Innocuous IFNγ induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production

Renu Jain,[1] Danielle M. Tartar,[1] Randal K. Gregg,[1] Rohit D. Divekar,[1] J. Jeremiah Bell,[1] Hyun-Hee Lee,[1] Ping Yu,[1] Jason S. Ellis,[1] Christine M. Hoeman,[1] Craig L. Franklin,[2] and Habib Zaghouani[1,3]

[1]Department of Molecular Microbiology and Immunology, [2]Department of Veterinary Pathobiology, and [3]Department of Child Health, University of Missouri, Columbia, MO 65212

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The role of Th17 cells in type I diabetes (TID) remains largely unknown. Glutamic acid decarboxylase (GAD) sequence 206–220 (designated GAD2) represents a late-stage epitope, but GAD2-specific T cell receptor transgenic T cells producing interferon γ (IFNγ) protect against passive TID. Because IFNγ is known to inhibit Th17 cells, effective presentation of GAD2 peptide under noninflammatory conditions may protect against TID at advanced disease stages. To test this premise, GAD2 was genetically incorporated into an immunoglobulin (Ig) molecule to magnify tolerance, and the resulting Ig-GAD2 was tested against TID at different stages of the disease. The findings indicated that Ig-GAD2 could not prevent TID at the preinsulitis phase, but delayed TID at the insulitis stage. More importantly, Ig-GAD2 sustained both clearance of pancreatic cell infiltration and β-cell division and restored normoglycemia when given to hyperglycemic mice at the prediabetic stage. This was dependent on the induction of splenic IFNγ that inhibited interleukin (IL)-17 production. In fact, neutralization of IFNγ led to a significant increase in the frequency of Th17 cells, and the treatment became nonprotective. Thus, IFNγ induced by an adjuvant free antigen, contrary to its usual inflammatory function, restores normoglycemia, most likely by localized bystander suppression of pathogenic IL-17–producing cells.

CORRESPONDENCE
Habib Zaghouani:
zaghouanih@health.missouri.edu

Abbreviations used: GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; IAA, insulin autoantibody; TID, type I diabetes.

Antigen-specific approaches have been defined that could prevent the development of type I diabetes (TID; for review see [1]). However, antigen-driven strategies that could counter the disease at more advanced stages have yet to be defined (1). As with many autoimmune disorders, TID most likely involves multiple autoantigens and diverse T cell specificities (2, 3). In addition, sequential spreading seems to orchestrate TID, with insulin being required for the initiation of the disease (4), whereas GAD-reactive T lymphocytes are more involved at later stages of TID (5, 6). Thus, for an antigen-specific therapy to be effective and practical against TID, it would have to target late-stage epitopes that could counter diverse aggressive T cell specificities. GAD2 peptide corresponding to amino acid sequence 206–220 of GAD is considered a late-stage epitope because its T cell reactivity is detected at an advanced stage of the disease (7). TCR transgenic T cells specific for GAD2 peptide were generated, but these produced both IFNγ and IL-10 and were protective against TID when tested in a transfer model of passive diabetes (8). Given this information, we reasoned that effective presentation of GAD2 peptide in vivo under noninflammatory conditions would possibly induce IFNγ- and IL-10–producing T cells that could protect against TID. Because IFNγ displays inhibitory activity against Th17 cells (9, 10), the approach could prove effective even at an advanced stage of the disease if Th17 cells play a pathogenic role in TID. To test these premises, GAD2 peptide was genetically inserted into the variable region of a heavy chain Ig gene, and the fusion gene was transfected into

R.K. Gregg's present address is Dept. of Basic Sciences, Philadelphia College of Osteopathic Medicine, Suwanne, GA 30024.
J. J. Bell's present address is Dept. of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.
P. Yu's present address is National Cancer Institute Metabolism Branch, Bethesda, MD 20892.
H.-H. Lee's present address is Division of Immunology, Karp Laboratories, Children's Hospital, Harvard Medical School, Boston, MA 02115.

a myeloma B cell line along with the parental light chain gene for expression as a complete Ig-GAD2. Because Igs internalize into APCs via Fcγ receptor (FcγR), the grafted GAD2 peptide will be efficiently dragged into the cells, where it accesses newly synthesized MHC class II molecules, and presentation will be significantly increased relative to free peptide, as was the case for other diabetogenic and encephalitogenic peptides (11–16). Moreover, because Igs are self-proteins, when injected into animals, presentation occurs without inflammation, leading to lack of costimulation and magnification of tolerance (12–14).

In an initial attempt, Ig-GAD2 was tested for prevention of TID before insulitis, but proved ineffective for delay of disease. However, when the treatment was administered at the insulitis stage, protection against TID was observed. More importantly, Ig-GAD2 given to hyperglycemic mice at the prediabetic stage was highly effective, leading to clearance of pancreatic cell infiltration, stimulation of β-cell division, and restoration of normoglycemia. Investigation of the mechanism underlying reversal of disease revealed the presence of splenic IFNγ-producing GAD2-specific T cells that were, indeed, responsible for reversal of disease because neutralization of IFNγ restored progression to overt diabetes. In parallel, the protected mice had reduced production of IL-17 cells in the spleen and pancreas relative to diabetic mice, and exogenous IL-17 reinstated progression to diabetes in the otherwise protected animals. Thus, splenic IFNγ likely interferes with supply of Th17 to the pancreas, leading to clearance of islet infiltration, stimulation of β cell division, and restoration of normoglycemia.

RESULTS

Treatment with Ig-GAD2 restores normoglycemia

The I-A^{g7}–restricted diabetogenic GAD2 peptide was genetically expressed on an Ig molecule, and the resulting Ig-GAD2 was used to test against TID. Similarly, the nondiabetogenic I-A^{g7}–restricted hen egg lysozyme (HEL) 11–25 sequence was also incorporated in an Ig, and the resulting Ig-HEL was used as control (16). The chimeras were then tested for presentation to a GAD2-specific T cell line. As indicated in Fig. 1, Ig-GAD2 was taken up by APCs, processed, and presented to GAD2-specific T cells much more efficiently than free GAD2 peptide (Fig. 1 A, B). The control Ig-HEL was unable to induce similar stimulation of the GAD2-specific T cells. Ig-GAD2 was then assayed for tolerogenic function by testing for prevention of TID in young NOD mice undergoing the initial phase of islet infiltration, which is referred to as the preinsulitis stage. The results in Fig. 1 C indicate that Ig-GAD2 had no significant long-term protective effect against TID relative to Ig-HEL or untreated mice. Knowing that insulin, but not GAD, is required for initiation of diabetes at the preinsulitis stage (4), the lack of protection might have been caused by the absence of activated GAD2-specific target T cells at this stage. We then tested Ig-GAD2 for suppression of diabetes at a later stage during insulitis. It has been shown that seroconversion to insulin autoantibody (IAA) production is indicative of ongoing insulitis (17, 18), and our own studies indicated that among the 83% of female NOD mice that seroconvert to IAA at the age of 8–11 wk, 84% develop overt

