Research article

NOD mice with knockout of both native insulin genes and a mutated proinsulin transgene, alanine at position B16 in preproinsulin (B16:A-dKO mice), do not develop diabetes. Transplantation of NOD islets, but not bone marrow, expressing native insulin sequences (tyrosine at position B16) into B16:A-dKO mice rapidly restored development of insulin autoantibodies (IAAs) and insulitis, despite the recipients’ pancreatic islets lacking native insulin sequences. Splenocytes from B16:A-dKO mice that received native insulin–positive islets induced diabetes when transferred into wild-type NOD/SCID or B16:A-dKO NOD/SCID mice. Splenocytes from mice immunized with native insulin B chain amino acids 9–23 (insulin B9–23) peptide in CFA induced rapid diabetes upon transfer only in recipients expressing the native insulin B9–23 sequence in their pancreata. Additionally, CD4+ T cells from B16:A-dKO mice immunized with native insulin B9–23 peptide promoted IAAs in NOD/SCID mice. These results indicate that the provision of native insulin B9–23 sequences is sufficient to prime anti-insulin autoimmunity and that subsequent transfer of diabetes following peptide immunization requires native insulin B9–23 expression in islets. Our findings demonstrate dependence on B16 alanine versus tyrosine of insulin B9–23 for both the initial priming and the effector phase of NOD anti-islet autoimmunity.

Introduction

In patients who develop organ-specific autoimmunity, an important question is why only certain organs are targeted (1–3). We believe that there may be single or multiple primary autoantigens that are specific to a target organ and trigger the autoimmune response, although there are examples of autoimmunity directed against autoantigens expressed in multiple tissues for some organ-specific autoimmune diseases. In type 1A diabetes, which is a pancreatic β-cell–specific autoimmune disease, insulin and the islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP) have been identified as β-cell–specific autoantigens (4–6).

For type 1A (immune-mediated) diabetes, insulin has been proposed as a key autoantigen (7, 8). Insulin has been shown to be a target of both T and B lymphocytes with the demonstration of both insulin autoantibodies (IAAs) and insulin-reactive T cells in patients with type 1A diabetes, in prediabetic subjects, and in animal models such as the NOD mouse (9–13). IAAs are frequently detected in sera of patients and NOD mice before and after diabetes onset (14, 15).

Kent et al. recently reported that CD4+ T cell clones isolated from pancreatic lymph nodes of patients with type 1 diabetes react with an insulin A chain peptide (amino acids 1–15) restricted by DR4 (16). In NOD mice, both CD4+ and CD8+ T cells derived from pancreatic lymph nodes and pancreatic islets show insulin reactivity (17, 18), and insulin-reactive T cell clones established from pancreatic islets show cytotoxicity to pancreatic β cells (19, 20). The observations that IAA levels are highest in the youngest children developing diabetes and usually precede the development of other autoantibodies (21, 22) and that insulin-reactive T cells are preferentially detected in younger NOD mice (23) have led to the hypothesis that insulin may be a crucial autoantigen in initiating islet autoimmunity. Supporting this hypothesis are the separate findings of Jaeckel et al. and French et al. that targeting T cells reacting with insulin results in dramatic prevention of type 1 diabetes in NOD mice (24, 25).

Among the insulin epitopes recognized by NOD islet-infiltrating T cells, insulin B chain amino acids 9–23 (insulin B9–23) is reported to be a key peptide (26). Anti–insulin B9–23 CD4+ TCR transgenes can induce (BDC12-4.1) (27) or prevent diabetes (2H6) (28). We recently reported that dual Ins1 and Ins2 knockout NOD mice that express a mutated insulin transgene—the wild-type tyrosine at amino acid 16 of the insulin B chain replaced with alanine—are protected from anti-islet autoimmunity and prevent diabetes (29). These mice lack both endogenous insulin genes and were rescued from an absolute insulin deficiency by a transgene expressing the alanine-to-tyrosine mutation in Ins2 under control of the Rat insulin promoter. Mice carrying the transgene express an altered form of Ins2 (alanine at position 16 of insulin B chain; B16:A). These results suggest that insulin, and specifically the insulin B9–23 sequence, may be essential for the initiation of the spontaneous diabetogenic autoimmune process of NOD mice.

