Supporting Information

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SI Experimental Procedures

Evaluation of Mice and Drug Administration. IPGTT, insulin tolerance test, and glucagon tolerance test were performed as described (1). Circulating insulin, C-peptide, or glucagon levels were measured by ELISA or RIA (Linco Research) kits, and serum cytokines by Multiplex kit (BioRad). Cytokines in culture supernatants were measured by ELISA (R&D Systems). Induction of diabetes was made by i.p. injection of STZ (40 mg/kg for four consecutive days) in CD1 mice. Metabolic cages were used before the termination of experiment. Some mice received BrdU-injection (i.p., 100 mg/kg) 6 h before the animals were killled. The pancreas was isolated and processed as described previously (1, 2).

Immunoblotting. We detected Akt and phospho-Akt by immunoblotting as described before (1). Primary antibodies (1:500) were from Cell Signaling, and HRP-conjugated secondary antibodies (1:2,000) were from Jackson ImmunoResearch. We quantified the band densities by using the ImageJ program.

INS-1 Culture, Islet Isolation, Apoptosis, and TUNEL Assays. INS-1 cells and mouse islets isolated by collagenase digestion were maintained in RPMI 1640 medium (1, 3). For induction of apoptosis, the INS-1 cells or islets were challenged with STZ (15 mM) or a mixture of cytotoxic cytokines (10 ng/mL IL-1β, 50 ng/mL TNF-α, 50 ng/mL IFN-γ; Sigma) in RPMI 1640 medium in the presence or absence of GABA (100 μM) FBS for 20 to 24 h. Apoptotic cells were identified by typical morphologic criteria of chromatin condensation and fragmentation using DAPI-nuclear staining or TUNEL labeling (TMR red; Roche/Applied Science). Insulin secretion in cultured INS-1 cells was performed as previously described (2, 3).

Pancreatic Tissue Preparation and Immunostaining. Freshly isolated pancreata were fixed overnight in formaldehyde–acetic acid solution as described previously (1, 2). Each pancreas was subsequently cut into 10 segments to account for the distribution of islets across the entire tissue. Following dehydration in ethanol and clearing with xylene, the pancreatic pieces were embedded in paraffin. Pancreatic sections (5 μm, crossing all 10 segments) were incubated with guinea pig anti-insulin and rabbit anti-glucagon (1:1,000; Dako) and detected with fluorescent (Cy3- and FITC-conjugated IgG) or biotinylated secondary antibodies, and, in the latter case, samples were incubated with avidin–biotin–peroxidase complex (Vector Laboratories) before chromogen staining of DAB (Sigma-Aldrich) or Fuchsin red (Dako) and subsequent hematoxylin counterstaining. Proliferative islet β-cells were identified in the pancreatic section, double-stained for insulin and BrdU or Ki67 by using anti-BrdU (1:200; Sigma) or anti-Ki67 (1:200; chromogen:DAB; Abcam), and relevant secondary antibodies (1:1,000; Jackson Labs). Apoptotic β-cells were identified in isolated mouse islets with insulin and TUNEL labeling (TMR red; Roche) of positive islet cells. DAPI nuclear staining was performed in isolated mouse islets, and the specific TUNEL staining was found to be only nuclear-localized. Results are expressed as the percentage of $BrdU^+$ (or TUNEL-positive) β-cells. Images were captured by a fluorescence microscope (Eclipse TE 200; Nikon).

Analysis of β-Cell Mass. Pancreatic images were visualized using ScanScope CS (Aperio Technologies) and analyzed by using the ImageScope program (Aperio Technologies). Entire pancreatic tissue areas from each of the pancreatic sections containing 10 segments were outlined, and the number of strong positive signals within those areas, representing insulin- or glucagon-positive cells, was determined as the function of the positive pixel count algorithm (Aperio Technologies). This algorithm was also used to detect the number of negative signals, which represented the total pancreatic area. Total β- and α-cell mass for each pancreas was determined as the product of the total cross-sectional β- or α-cell areas over total tissue area and the weight of the pancreas before fixation as we described previously (1).