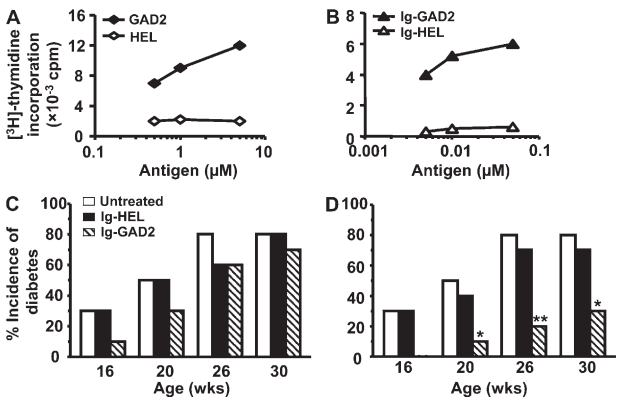

Figure 1. Ig-GAD2 treatment given at the insulitis-positive stage reverses T1D. (A and B) Presentation of Ig-GAD2 to T cells. NOD splenic APCs were incubated with free peptides (A) or Ig chimeras (B), and 1 h later GAD2-specific T cells were added. Activation was assessed by [^{3}H]thymidine incorporation. HEL peptide and Ig-HEL were included as negative controls. (C and D) Percentage of mice free of diabetes upon treatment with Ig-GAD2 or the control Ig-HEL at the preinsulitis (C) and insulitis (D) stage, respectively. All mice were monitored for blood glucose from 12 to 30 wk of age. *, $P < 0.05$; **, $P < 0.01$ compared with untreated mice. A mouse is considered diabetic when blood glucose level is ≥ 300 mg/dl for two consecutive weeks. An untreated group was included in all experiments for comparison purposes. At least 10 mice were included in each experimental group.

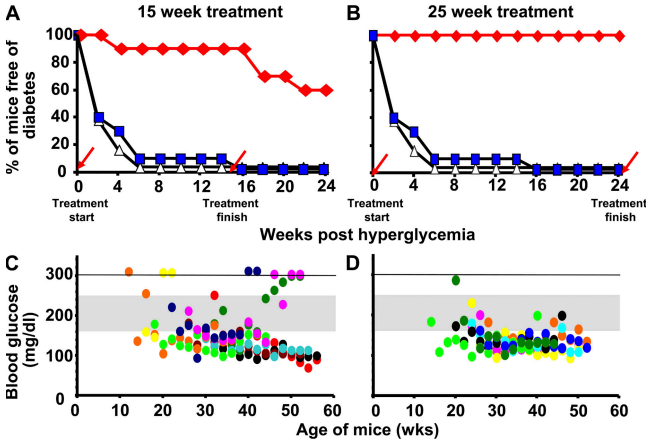

Figure 2. Ig-GAD2 treatment given at the prediabetic stage reverses T1D. (A and B) Percentage of mice free of diabetes upon treatment with Ig-GAD2 or the control Ig-HEL at the hyperglycemic stage for 15 (A) or 25 wk (B). Arrows indicate the beginning and end of treatment. (C and D) Individual blood glucose levels of Ig-GAD2 treated mice are shown from the week of diagnosis of hyperglycemia up to 52 or 56 wk of age for 15 (C) and 25 wk (D) treatment regimens. Each dot represents a different mouse. A mouse is considered hyperglycemic or diabetic when blood glucose level is between 160–250 or ≥ 300 mg/dl for two consecutive weeks, respectively. The shaded area indicates the hyperglycemic range of blood glucose levels and the line depicts the diabetic level. An untreated group was included in all experiments for comparison purposes. At least 10 mice were included in each experimental group.

diabetes (16). Ig-GAD2 was then tested for delay of TID upon IAA-seroconversion. An initial regimen consisting of 300 μg of Ig-GAD2 at week 1, 2, and 3 upon IAA seroconversion indicated that 50% of mice were protected against diabetes up to 30 wk of age (unpublished data). This was promising, as the same regimen did not protect at the preinsulitis stage, and it prompted us to test a prolonged regimen for suppression of diabetes. As indicated in Fig. 1 D, administration of Ig-GAD2 into insulitis-positive (IAA^+) mice delayed T1D, and most of the animals (7 out of 10) remained free of disease by week 30 of age. Ig-HEL–treated animals, like the untreated group, were not significantly protected (Fig. 1 D). These results indicate that Ig-GAD2 protects against T1D at a later, rather than earlier, stage of the disease. We then evaluated Ig-GAD2 at the more advanced hyperglycemic stage. Accordingly, blood glucose levels were monitored beginning at week 12 of age, and mice displaying hyperglycemia between the ages of 14 to 30 wk were subjected to a daily injection of Ig-GAD2 for 5 d, and then a weekly injection for either 15 or 25 wk. The results show that 90% of the mice under the 15-wk Ig-GAD2 regimen were protected against diabetes throughout the 15 wk of treatment (Fig. 2 A). However, only 60% of the mice remained disease-free for the 10 wk after cessation of treatment. Untreated and Ig-HEL recipient mice became diabetic by the fifth week of hyperglycemia. When the regimen was extended to 25 wk, 100% of the Ig-GAD2–treated animals were protected (Fig. 2 B), and normoglycemia was restored in all mice. This status persisted throughout the duration of the study (mice aged 52–56 wk). The weekly blood glucose level of individual mice shows a consistent pattern of return to normoglycemia for 6 out of 10 mice in the 15-wk treatment regimen, and all 10 animals in the 25-wk regimen (Fig. 2, C and D). A detailed description of the day of onset, as well as the level of blood glucose at the beginning and termination of the hyperglycemic treatment regimen, is provided in Table I. These results demonstrate that protection against the disease by Ig-GAD2 occurs at the onset of insulitis, whether this manifests at an early or an older age. Overall, this antigen-specific single-epitope therapy by Ig-GAD2 restores normoglycemia in prediabetic mice, a stage at which GAD2-specific T cells could be targeted.

Treatment with Ig-GAD2 increases the number of healthy pancreatic islets

To determine whether the restoration of normoglycemia by Ig-GAD2 is caused by interference with cell infiltration, the mice were subjected to histopathologic analysis upon

Table I. Blood glucose (BG) level at the onset of hyperglycemia and at termination of treatment regimen[a]

Mouse ID	Age at onset of hyperglycemia (wk)	Blood glucose level before treatment (mg/dl)[b]	Blood glucose level after termination of treatment (mg/dl)[c]
15-wk treatment regimen			
106.1	30	161	133
106.7	30	165	113
119.2	30	180	285[d]
119.8	26	182	112
119.9	28	165	128
190.1	12	250	118
191.2	18	179	99
192.2	26	212	134[d]
192.3	16	160	308[d]
196.1	22	222	150[d]
25-wk treatment schedule			
220.1	26	160	135
221.12	24	174	120
206.2	24	231	121
203.1	24	181	110
232.3	20	173	140
236.6	14	185	116
225.9	26	163	120
237.8	20	250	130
227.7	28	180	137
244.4	16	171	123

[a]The results illustrated in this table were from the mice treated with either the 15- or 25-wk Ig-GAD2 treatment regimen and described in Fig. 2. The hyperglycemia onset represent the week during which the mice showed, for the first time, a blood glucose level of 160–250 mg/dl.
[b]Blood glucose level obtained the second week of hyperglycemia.
[c]Blood glucose level obtained at the termination of treatment regimen.
[d]Mice that became diabetic before or within 7 wk of treatment termination.

