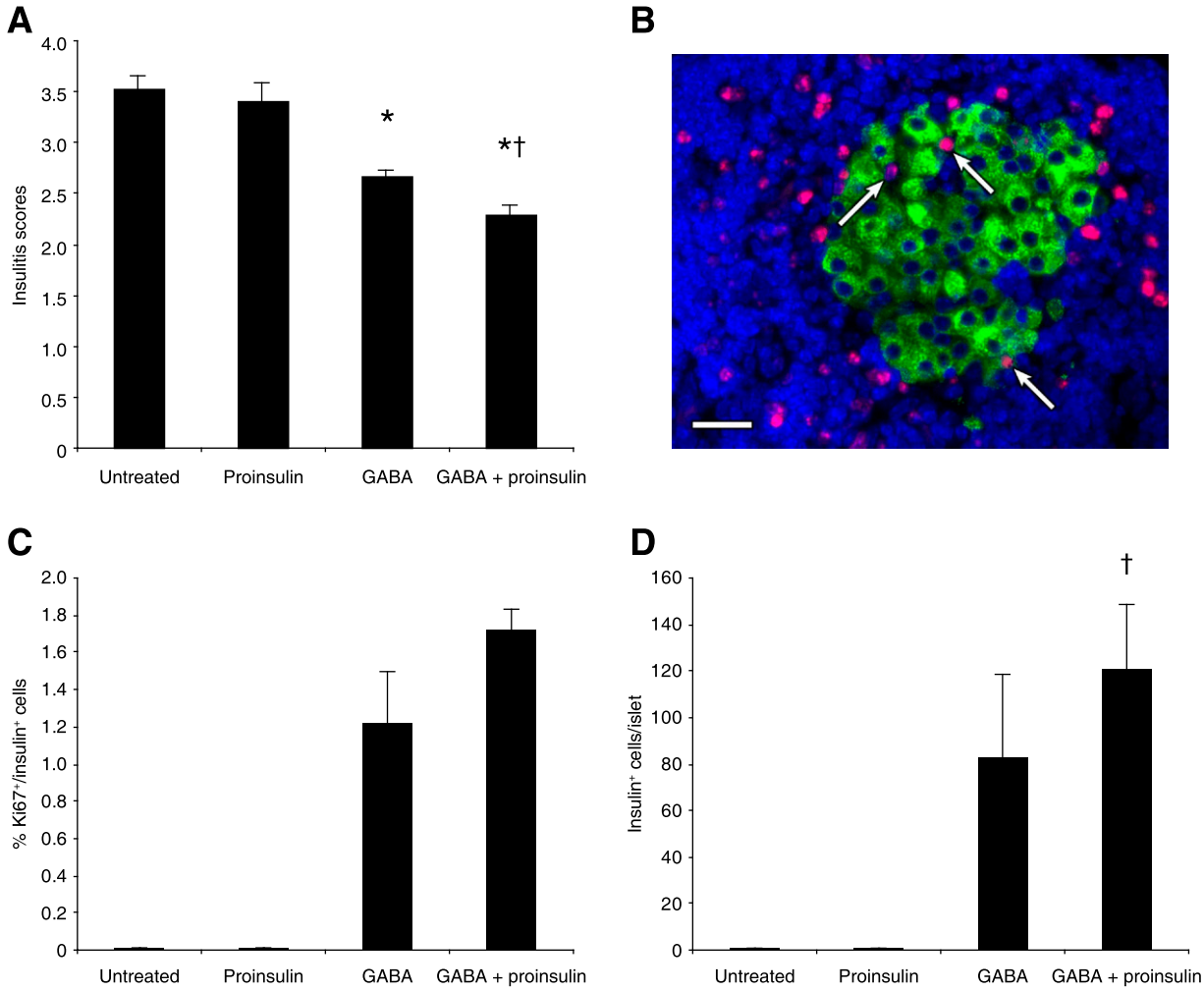


**Figure 3—**Effect of monotherapy and combined therapy on insulitis, β-cell replication, and the percentage of insulin$^+$ cells per islet in newly diabetic NOD mice. Newly diabetic NOD mice were randomized to receive water without GABA (untreated) or water containing GABA (20 mg/mL) and/or intraperitoneal proinsulin/alum ($n$ = 6 mice per group). Additionally, their water contained BrdU. After 10 days, their pancreata were analyzed for insulitis or β-cell replication. At least 12–15 islets from two sections (with an interval of 150 μm) of each pancreatic tissue were examined in a blinded manner. *A*: Insulitis scores. *$P < 0.05$ vs. untreated, †$P < 0.05$ vs. the GABA monotherapy group. *B*: Representative image of anti-Ki67 (red) and anti-insulin (green) staining cells in an islet from a mouse that had been treated with combined therapy (original magnification ×400). Arrows indicate Ki67$^+$insulin$^+$ cells. Scale bar = 50 μm. *C*: The percentages of Ki67$^+$insulin$^+$ β-cells in total insulin$^+$ β-cells. Data are expressed as the mean ± SEM of the percentages of Ki67$^+$insulin$^+$ islet cells in different groups of mice. Similar results were observed using BrdU/insulin immunostaining (data not shown). *D*: The number of insulin$^+$ cells per islet. †$P < 0.05$ vs. the GABA monotherapy group.

GABA monotherapy, and to a greater extent combined therapy, promoted β-cell replication and preserved insulin$^+$ cells in newly diabetic NOD mice. Our findings provide a proof-of-principle that combining ABT with an immunoregulator can effectively restore normoglycemia in newly diabetic mice. GABA appears to be safe for human consumption (20–22) and does not cause general immunosuppression (10). GABA-Rs are expressed by human immunocompetent cells and β-cells. Activation of GABA-Rs can inhibit human T-cell proliferation in vitro (8,15) and can promote human β-cell replication and survival in transplanted islets (19). Therefore, the use of GABA as an immunoregulator in combination with ABTs or other candidate immunotherapeutics, may hold promise for T1D intervention in humans.

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**Duality of Interest.** J.T. and D.L.K. are inventors of GABA-related patents. D.L.K. serves on the scientific advisory board of Diamyd Medical. No other potential conflicts of interest relevant to this article were reported.

**Author Contributions.** J.T. conceived, designed, and performed the experiments; analyzed the data; and wrote the manuscript. H.D. performed the experiments and analyzed the data. A.V.N. and Z.C. performed the experiments. D.L.K. conceived and designed the experiments, and wrote the manuscript. J.T. and D.L.K. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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