Supporting Information

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SI Materials and Methods

Immunization of NOD Mice. To favor induction of R3-specific antibodies, groups of five female NOD mice were immunized four times at 3-wk intervals with two versions of the insulin peptide bound to IA^{g7} in R3, both linked covalently to the N terminus of the IA^{g7} β-chain via a flexible protein linker. The first immunization was with 100 μg of IA^{g7} bound to peptide R3:RAE emulsified in incomplete Freund's adjuvant (IFA), administered s.c. The second immunization was with 100 μg of IA^{g7} bound to R3:REss, also given s.c. in IFA. This variant has a cysteine substitution at position 62 of the IA^{g7}α-chain that allows the formation of a disulfide bond with the B19(p6) cysteine of the peptide. We also cleaved the peptide linker in this protein with thrombin to free the C terminus of the peptide and reduce the possibility of generating antibodies whose binding depended on this nonnative linker of the complexes. The third immunization was a repeat of the first injection, and the final boost was with 100 μg of a mixture of the two proteins without IFA given i.p. for the final boost.

Hybridoma Generation and Screening. To generate hybridomas, dispersed splenocytes and draining lymph node cells were harvested from single animals and mixed with the fusion partner Sp2/0-Ag14 (American Type Culture Collection) at a ratio of 5:1. After washing with HBSS to remove FBS, 1 mL of 40% (wt/vol) PEG 8000 was added to the cell pellet drop wise over a period of 30 s. After an additional 30 s, 1 mL of FBS-free minimum essential medium for suspension culture (SMEM GIBCO 11380-037) was added with mixing over a total of 30 s. Finally 2 mL, 3 mL, 4 mL, 5 mL, and another 5 mL of FBS-free media was added drop wise with mixing to the cell pellet. The cells were then incubated at 37 °C for 10 min to induce complete fusion, washed with balanced salt solution, and then resuspended in SMEM containing 10% FBS at a density of 4 × 106 cells per mL and 100-μL aliquots were distributed in flat-bottom 96-well plates. The next day, 100 μL of 2× HAT selection medium (Hypoxanthine H,10−4 M; Aminopterin A, 0.4 × 10−6 M; Thymidine T, 1.6 × 10−5 M) was added. Supernatants from wells containing growing hybridomas were screened by ELISA with plates coated with IA^{g7} bound to the R3:RE peptide or with control complexes, IA^{g7}–HEL and IAb–3K. Antibodies that bound IA^{g7}–insulin complex, but not the control complexes, were selected for cloning by limiting dilution. The clone with the strongest binding to the IA^{g7}–insulincomplex, mAb287, was chosen for all subsequent experiments. The mAb287 cells were subsequently adapted to grow in serum-free medium (GIBCO12045-076) supplemented with cholesterol (GIBCO 12531) and pluronic F-68. mAb287 antibody was affinity purified from the culture medium by using protein A Sepharose (GE 17-0618).