completion of the treatment regimen. As indicated in Fig. 3, most of the islets in hyperglycemic and diabetic mice exhibited intrainsulitis (Fig. 3 A, 1, 2, and 3), the majority of islets in treated mice were not inflamed (Fig. 3 A, 4) or had only mild periinsulitis (Fig. 3 A, 5 and 6). Moreover, enumeration of the islets indicated that the treated animals had a significantly greater number of total islets than the hyperglycemic or diabetic mice (Fig. 3 B). The number of insulin-positive islets also increased from 14 per pancreas at the prediabetic (hyperglycemic) stage to 29 per pancreas upon treatment with Ig-GAD2. Also, the 15-wk group had a higher number of islets with periinsulitis (38 vs. 30%) or no insulitis (35 vs. 17%) relative to the hyperglycemic stage (Fig. 3 C). On the other hand, the number of islets with severe and mild intrainsulitis were reduced in the treated versus hyperglycemic mice (8 and 19% vs. 22 and 31%, respectively). Surprisingly, in the 25-wk treatment group, although the total number of islets was reduced to that of the hyperglycemic stage, most of these islets exhibited no insulitis (60%), periinsulitis (28%), or mild intrainsulitis (12%; Fig. 3 C). Overall, the treatment with Ig-GAD2 led to a significant increase in the number of noninflamed (healthy) islets.

Ig-GAD2–treated mice exhibit pancreatic β-cell division

The increase in the number of healthy islets in the treated mice could be caused by regression of cell infiltration and/or formation of new β cells. To address this premise, the treated mice were injected with the proliferation indicator BrdU and killed, and pancreatic sections were double stained with anti-insulin and -BrdU antibodies and analyzed for BrdU incorporation

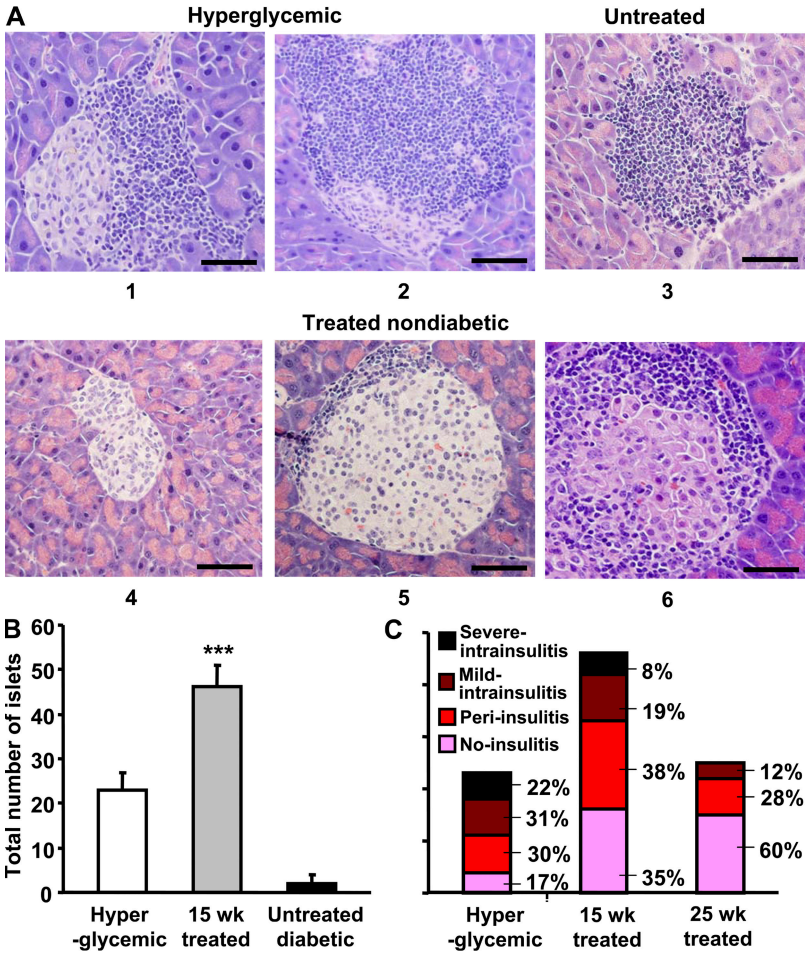

Figure 3. Ig-GAD2 treatment diminishes insulitis and increases the total number of islets. (A) Pancreatic histology. Four sections per pancreas (8 μm thick each cut 100 μm apart) from 5 hyperglycemic (A, 1 and 2), untreated diabetic (A, 3) or 15-wk Ig-GAD2–treated nondiabetic (A, 4, 5, and 6) mice were stained with hematoxylin and eosin and analyzed at 400× magnification. For the hyperglycemic and untreated diabetic mice sections were made the second week of diagnosis. For the treated nondiabetic mice, histology was performed 7 wk after the last treatment. (B) Total islets per pancreas as determined by hematoxylin and eosin staining from the three groups of mice described in A. Only structures with visible islet cells and incomplete infiltration were counted. (C) Islets from hyperglycemic, 15 and 25 wk Ig-GAD2–treated mice were scored as described in Materials and methods, and the percentages represent the number of islets of a given score over the total number of islets from B. The sections were made at the time indicated in A, and 2 d after the last Ig-GAD2 injection for the 25 wk–treated group. ***, P = 0.0001 for the total number of islets in 15 wk–treated versus hyperglycemic group. Error bars indicate the SD. Bars, 25 μm.

and insulin production. BrdU staining was visible in the highly proliferative luminal intestinal cells used as control, but these had no staining with anti-insulin antibody (Fig. 4 A). Islets of nondiabetic 5-wk-old mice were positive for insulin, but did not incorporate BrdU, suggesting that these insulin-producing β cells were not dividing (Fig. 4 B). Thus, under normal circumstances, insulin production emanates from existing β cells whose nuclei do not incorporate BrdU, giving a minimal number of BrdU/insulin double-positive ($BrdU^+/insulin^+$) β cells (Fig. 4 E). The hyperglycemic mice showed very few insulin-producing β cells and no BrdU incorporation (Fig. 4 C), resulting in an insignificant number of $BrdU^+/insulin^+$ cells (Fig. 4 E). In contrast, islets from the 25-wk treatment group showed $insulin^+$ β cells that were either $BrdU^-$ (residual β cells) or $BrdU^+$ (newly formed β cells; Fig. 4 D). Notably, the number of these insulin-producing dividing β cells was significantly increased in all 10 mice in which treatment restored normoglycemia (Fig. 4 E). Interestingly, the total number of dividing cells producing insulin ($BrdU^+/insulin^+$) was low, and it may not solely account for the restoration of normoglycemia. $BrdU^-/insulin^+$ residual islet cells, which amounted to 81 cells per pancreas, may have also contributed to the control of blood glucose levels, and these likely represent a combination of newly formed and residual β cells that were rescued by regression of infiltration. There was a minimal number of dividing β cells ($BrdU^+/insulin^+$) in the normal and hyperglycemic groups, despite the presence of 927 and 50 $BrdU^-/insulin^+$ β cells, respectively. These results indicate that treatment with Ig-GAD2 reduces cell infiltration, leading to rescue of residual and formation of new β cells.