Isolation of Spleen Cell Subpopulations and Flow Cytometry Analysis. Spleen cell isolation, in vitro culture, magnetic cell sorting to isolate subpopulations, and flow cytometry analysis were performed as described (4). The nonadherent cells were purified by magnetic sorting into $CD4^+$ and $CD8^+$ T cells (>95% pure; R&D Systems). The macrophages (>90% pure) were prepared from the adherent fraction. The antigen-presenting cells were prepared by irradiating adherent spleen cells. We performed phenotypic analysis of isolated splenocytes by multicolor flow cytometry by using the following fluorescence-conjugated antibodies: anti-CD4-PE, anti-CD25-PerCP-Cy5.5, anti-Foxp3-FITC and the corresponding isotype controls (eBiosciences), and anti-Nrp1 and anti-TGFβRI antibodies (R&D Systems) labeled with Alexa Fluor 647 or 488, respectively (Molecular Probes).

Lymphocytic Stimulation in Vitro. Splenic T cells (5×10^4 cells per well) were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies (2 μg/mL each) in the presence or absence of 1 mM GABA, with or without 100 μM picrotoxin. Proliferation of the $CD8^+$ T cells (5×10^4/well) from transgenic TCR-8.3 NOD mice was induced by a synthesized peptide mimotope KYNKANAFL (1 μM). The splenic T cells from saline solution- or GABA-treated mice were cocultured with irradiated antigen-presenting cells (1×10^5) from the adherent fraction in the presence of the peptide alone or peptide plus GABA (200 μM) for 72 h. Cell proliferation was measured by MTT assay. The macrophage-rich splenic adherent fraction was stimulated with 1 μg/mL LPS and 3 ng/mL IFN-γ, in the absence or presence of GABA. The conditioned medium was collected for cytokine ELISA.

Electrophysiology. INS-1 cells were cells were bathed in the standard extracellular solution and intracellular solution at 22 °C as described (5). Patch-clamp recordings of INS-1 cells were performed under voltage- or current-clamp mode by using an Axopatch 700A amplifier controlled by pClamp9 (Axon Instruments). The EC_{50} of GABA in INS-1 cell is 22.3 μM (5). The pipette resistance acquired under voltage- or current-clamp model was 4 to 6 MΩ or 20 to 30 MΩ, respectively. We perfused GABA (100 μM) alone or with bicuculline (100 μM) focally onto the cells by using a gravity-driven perfusion system. Electrical signals were filtered at 1 kHz and digitized at 2 kHz.

Intracellular Ca^{2+} Measurement. Intracellular Ca^{2+} was measured using Fura-2 AM (Molecular Probes). INS-1 cells or isolated mouse islet β-cells were preloaded with Fura-2 AM (2 μM), washed, and transferred to a thermal-controlled chamber and perfused with GABA or muscimol, with or without bicuculline focally in the recording solution (5) while the recordings were made with an intensified CCD camera. The fluorescent signal was recorded with a time-lapse protocol, and the fluorescence intensity (i.e., Poenie–Tsien) ratios of images were calculated by using ImagePro-5.

1. Wang Q, Brubaker PL (2002) Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. *Diabetologia* 45:1263–1273.
2. Soltani N, Kumar M, Glinka Y, Prud'homme GJ, Wang Q (2007) In vivo expression of GLP-1/IgG-Fc fusion protein enhances beta-cell mass and protects against streptozotocin-induced diabetes. *Gene Ther* 14:981–988.
3. Xu E, et al. (2006) Intra-islet insulin suppresses glucagon release via GABA-GABAA receptor system. *Cell Metab* 3:47–58.
4. Glinka Y, Chang Y, Prud'homme GJ (2006) Protective regulatory T cell generation in autoimmune diabetes by DNA covaccination with islet antigens and a selective CTLA-4 ligand. *Mol Ther* 14:578–587.
5. Dong H, et al. (2006) Gamma-aminobutyric acid up- and downregulates insulin secretion from beta cells in concert with changes in glucose concentration. *Diabetologia* 49:697–705.