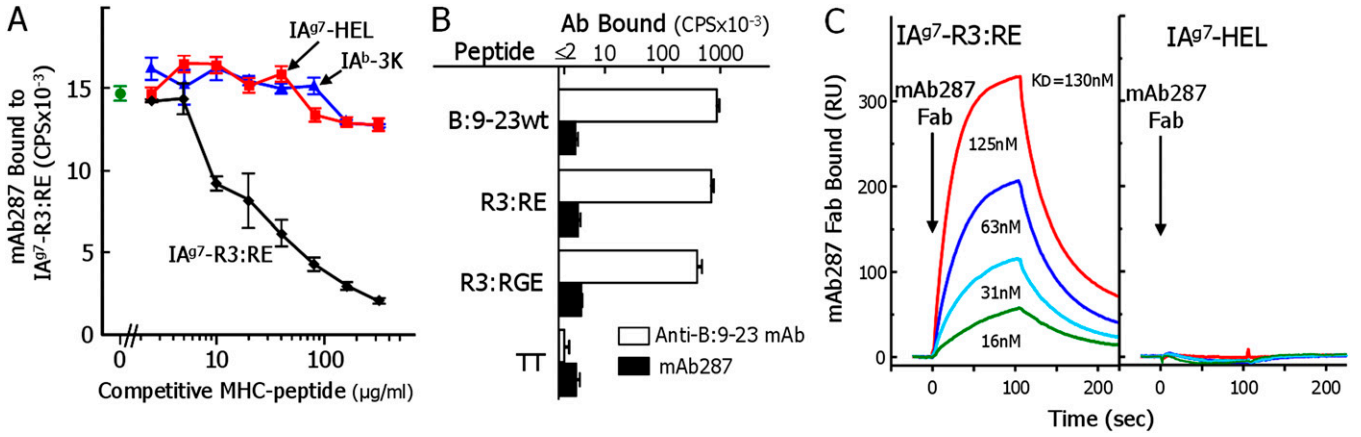

Fig. S1. Additional experiments to establish the specificity of mAb287. (*A*) Purified mAb287 (1 ng/mL) was preincubated with various concentrations of IA^{g7}–R3:RE (black line), IA^{g7}–HEL (red line), or IA^{b}–3K (blue line). The competition mixtures were added to IA^{g7}–R3:RE-coated ELISA plates and the bound mAb287 measured as described in *Materials and Methods*. Each bar is the average of the results of three independent experiments ± SEM. (*B*) ELISA plates were coated with amino acid 9–23 peptide of the β-chain (B:9–23), R3:RE, R3:RGE, or TT soluble peptides, and binding of mAb287 or the B:9–23–specific mAb, AIP-46.12, (1 μg/mL) was measured as described in *Materials and Methods*. The experiment was repeated twice with similar results. (*C*) As described in *Materials and Methods*, the binding kinetics of various concentrations of Fab fragments of mAb287 to immobilized IA^{g7}-R3:RE (*Left*) or IA^{g7}-HEL (*Right*) were recorded by using surface plasmon resonance. The overall affinity of the mAb was calculation with software supplied with the instrument. Similar results were obtained in a second experiment.

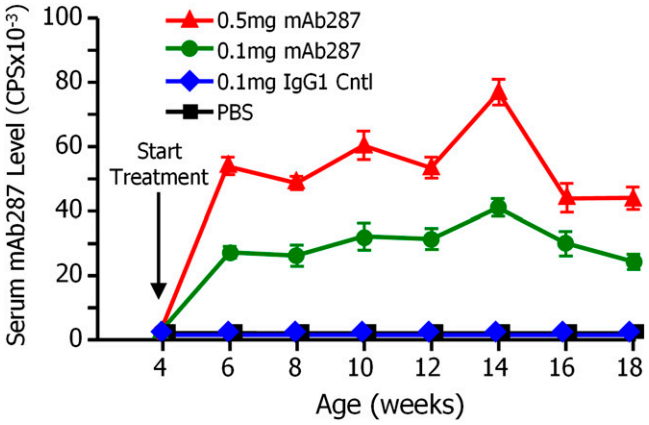

Fig. S2. Serum levels of mAb287 in treated mice. Serum samples were obtained from each mouse by retroorbital bleed at baseline (4 wk), after two therapeutic/control injections (6 wk), and thereafter at 4-wk intervals, with a 3-d delay between antibody administration and bleed. Circulating mAb287 was measured by using IA^{97}–R3:RE-coated plates as described in *Materials and Methods*. Mean values of mAb287 levels were shown.

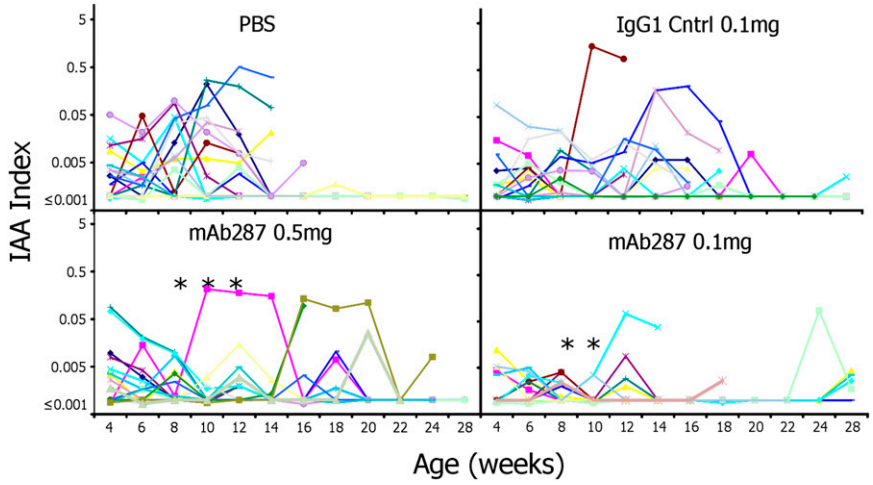

Fig. S3. The development of insulin autoantibody (IAA) in nonobese diabetic (NOD) mice with four different treatments. mAb287 mice (0.5 mg) had lower mIAA values at 8, 10, and 12 wk, and 0.1-mg dose group mice had lower mIAA at 8 and 10 wk compared with PBS group at the same age. *y* axis shows the index of IAA in the standard fluid phase IAA radioassay (1). $*P < 0.05$ compared with PBS group by Mann–Whitney analysis.

1. Yu L, et al. (2000) Early expression of antiinsulin autoantibodies of humans and the NOD mouse: Evidence for early determination of subsequent diabetes. *Proc Natl Acad Sci USA* 97(4):1701–1706.