Ig-GAD2–treated mice produce protective IFNγ

Previous studies indicated that Ig-GAD1, which is an Ig chimera carrying GAD524-543, and Ig-INSβ carrying insulin 9–23 aa residues induce T regulatory (T reg) cells and prevent T1D only when given in an aggregated, but not soluble, form (15, 16). This is because aggregated, but not soluble, Ig chimeras cross-link FcγR on APCs, induce IL-10 by the presenting cells, and expand T reg cells (15, 16). In this study, only soluble Ig-GAD2 was used for treatment. Despite the fact that soluble Ig-GAD2 does not induce the production by APCs of the T reg cell growth factor IL-10 (19) and is predicted not to expand T reg cells, it was tested for expansion of T reg cells in hyperglycemic mice before and after treatment with Ig-GAD2. The results indicated that the percentage of $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+FoxP3^+$ T cells in the spleen, as well as in the pancreatic lymph nodes, were similar before and after treatment (Table II). This suggests that T reg cells play a minimal role in disease reversal by soluble Ig-GAD2.

For Ig-GAD2 therapy, it is logical to contemplate that the resolution of the inflammatory infiltration is caused by modulation of GAD2-reactive diabetogenic T cells. Because T1D is likely to involve multiple autoantigens, the restoration of normoglycemia would require modulation of diverse T cell specificities. Thus, the plausible hypothesis postulates that

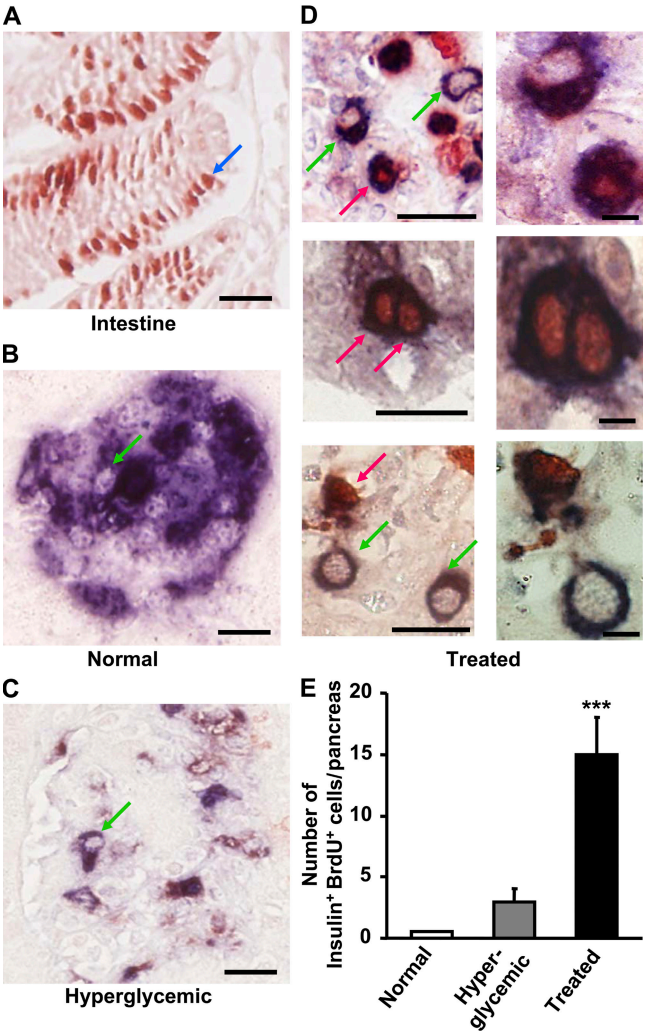

Figure 4. β Cells from mice treated with Ig-GAD2 incorporate BrdU. Mice (10 per group) were given 100 mg/kg BrdU i.p. and killed 3 h later. Sections of the small intestine or pancreas were stained with anti-insulin and -BrdU antibodies, and then analyzed for insulin production (blue cytoplasmic rim) and BrdU incorporation (red nuclei) at 400× magnification. Blue arrows indicate $BrdU^+$ cells, green arrows indicate $insulin^+$ cells, and red arrows indicate $BrdU^+/insulin^+$ cells. Intestinal lumen (A) and β cells (D) from mice recipient of the 25-wk Ig-GAD2 regimen. (B) Beta cells from 5-wk-old nondiabetic NOD mice. (C) β Cells from hyperglycemic mice. (E) Total number of $insulin^+/BrdU^+$ cells in nondiabetic (normal), hyperglycemic, and Ig-GAD2–treated nondiabetic NOD mice. ***, P = 0.0001, treated group compared with hyperglycemic group. Error bars indicate the SD of 10 pancreata. Bars: (A–C) 25 μm; (D, left) 20 μm; (D, right) 5 μm D.

recovery from the disease involved localized bystander suppression. To test this premise, the splenic cells from recovered mice were stimulated with GAD2 peptide and assessed for both suppressive and inflammatory cytokines. The results indicate that although no measurable IL-4 or TGFβ was detected (not depicted), there was significant IFNγ and IL-10 production by these cells relative to the control HEL peptide (Fig. 5 A). Moreover, intracellular cytokine analysis of CD4 and Vβ8.2

Table II. Reversal of T1D by Ig-GAD2 does not significantly increase expression of phenotypic markers associated with T reg cells[a]

	$CD4^+CD25^+FoxP3^+$		$CD4^+CD25^+CD62L^+$	
	Untreated	Treated	Untreated	Treated
SP	4.9	3.3	2.0	1.9
PLN	5.0	5.5	3.0	4.4

[a]Spleen and pancreatic lymph node cells from Ig-GAD2–treated and control untreated mice were stained with anti-CD4, -CD25, and -CD62L or -FoxP3 antibody. The cells were gated on $CD4^+$ cells and analyzed for CD25, FoxP3, and CD62L expression by flow cytometry.

T cells indicated that the majority of the T cells produced only IFNγ, with fewer cells stained positive for both IL-10 and IFNγ (Fig. 5 B). Indeed, upon stimulation with GAD2 peptide, a significant increase (four- to sevenfold) in the number of CD4/Vβ8.2 T cells producing IFNγ was observed in the Ig-GAD2–treated versus untreated mice. Because IL-10 is known for its anti-Th1 suppressive function (20–22), we suspected that protection against the disease involves the function of these IL-10/IFNγ–producing cells. To our surprise, however, when in vivo cytokine neutralization was performed along with Ig-GAD2 treatment, the recovery persisted with anti–IL-10 treatment, but was nullified by removal of IFNγ (Fig. 5 C). Isotype-matched rat IgG had no effect on the disease (Fig. 5 C). These observations indicate that IFNγ, contrary to its well-defined inflammatory function, is likely involved in modulation of inflammation and restoration of normoglycemia.