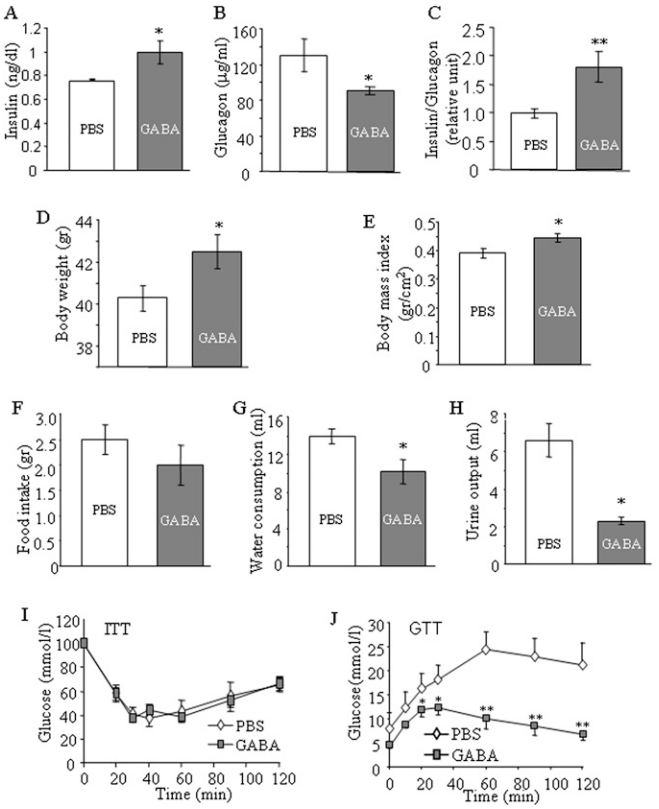

Fig. S1. GABA prevents diabetes in MDSD mice in association with improved glucose homeostasis. Insulin (*A*) and glucagon (*B*) levels were measured in serum of saline solution- or GABA-treated MDSD mice by RIA (53 d after first STZ injection); (*C*) the ratio of insulin to glucagon is calculated. (*D*) The body weight of the two groups of mice was measured at the end of the feeding course of 60 d. (*E*) Body mass index as a function of weight to length is calculated. Metabolic studies using metabolic cages show the food intake (*F*), water consumption (*G*), and urine output (*H*) in GABA-treated or saline solution-treated MDSD mice. (*I*) Insulin tolerance test (ITT) and (*J*) glucagon tolerance test (GTT) conducted at the end of the feeding course are shown. (*$P < 0.05$ and **$P < 0.01$; $n = 5–8$.)

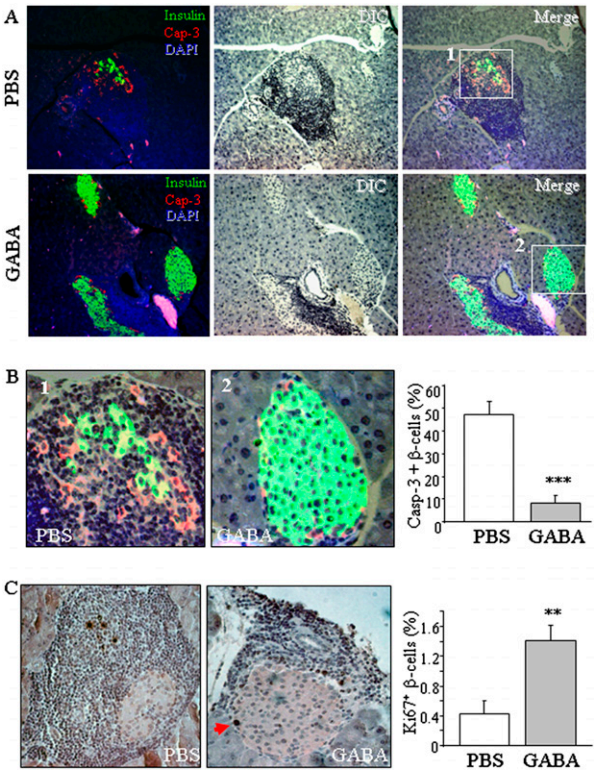

Fig. S2. GABA suppresses apoptosis and enhances proliferation of β-cells in NOD mice. (*A*) Pancreatic sections from saline solution- and GABA-treated NOD mice were stained for insulin (green), procaspase-3 (red), nuclei (DAPI, blue), and with H&E for morphologic analysis. The fluorescent images were merged with differential interference contrast image (DIC) and show the insulin-positive islet cells and procaspase-3–positive β-cells and infiltrating lymphocytes within islet structure. (*B*) Higher magnification of the islets (insets 1 and 2 in *A* and *B*); bar graph shows the ratio of caspase-3–positive β-cells from the two groups of mice. (*C*) Evaluation of islet β-cell (pink) proliferation by Ki67 staining (brown) in NOD mice at 13 wk of age; the arrow indicates Ki67$^+$ β-cell, and the bar graph shows the counting results in the two groups of mice. (*$P < 0.05$; **$P < 0.01$; $n = 5$.)