Given the prevention of diabetes in NOD mice lacking native insulin B9–23 sequences, one obvious question arises: In which tissues would expression of the native insulin sequence restore anti-insulin autoimmunity? Besides the target pancreatic β cells, preproinsulin is reported to be expressed in thymic epithelial, thymic dendritic, and a subset of peripheral dendritic cells, potential sites at which preproinsulin expression may modulate insulin autoimmunity (30–32). With the creation of multiple NOD strains lacking native insulin genes, including strains with the NOD/SCID mutation, it is now possible to transplant islets

Nonstandard abbreviations used: B16:A, alanine at position 16 of insulin B chain; B16:A-dKO, double Ins1 and Ins2 knockout NOD mice with a mutated B16:A preproinsulin transgene; B16:Y, tyrosine at position 16 of insulin B chain; B16:Y-dKO, double Ins1 and Ins2 knockout NOD mice with a mutated B16:Y preproinsulin transgene; IAA, insulin autoantibody; insulin B9–23, insulin B chain amino acids 9–23.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 117:1835–1843 (2007). doi:10.1172/JCI31368.
Bone marrow transplant with native B16:Y insulin genes is not sufficient to restore insulin autoimmunity. Four-week-old irradiated B16:A-dKO (A) or wild-type B16:Y mice (B) were transplanted (Tx) with bone marrow derived from 4-week-old wild-type NOD mice. Native insulin-positive B16:Y bone marrow cells did not induce IAAs in B16:A-dKO mice but did induce IAAs in B16:Y mice. Each line represents an individual recipient. The y axis represents the micro-IAA assay (mIAA) index in log scale.

Figure 1
Bone marrow transplant with native B16:Y insulin genes is not sufficient to restore insulin autoimmunity. Four-week-old irradiated B16:A-dKO (A) or wild-type B16:Y mice (B) were transplanted (Tx) with bone marrow derived from 4-week-old wild-type NOD mice. Native insulin-positive B16:Y bone marrow cells did not induce IAAs in B16:A-dKO mice but did induce IAAs in B16:Y mice. Each line represents an individual recipient. The y axis represents the micro-IAA assay (mIAA) index in log scale.

Figure 2
Development of IAAs after B16:Y NOD/SCID islet transplant. (A) B16:A-dKO mice, when transplanted with native B16:Y insulin NOD/SCID islets, developed IAAs. (B) Mice receiving B16:A-dKO islets did not express insulin antibodies after transplant, with the exception of 1 mouse. Each line represents an individual mouse. The y axis represents the micro-IAA assay index in log scale.

Results
Transplantation of bone marrow with native insulin genes does not restore insulin autoimmunity. Preproinsulin is reported to be expressed in thymic epithelial, thymic dendritic, and a subset of peripheral dendritic cells (30–32). In order to determine whether native insulin gene expression in hematopoietic cells is sufficient to induce anti-islet autoimmunity, 1 × 10⁷ bone marrow cells derived from 4-week-old wild-type B16:Y NOD mice were transplanted into irradiated 4-week-old double Ins1 and Ins2 knockout mice with a mutated B16:A preproinsulin transgene (B16:A-dKO mice). None of the 5 B16:A-dKO recipients developed IAAs (Figure 1A). In contrast, 6 of 7 B16:Y NOD recipients of wild-type B16:Y bone marrow expressed anti-IAAs (Figure 1B). Thus, bone marrow cells carrying the native B16:Y insulin sequence do not restore anti-insulin autoimmunity in B16:A-dKO mice.