Ig-GAD2 treatment interferes with IL-17 production in an IFNγ-dependent fashion

Th17 cells represent a newly defined subset of pathogenic T cells whose development can be facilitated by TGFβ and

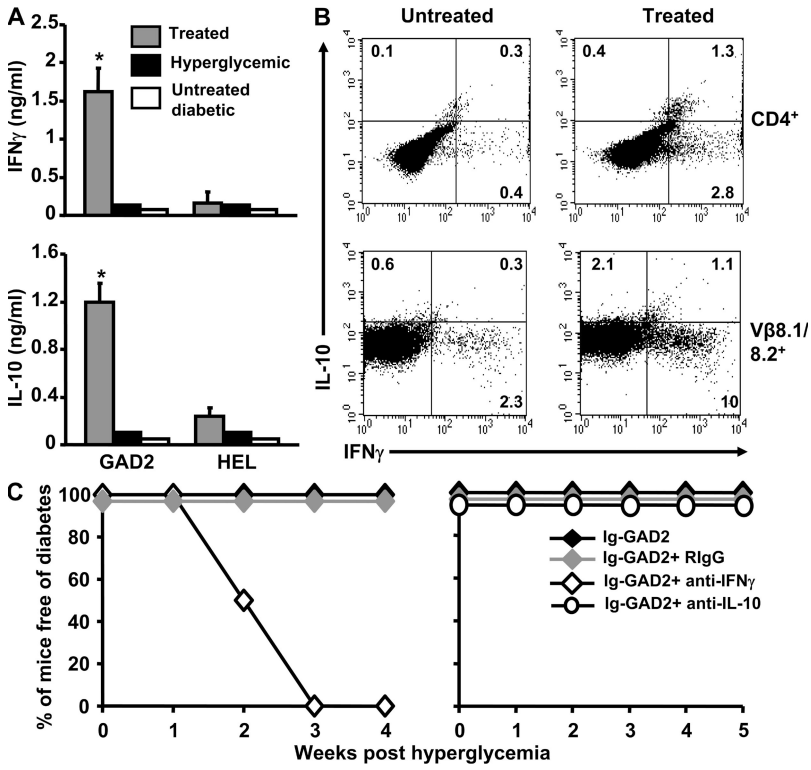

Figure 5. Treatment with Ig-GAD2 induces IFNγ that sustains protection against diabetes. (A) Splenocytes from hyperglycemic mice recipient of the 25-wk Ig-GAD2 treatment regimen were stimulated in vitro with GAD2 and the control HEL peptide and IFNγ and IL-10 were measured by ELISA as described in Methods. Diabetic as well as untreated hyperglycemic mice were included for control purposes. Each bar represents the mean ± SD of three independent experiments. *, P = 0.01 when stimulation by GAD2 is compared with HEL peptide. (B) Intracellular IL-10 and IFNγ production by splenic CD4 (top) or Vβ8.1/8.2 (bottom) T cells from the 25-wk–treated (right) and hyperglycemic untreated (left) mice. This was done by intracellular staining upon stimulation with GAD2 peptide, as indicated in the Materials and methods. Data are representative of three independent experiments. (C) Percentage of mice free of diabetes upon in vivo neutralization of IFNγ (left) or IL-10 (right) during treatment with Ig-GAD2 at the hyperglycemic stage. Anti-IFNγ (R4-6A4), anti-IL-10 (JES5-2A5), or isotype control rat IgG were given to mice (500 μg/mouse per injection) i.p. every 3 d for 4 consecutive weeks, beginning with the first injection of Ig chimeras. The mice received a total of nine antibody injections. At least eight mice were included in each experimental group.

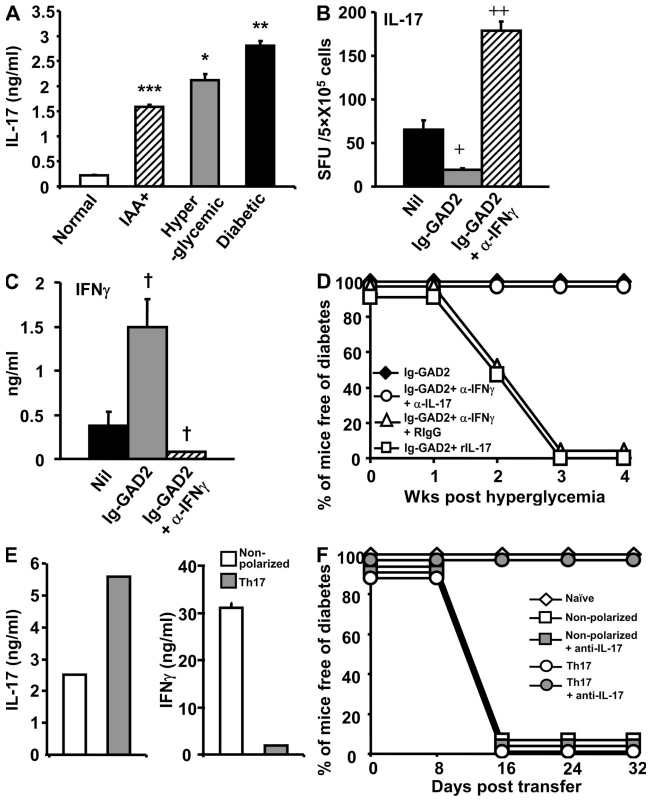

Figure 6. Neutralization of IFNγ during treatment with Ig-GAD2 restores IL-17 production. (A) IL-17 response from the splenocytes of preinsulitis (normal), insulitis-positive (IAA+), hyperglycemic, and diabetic mice upon in vitro stimulation with anti-CD3 antibody. Data are representative of three independent experiments. ***, P = 0.0004, insulitis-positive versus normal; *, P = 0.02, hyperglycemic versus insulitis-positive; **, P = 0.005, diabetic versus hyperglycemic group. IL-17 (B) and IFNγ (C) response from splenocytes of mice recipient of anti-IFNγ during treatment with Ig-GAD2 at the hyperglycemic stage. Splenocytes were harvested when the mice became diabetic on the fourth week of treatment; they were stimulated in vitro with GAD2 peptide, and their responses were measured by ELISPOT and ELISA, respectively. Nil (diabetic) and Ig-GAD2–treated groups were included as controls. Data are representative of three independent experiments. †, P = 0.01, treated versus nil group; ††, P = 0.001, Ig-GAD2 + anti-IFNγ versus Ig-GAD2 group. †, P = 0.04, Ig-GAD2 versus nil group; †, P = 0.02, Ig-GAD2 + anti-IFNγ versus Ig-GAD2 group. (D) Percentage of mice free of diabetes upon administration of recombinant IL-17 or neutralization of both IFNγ and IL-17 during treatment with Ig-GAD2 at the hyperglycemic stage. IL-17 was administered (1 μg/mouse per injection) i.p. daily for 5 consecutive days, beginning with the first injection of Ig-GAD2. Subsequently, the mice received an injection of rIL-17 every week, along with Ig-GAD2. An injection of anti-IFNγ (R4-6A4; 500 μg/mouse) and anti-IL-17 (TC11-18H10; 200 μg/mouse) was given on the first day of treatment with Ig-GAD2 after diagnosis of hyperglycemia. Four additional injections were given at 4-d intervals. At least eight mice were included in each experimental group. (E and F) Th17-polarized cells induce diabetes. (E) IL-17 (left) and IFNγ (right) responses from the nonpolarized and Th17 polarized splenocytes were measured by ELISA. Each bar represents the mean ± SD of triplicate wells. (F) Percentage of mice free of diabetes upon adoptive transfer of 10×10^6 naive, nonpolarized and Th17-polarized cells in NOD.scids (4–6 wk old). Additional groups received IL-17 neutralizing antibody, along with Th17-polarized and nonpolarized cells for control purposes. Anti–IL-17 antibody (TC11-18H10; 200 μg/mouse) was given on the day of transfer, and two additional injections were given at day 4 and 16 after transfer.