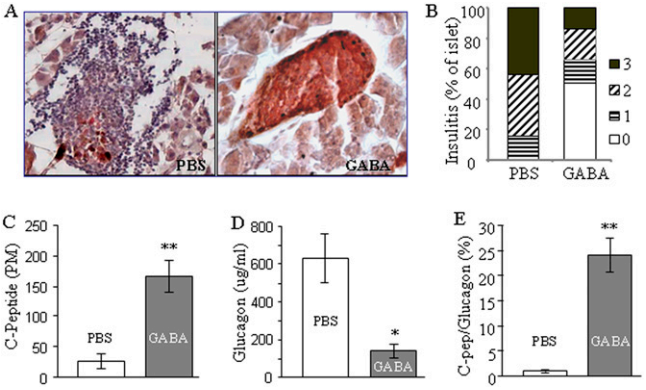

Fig. S3. GABA eliminates insulitis in TCR-8.3 NOD mice. (*A*) We stained the pancreatic section of TCR-8.3 NOD mice, treated with PBS solution or GABA, by insulin (brown) and glucagon (black) with H&E counterstaining, which showed that insulitis is suppressed in the GABA-treated mice. (*B*) The insulitis scores were determined as follows: 0, no lymphocyte infiltration; 1, mild periinsular lymphocyte infiltration; 2, moderate lymphocyte infiltration (i.e., granulation of <50%); and 3, severe massive lymphocyte infiltration (i.e., granulation of > 50%). GABA administration increased circulating C-peptide (*C*) and lowered glucagon (*D*) levels, leading to an increased ratio of C-peptide to glucagon (*E*). (*$P < 0.05$ and **$P < 0.01$; $n = 5$.)

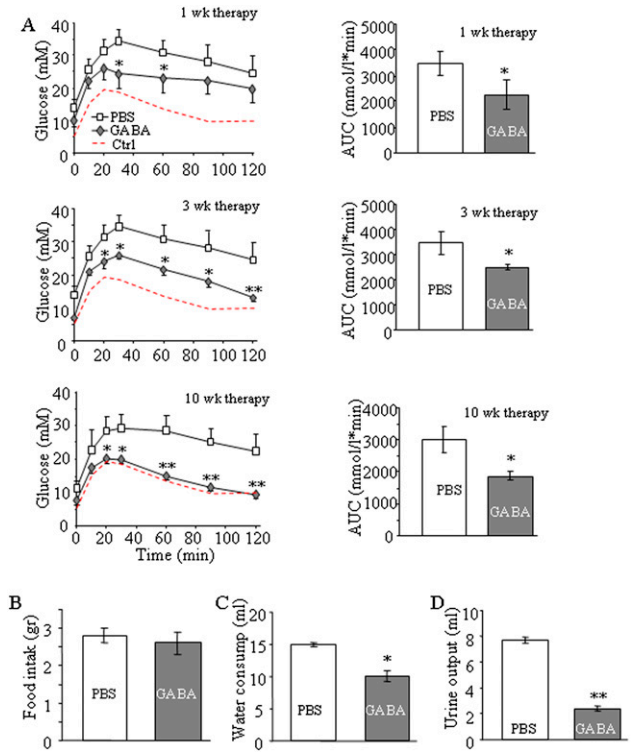

Fig. S4. GABA reverses established diabetes in MDSD mice. (*A*) IPGTTs were performed at 1 wk, 3 wk, and 10 wk after initiation of GABA administration in established diabetic MDSD mice (blood glucose > 30 mM). Metabolic studies show food intake (*B*), water consumption (*C*), and urine output (*D*) in saline solution- and GABA-treated MDSD mice. (*$P < 0.05$ and **$P < 0.01$; $n = 5$.)