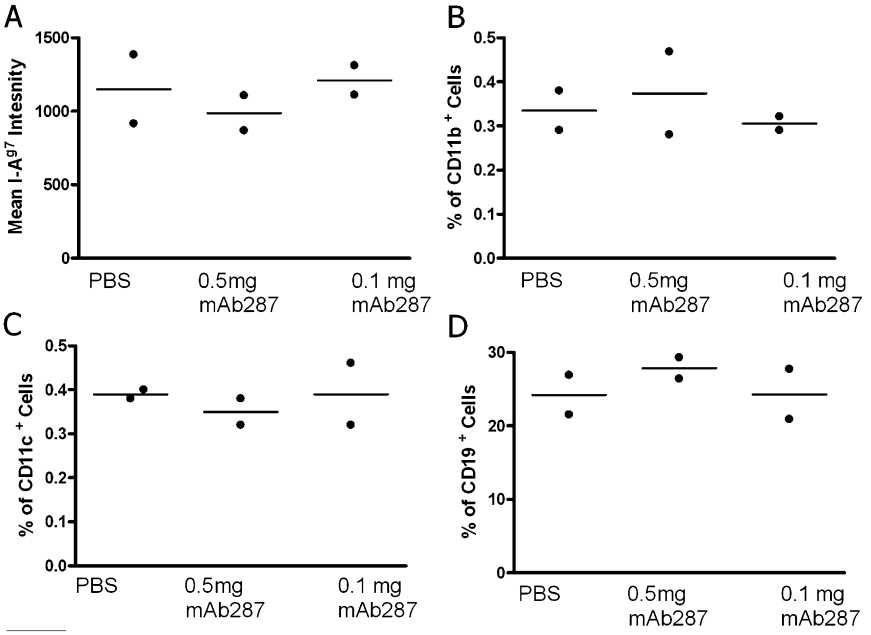

Fig. S4. Treatment with mAb287 has no effect on IA^{g7} expression or APC distributions. Representative animals (n = 2) from the PBS and low and high treatment groups were killed at 10 wk of age. Splenocytes were prepared and stained with antibodies to IA^{g7}, CD11b, CD11c, and CD19, respectively. The mean fluorescence intensities of IA^{g7} staining (*A*), and percentages of CD11b- (*B*), CD11c- (*C*), and CD19- (*D*) positive cells in the live cell gates are shown.

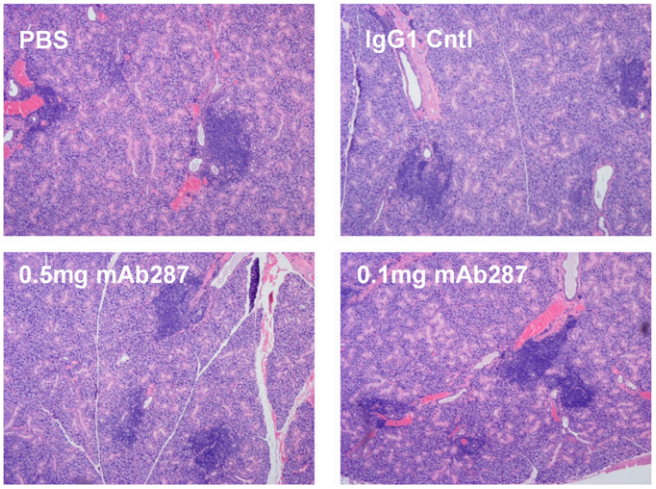

Fig. S5. Treatment with mAb287 does not affect the development of sialitis. Representative photomicrographs of salivary glands of H&E stain at 4× magnification showing sialitis was found in NOD mice all four groups at 30 wk. Salivary glands were harvested and fixed with 10% (vol/vol) formalin buffer for H&E staining.

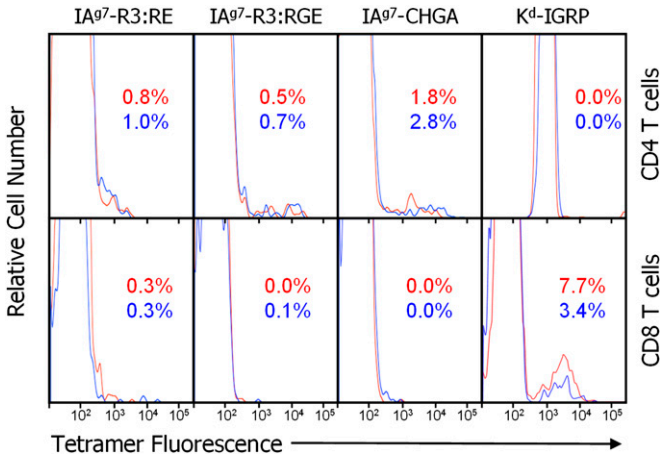

Fig. S6. Antigen-specific T cells detected in the pancreases of control and mAb287-treated mice. Pooled islet cells from the pancreases of the control (red) and mAb287 (blue) treated mice described in Fig. 3 *B–D* were gated on either live B220^{-}CD4^{+} or live B220^{-}CD8^{+} T cells and analyzed for the binding of IA^{97}-insulin R3:RE, IA^{97}-insulin R3:RGE, IA^{97}-ChgA, and K^{d}-IGRP tetramers. The percentages of specific tetramer+ T cells for the IA^{97} tetramers were calculated by subtracting the values obtained with CD8 T cells from those obtained with CD4 T cells and vice versa for the K^{d} tetramer.