IL-6 or interfered with by IFNγ or IL-27 (9, 10, 23–26). Because Ig-GAD2 treatment induces IFNγ, we sought to determine whether restoration of normoglycemia involves interference with IL-17 production. Accordingly, we began by assessing whether IL-17 is produced by NOD T cells, and followed the pattern of its secretion during disease progression. Fig. 6 A shows that stimulation with anti-CD3 antibody did not induce measurable IL-17 by splenocytes from normal 4-wk old mice. However, IL-17 was evident upon IAA-seroconversion and increased significantly when the mice progressed to hyperglycemia and diabetes. The treatment with Ig-GAD2 at the hyperglycemic stage significantly reduced the frequency of GAD2-specific IL-17–producing cells as measured by spot formation (Fig. 6 B). However, neutralization of IFNγ by administration of anti-IFNγ antibody along with Ig-GAD2 restored even higher frequency of Th17 cells. This Th17 restoration is likely caused by complete neutralization of IFNγ because IFNγ-producing Th1 cells could not be detected by ELISPOT (not depicted) and no measurable IFNγ cytokine was found by ELISA (Fig. 6 C). It is thus likely that the restoration of diabetes by neutralization of IFNγ during treatment with Ig-GAD2 (Fig. 5 C) is caused by restoration of Th17. In fact, administration of rIL-17 along with Ig-GAD2 treatment nullifies tolerance and restores diabetes (Fig. 6 D). Moreover, administration of both anti-IFNγ and –IL-17, but not anti-IFNγ and rat IgG, simultaneously protects against T1D (Fig. 6 D), further confirming the interplay between IFNγ and IL-17. To ensure that Th17 cells can be diabetogenic, we chose the BDC2.5 TCR transgenic T cells (27) for polarization with anti-CD3 and -CD28 antibodies and tested for transfer of diabetes into NOD.scid mice. The rationale for this choice instead of Ig-GAD2–induced Th17 cells lies in the fact that the BDC2.5 cells are well characterized and represent a homogeneous population in which the number of cells to be transferred can be precisely controlled. In addition, the Ig-GAD2/NOD model represents a polyclonal system in which the different subsets of T cells cannot be separated. Thus, BDC2.5 T cells were stimulated with anti-CD3 and -CD28 antibodies in the presence or absence of Th17 polarizing factors, and the cells were tested for transfer of diabetes into NOD.scid mice.

As indicated in Fig. 6 E, the Th17-polarized cells had enhanced levels of IL-17 compared with nonpolarized cells, but no measurable IFNγ, whereas nonpolarized cells produced significant IFNγ. These results indicate that the polarization to Th17 was significant under the chosen conditions. Furthermore, when the polarized cells were transferred into NOD.scid mice, diabetes manifested within 16 d after transfer, as with activated, but not polarized, T cells (Fig. 6 F). Diabetes did not occur when the transfer was made with naive BDC2.5 cells. In addition, when IL-17 was neutralized by injection of anti–IL-17

antibody in the mice recipient of Th17-polarized cells, the disease did not manifest. However, neutralization of IL-17 did not protect against diabetes transferred by nonpolarized cells. These results indicate that Th17 cells producing IL-17 can transfer diabetes into naive mice. The results are thus interpreted to indicate that Ig-GAD2 mobilizes IFNγ-producing splenic Th1 cells that interfere with IL-17–producing diabetogenic lymphocytes to reduce inflammation, sustain islet formation, and restore normoglycemia.

Treatment with Ig-GAD2 sustains long-lasting production of IFNγ in the spleen and nullifies IL-17 in the pancreas

At the hyperglycemic stage, most of the pathogenic T cells likely reside in the pancreas as differentiated cells that have already been exposed to antigen (28, 29). Because IFNγ has been suggested to interfere with the differentiation of naive cells into Th17 (9, 10), it is likely that IFNγ Th1 cells operate their interference with Th17 in the spleen or pancreatic lymph nodes rather than the pancreas. Analysis of the dynamics of both populations at the beginning, as well as at the end, of Ig-GAD2 treatment indicated that during the initial phase of hyperglycemia, IFNγ-producing Th1 cells are mostly located in the spleen (Fig. 7 A), whereas Th17 cells reside in the pancreas (Fig. 7 B). However, at the end of the treatment, Th1 cells remain in the spleen (Fig. 7 C), whereas Th17 cells are undetectable in any organ (Fig. 7 D). These results suggest that Ig-GAD2 induces IFNγ in the spleen, which likely interfere with differentiation of naive cells into Th17 cells, resulting in a diminished supply of these cells to the pancreas.

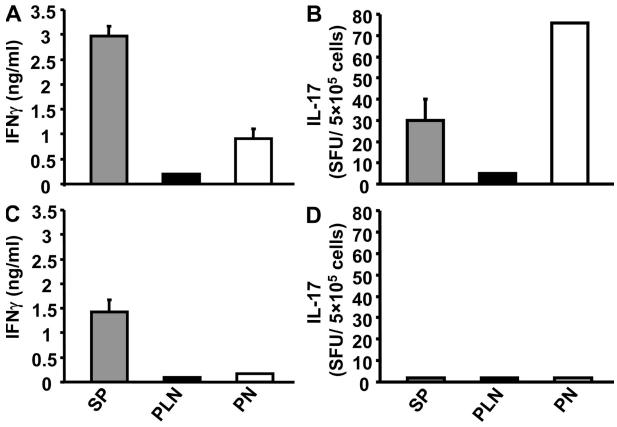

Figure 7. Splenic IFNγ induced by Ig-GAD2 treatment diminishes splenic and pancreatic IL-17 causing reversal of diabetes. IFNγ and IL-17 cytokine responses of splenic, pancreatic, and pancreatic lymph node cells from mice treated with Ig-GAD2 for 1 wk (A and B) or 25 wk (C and D) starting from the week of hyperglycemia diagnosis. The cells were stimulated with GAD2 peptide, and the responses were measured by ELISA for IFNγ and ELISPOT for IL-17, as indicated in Materials and methods. Each bar represents the mean ± SD of two independent experiments.

DISCUSSION

Treatment with anti-CD3 antibody alone has been shown to reverse diabetes, but disease reoccurrence has been observed (30–32). This justifies the search for new strategies, and the combination of anti-CD3 antibody with antigen-specific therapy did overcome rebounding of disease (33). Because T1D involves multiple autoantigens that likely manifest their activities at different stages of the disease, it has been difficult to define antigen-based regimens that could reverse the disease process at an advanced stage (1). Because Igs have proven powerful for enhancing tolerogenic function of peptides (12, 16) and GAD2 peptide was defined as a late-stage protective epitope (5–8), we incorporated GAD2 peptide onto an Ig molecule and tested the resulting Ig-GAD2 for protection, as well as reversal of advanced T1D process. Surprisingly, Ig-GAD2 was not protective at the preinsulitis stage (Fig. 1 C), but delayed the disease when it was administered upon IAA seroconversion (Fig. 1 D) (14). This differential efficacy may be related to delayed spreading of GAD2-specific T cells that become available for targeting at an advanced stage of the disease (7). This has prompted us to test for reversal of T1D at the prediabetic stage, when blood glucose has reached an abnormal level. Again, Ig-GAD2 was able to restore long-lasting normoglycemia in most of the animals (Fig. 2, A and C), and when the regimen was extended to 25 wk (Fig. 2, B and D) all mice were protected. To date, we have tested >30 mice with the 25-wk regimen and all animals maintained normoglycemia until they were terminated at 52 or 56 wk of age for other use. At the histology level, the 15-wk regimen reduced infiltration, and this was accompanied by an increase in the total number of islets relative to the beginning of the treatment (Fig. 3 B). However, with the 25-wk regimen, the number of islets dropped back to where it was at the hyperglycemic stage, but the majority of these were healthy islets. We believe that both eradication of infiltration from slightly infiltrated islets as well as formation of new islets were part of the repair process. The initial increase and the return of the number of islets may reflect dynamics in which new islets were formed while severely infiltrated islets were eliminated and those mildly infiltrated were cleaned up. This is drawn from Fig. 3 C, which illustrates the evolution of status and number of islets during the treatment and is supported by the BrdU incorporation observed with insulin-producing β cells (Fig. 4). There was, in fact, a substantial increase of dividing cells that were simultaneously producing insulin, in addition to cells that were producing insulin without evidence of division (Fig. 4). The formation of new islets has been reported before, but whether these are the product of stem cells maturation, origination from splenic cells, or division of residual β cells remains unknown (34–38). It has been shown that physical damage of islets stimulates β cell division (38). In Ig-GAD2–treated mice, upon clearance of inflammatory cells the damaged islets may likewise spontaneously initiate division of β cells. However, given that exogenous compounds such as CFA (35–37) and now Ig-GAD2 allow for β cell division, it may be that clearance of inflammatory cells minimizes cell division inhibitory factors, leading to proliferation of residual β cells or differentiation of β cell progenitors. The precise mechanism underlying β cell proliferation is of great interest, and