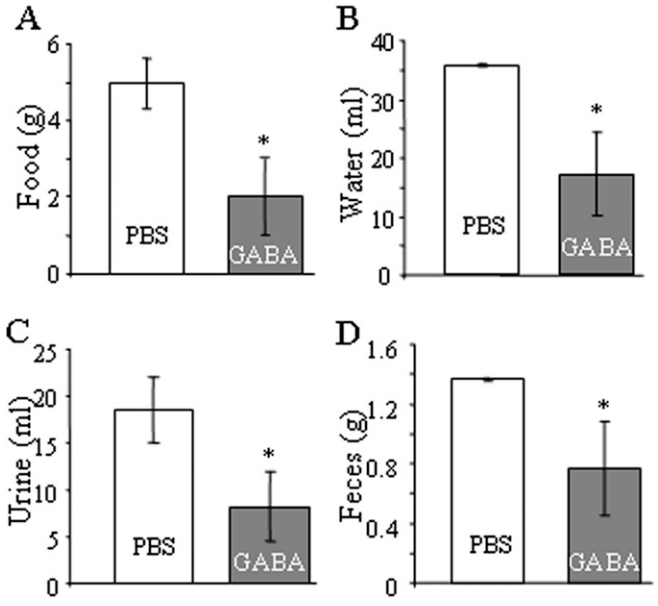

Fig. S5. GABA improves metabolic conditions in NOD mice. Metabolic studies show food intake (*A*), water consumption (*B*), urine output (*C*), and feces output (*D*) in established diabetic NOD mice (blood glucose > 20 mM) treated with saline solution or GABA for 50 d.

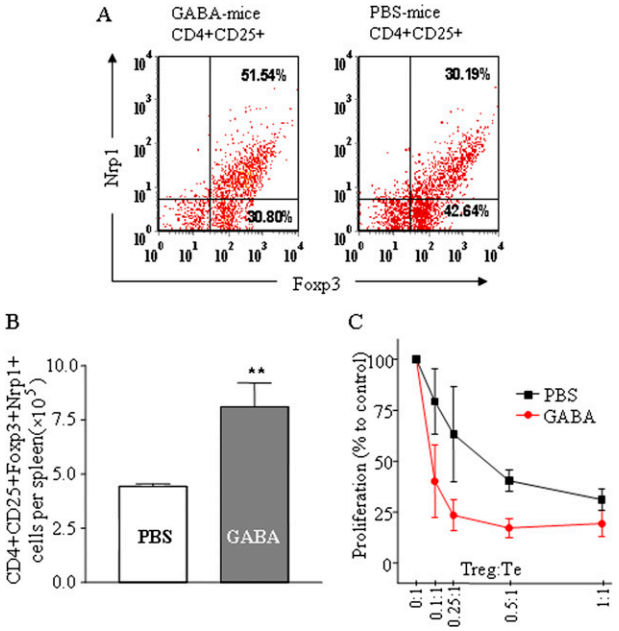

Fig. S6. GABA treatment increases Treg numbers and suppressive activity in the spleen of NOD mice. (*A*) Coexpression of Nrp1 and Foxp3 by $CD4^+CD25^+$ cells; the $Nrp1^+$ and $Foxp3^+$ populations are gated on the isotype controls. A representative result shows that GABA induces a shift toward a greater proportion of $CD4^+CD25^+$ Treg cells that coexpress both Foxp3 and Nrp1. (*B*) GABA treatment increased the total number of $CD4^+CD25^+Foxp3^+Nrp1^+$ cells in the spleen of NOD mice ($n = 5$ per group; $^*P < 0.05$). (*C*) Magnetically sorted $CD4^+CD25^+$ cells (Treg) from the spleen of GABA-treated NOD mice or control mice ($n = 4$ per group) were more effective at suppressing the proliferation of the effector $CD4^+$ T cells (Te) from a syngeneic nondiabetic mouse. Proliferation of Te was induced by the plate-bound anti-CD3 and anti-CD28 antibodies (results represent the mean ± SEM).

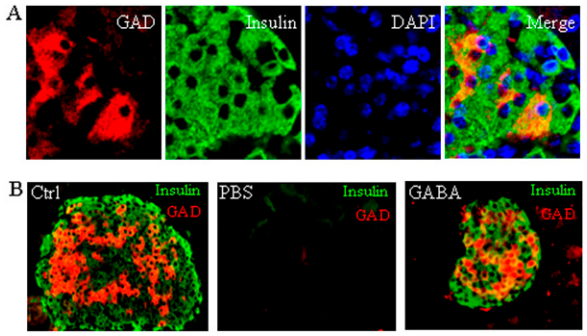

Fig. S7. GABA treatment protects against a reduction of β-cell GAD. (*A*) Double staining for insulin (green) and GAD (red) using anti-GAD/65/67 antibody (AB1511; Millipore; this antibody detects both GAD56 and GAD67) in pancreatic sections from NOD mice before the onset of diabetes. (*B*) Pancreatic GAD65/67–insulin staining from control (Ctrl), PBS solution-treated (PBS), and GABA-treated (GABA) TCR-8.3 NOD mice at 13 wk of age. ($^*P < 0.05$; $n = 5$.)