effort is being made to determine how Ig-GAD2 leads to β cell division. What is important here is that a single-epitope treatment could lead to eradication of infiltration involving diverse T cell specificities. One has to assume that there must be at least a local bystander suppression that targets GAD2-specific T cells and unrelated neighboring cells. When the mice treated with Ig-GAD2 were tested for cytokine production, we expected to see suppressive or Th2-associated cytokines, which usually drive bystander suppression. The results, however, showed that there was IL-10 production, but that this was accompanied by IFNγ (Fig. 5). The other surprise was that neutralization of IFNγ, but not IL-10, nullifies the therapeutic action of Ig-GAD2 and restores diabetes (Fig. 5). These findings provide support to prior observations showing that TCR transgenic IFNγ-producing GAD2-specific T cells prevent the onset of diabetes in an animal model of disease transfer (8). The question then is how can a well-defined inflammatory cytokine such as IFNγ mediate suppression of diabetes, which likely involves diverse T cell specificities? Given the recent observations indicating that IFNγ could interfere with differentiation of naive cells into Th17 (9, 10), and that IL-17, which is the product of Th17, displays pathogenic functions (39), we sought to test whether progression to diabetes involves the activity of Th17 and if so whether treatment with Ig-GAD2 affects these pathogenic T cells. Indeed, an increase of IL-17 was observed in NOD mice as they progressed toward diabetes (Fig. 6 A), and treatment with Ig-GAD2 reduced the frequency of IL-17–producing Th17 cells (Fig. 6 B). However, neutralization of IFNγ by anti-IFNγ antibody restored IL-17 production (Fig. 6 B). In support of this Ig-GAD2–induced IFNγ/IL-17 interplay is the observation that administration of rIL-17 with Ig-GAD2 nullified the therapeutic effect of Ig-GAD2. Also, neutralization of both IFNγ and IL-17 support protection, further justifying the IFNγ/IL-17 interplay. Moreover, polarized BDC2.5 Th17 cells transferred diabetes to NOD.scid mice, and neutralization of IL-17 inhibited such disease transfer (Fig. 6 F). Finally, IFNγ is mostly produced in the spleen, which provides a noninflammatory environment (Fig. 7, A and B) and likely acts to inhibit differentiation of naive cells into Th17 in this organ, leading to a diminished supply of pathogenic Th17 cells into the pancreas. In fact, upon treatment with Ig-GAD2, Th17 cells become undetectable in the spleen or pancreas, whereas IFNγ remained significant in the spleen to sustain a long-lasting inhibition of Th17 differentiation (Fig. 7, C and D). It is known that IFNγ signaling through IFNγ receptor (IFNγR), in conjunction with other inflammatory cytokines, interferes with β cell growth and induces apoptosis (40, 41). In the Ig-GAD2 treatment, the fact that IFNγ is produced in the spleen may play dual beneficial roles. It inhibits differentiation of pathogenic Th17 cells, allowing for clearance of infiltration and termination of islet inflammation and by being away from the islets its interference with β cell growth and death is prevented, hence proliferation of β cells. This also provides support to the dual pathogenic/protective role IFNγ plays in diabetes, which likely depends on the site of production and T cell differentiation (42). In fact, this goes well with the observation that neutralization of IL-17 did not protect against diabetes transferred by IFNγ-producing Th1 BDC2.5 cells, as these lymphocytes could home to the pancreas, where their IFNγ drives apoptosis of β cells (Fig. 6, E and F).

Overall, we suggest that adjuvant-free Ig-GAD2 induced the production of IFNγ in a noninflamed lymphoid organ, leading to inhibition of differentiation of naive cells into Th17 cells, culminating in diminished infiltration, formation of β cells and reversal of the diabetic process. The presence of IFNγ would inhibit differentiation of neighboring naive cells, thus suppressing diverse T cell specificities. For effective bystander suppression to occur, it may be that Th1 cells migrate to the PLN and inhibit differentiation of diverse T cells into Th17 cells. However, because Th1 cell were not detected in this organ, the likely alternative is that APCs loaded with β-cell antigens circulate from the pancreas to the spleen and subject diverse T cells to inhibition of differentiation by local IFNγ-producing GAD2-specific Th1 cells. Administration of exogenous IFNγ may protect against diabetes if targeted to the site of T cell differentiation during antigen stimulation, but away from the islets. It is also important to mention that the regimen is effective at late stages, but not before insulitis, possibly because availability of GAD2-specific T cells and production of IFNγ are delayed. In fact, Ig-INSβ was able to delay the disease when given at the preinsulitis stage (16), but was unable to counter the disease once the mice had progressed to the hyperglycemic stage (not depicted). Again, this supports the dynamics of different epitopes during disease initiation and progression.

Collectively, the findings suggest that this antigen-specific immunomodulation targets diverse pathogenic T cells to halt inflammation and drive an islet repair process that restores long-lasting normoglycemia.

MATERIALS AND METHODS

Mice

NOD (H-2^{g7}), NOD.BDC2.5, and NOD.scid mice were used according to the guidelines of the University of Missouri Columbia Animal Care and Use Committee.

Peptides

All peptides used in this study were purchased from Metabion and purified by HPLC to >90% purity. Glutamic acid decarboxylase 2 (GAD2) peptide corresponds to aa residues 206–220 (TYEIAPVFVLLEYVT) of GAD-65 (7). Hen egg lysozyme (HEL) peptide encompasses a nondiabetogenic epitope corresponding to aa residues 11–25 (AMKRHGLDNYRGYSL) of HEL (43). GAD2 and HEL peptides are presented to T cells in association with I-A^{g7} MHC class II molecules.

Ig chimeras

Ig-GAD2 and Ig-HEL express GAD2 and HEL peptide, respectively. This was accomplished by inserting the corresponding nucleotide sequence in place of the diversity segment within the complementarity determining region 3 (CDR3) of the heavy chain variable region of the 91A3 IgG2b, κ Ig (13–16). The fusion heavy chain gene was then transfected along with the parental κ light chain gene for expression as a complete self-Ig molecule, as previously described (11, 13–16). Large-scale cultures of transfectoma cells were performed in DME media containing 10% iron-enriched calf

serum (BioWhittaker). Purification of the chimeras used separate columns of rat anti–mouse κ chain mAb coupled to CNBr-activated 4B Sepharose (GE Healthcare).

Islet cell purification

This was done according to a standard islet purification procedure (44). In brief, the pancreata were digested with collagenase type IV (Invitrogen), and islets were separated on a ficoll gradient (GE Healthcare).

T cell line and proliferation assay

A T cell clone specific for GAD2 peptide was generated in NOD mice as previously described (15). For presentation of Ig-GAD2, irradiated (3,000 rads) NOD female splenocytes (5×10^5 cells/50 μl/well) were incubated with graded amounts of either free peptide or Ig chimeras (100 μl/well), and 1 h later the GAD2-specific T cells (5×10^4 cells/well/50 μl) were added. Proliferation was measured by [^{3}H]thymidine incorporation assay.

Assessment of insulin autoantibody (IAA) seroconversion, hyperglycemia, and diabetes

Serum IAA was detected by ELISA using porcine insulin as antigen, as previously described (16). Assessment of blood glucose levels used test strips and an Accu-Chek Advantage monitoring system. A mouse is considered hyperglycemic or diabetic when the blood glucose level is 160–250 mg/dl or 300 mg/dl, respectively, for 2 consecutive weeks.

Ig-GAD2 treatment regimens

Treatment at the preinsulitis stage. Mice are given an i.p. injection of 300 μg Ig-GAD2 or Ig-HEL in 300 μl PBS at 4, 5, and 6 wk of age, a stage at which islet infiltration has begun and that is referred to as preinsulitis. The mice were monitored for blood glucose level up to 30 wk of age.

Treatment at the insulitis (IAA$^+$) stage. Mice are tested for IAA, and those who seroconvert between the ages of 8–11 wk are given a weekly i.p. injection of 300 μg of Ig-GAD2 or Ig-HEL in 300 μl PBS up to week 12. Subsequently, the mice received another 300 μg of Ig-chimera every 2 wk until the age of 24 wk. These mice were monitored for blood glucose level beginning at week 12 until 30 wk of age.

Treatment at the hyperglycemic stage. Mice began blood glucose level monitoring at 12 wk of age, and those who displayed a level of 160–250 mg/dl for 2 consecutive weeks between the ages of 14–30 wk were considered hyperglycemic. These mice were then subjected to a daily i.p. injection of 500 μg Ig-GAD2 or Ig-HEL for 5 d. Subsequently, the mice received another 500 μg of Ig-chimera every week for 15 or 25 consecutive weeks, and blood glucose levels were continuously monitored until 56 wk. These treatments are referred to as 15- and 25 wk-treatment regimen, respectively.

Histology

Pancreata were harvested from NOD females, fixed in 10% formalin, and embedded in paraffin. Sections of 8-μm thickness were cut 100 μm apart to prevent double counting the same islet. Four sections per pancreas were stained with hematoxylin and eosin and analyzed by light microscopy. Insulitis scoring was performed according to the following criteria: severe insulitis, 50% or higher of the islet area is infiltrated; mild insulitis, <50% of the islet area is infiltrated; periinsulitis, infiltration is restricted to the periphery of islets; and no insulitis, absence of cell infiltration.

Immunohistochemistry

Evaluation of cell division by insulin-producing β cells was done as follows: Ig chimera–treated mice were injected i.p. with 100 mg/kg of BrdU in PBS (Sigma-Aldrich), 3 h before euthanasia. Pancreata and intestine were harvested and fixed, and sections were prepared as described in the previous section. For assessment of insulin production, the sections were stained with primary guinea pig anti-insulin antibodies, incubated with biotinylated goat anti–guinea pig antibodies, and visualized by saturation with Streptavidin-alkaline phosphatase using the chromagen, 5-Bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium. For detection of BrdU incorporation, the sections were counter stained with biotinylated anti-BrdU antibody (Zymed), treated with Streptavidin-horseradish peroxidase, and visualized with the chromagen 3-amino-9-ethylcarbazole.

Cytokine assays

Splenocytes (5×10^5 cells/well) were incubated with 30 μg/ml of free peptide or 5 μg/ml anti-CD3 antibody for 48 h, and cytokines in the supernatant were measured by ELISA and ELISPOT, as previously described (45).

Flow cytometry

For staining of CD4, CD25, and CD62L, cells were harvested from spleens and pancreatic lymph nodes and incubated with anti-CD4-PE, biotin-conjugated anti-CD25 (or isotype control biotin-conjugated rIgM), and anti-CD62L-FITC (or isotype control rIgG2a-FITC) for 30 min at 4°C. Subsequently, the cells were washed and stained with PerCP-conjugated streptavidin for 30 min at 4°C. The cells were washed, fixed with 4% formaldehyde for 20 min at room temperature, and then analyzed. All antibodies were purchased from BD PharMingen.

For intracellular Foxp3 staining, cells from spleens and pancreatic lymph nodes were first stained with anti-CD4-PE and biotin-conjugated anti-CD25 antibodies. This was followed by PerCP-conjugated streptavidin staining. The cells were fixed with Fix/Perm buffer (eBioscience), washed with permeabilization buffer (eBioscience), and stained with anti-Foxp3-FITC antibody (clone FJK-16s; eBioscience), or isotype control rIgG2a-FITC.

For intracellular cytokine analysis of IL-10 and IFNγ, the splenic cells (2×10^6 cell/ml) were stimulated with free peptide (30 μg/ml) for 6 h followed by 10 h incubation with brefeldin A (10 μg/ml) to block cytokine secretion and facilitate intracellular accumulation. The antibodies used were PerCP-anti-CD4 (RM4-5), biotin-anti-Vβ8.1/8.2, PE-anti-IFNγ (XMG1.2), and FITC-anti-IL-10 (JESS-16E3; all from BD Biosciences). Isotype-matched controls were included in all experiments. Events were collected on a FACScan flow cytometer and analyzed with CellQuest software (Becton Dickinson).

T cell polarization

Naive splenocytes were isolated from 4-wk-old NOD.BDC2.5 transgenic mice and activated with soluble anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) antibodies for 3 d in 10% FCS-DME media under Th17 polarizing (TGFβ [3 ng/ml], IL-6 [20 ng/ml], anti-IFNγ antibody [10 μg/ml], and anti–IL-4 antibody [10 μg/ml]) and nonpolarizing conditions. Supernatant from activated cells was tested for IFNγ and IL-17 by ELISA, and the cells were used for adoptive transfers.

Adoptive transfer experiments

For disease transfer by Th17, 10×10^6 naive, nonpolarized and Th17-polarized cells were injected i.v into NOD.scid (4–6-wk-old) mice. Additional groups of mice received IL-17–neutralizing antibody along with the T cell transfer to serve as controls. Anti-IL-17 antibody (TC11-18H10; 200 μg/mouse) was given on the day of transfer, and 2 additional injections were given at day 4 and 16 after transfer.

Statistical analysis

The χ^2 test was used for incidence of diabetes analysis among experimental and control groups. For the rest of the experiments, P values were calculated with the two-tailed Student's unpaired *t* test.

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