Transplantation of native B16:Y insulin–bearing islets induces development of IAAs. Using islet transplantation, we sought to determine whether native insulin expression in transplanted islets is sufficient to induce anti-islet autoimmunity in B16:A-dKO mice. Two hundred islets from native B16:Y insulin–bearing NOD/SCID mice were transplanted under the kidney capsule of 4-week-old B16:A-dKO mice. High IAA levels developed within 3 weeks of transplantation in 11 of 13 B16:Y islet recipients (Figure 2A). In contrast, 1 of 8 mice transplanted with B16:A-dKO islets developed IAAs after transplant, and only at a low titer (P < 0.0001; Figure 2B). Therefore, islet expression of insulin with the native insulin B9–23 sequence, even with ectopic expression under the kidney capsule, is sufficient to rapidly induce IAAs in NOD mice lacking native insulin B9–23 sequences.

Induction of insulitis by B16:Y islet transplant. The B16:Y islet grafts (Figure 3, A and C), but not the B16:A-dKO islet grafts (Figure 3, B and D), were completely destroyed by lymphocytic infiltration. This suggests that wild-type insulin, with the native B16:Y insulin B9–23 sequence, in transplanted islet grafts is recognized by lymphocytes of B16:A-dKO mice and that these lymphocytes are able to kill the native insulin–positive islet cells. In addition to analyzing the histology of the transplanted islet grafts, we also analyzed insulitis of the endogenous pancreatic islets of the B16:A-dKO recipient mice. All 5 recipients of B16:A islets that were sacrificed fewer than 20 weeks after transplant showed insulitis (Figure 3E) but not recipients of B16:A islets (Figure 3F). Pancreatic insulitis, however, was transient: 5 of 10 mice receiving the same B16:Y islet transplants that were sacrificed more than 35 weeks after islet transplant had insulinat. Insulitis at less than 20 weeks after transplant was significantly more severe than at more than 30 weeks after transplant (P = 0.02, χ² test; Figure 3G). Pancreatic insulitis of B16:A-dKO mice receiving B16:Y islets was more severe than that of mice receiving B16:A-dKO islet grafts (P < 0.03; χ² test; Figure 3G). Importantly, insulitis of mice receiving B16:A-dKO islets did not differ from unmanipulated B16:A-dKO mice, which did not receive islet transplants. These results indicate that transient insulitis of the B16:A pancreas induced by islet transplants is specific to the native B16:Y insulin sequence of donor islets.

While transplantation of B16:Y islets into B16:A-dKO mice induced not only graft destruction but also lymphocytic infiltration of the recipients’ pancreatic islets, diabetes was not induced. None of the B16:A-dKO mice that received B16:Y islet transplants became diabetic (0 of 11, followed up to 35 weeks). Even though insulitis was induced in the host pancreas, it was
B16:Y islets to B16:A-dKO recipients

Figure 3
Native B16:Y islet transplants induce graft insulitis and severe transient insulitis in endogenous pancreatic islets. (A–D) B16:Y NOD/SCID (A and C) and B16:A-dKO (B and D) islets were transplanted under the kidney capsule of B16:A-dKO mice. (A and B) H&E stain. (C and D) Insulin stain. Two weeks after transplant, the B16:Y NOD/SCID islet graft showed very little insulin staining and severe lymphocytic infiltration, whereas the B16:A-dKO islet graft was intact. (E and F) Endogenous pancreatic islets of B16:Y islet recipients (E), but not B16:A-dKO islet recipients (F), showed marked lymphocytic infiltration 18 weeks after islet transplant. Original magnification, ×100 (A, B, E, and F), ×200 (C and D). (G) More than 10 pancreatic islets from each B16:A-dKO mouse receiving B16:Y NOD/SCID or B16:A-dKO islet transplant were evaluated for lymphocytic infiltration less than 20 weeks or more than 30 weeks after transplant (n = 5–10). Pancreatic islets from an age-matched unmanipulated B16:A-dKO mouse were also evaluated. The y axis represents the mean ± SD of the insulitis score.

These results indicate that the diabetogenic splenocytes are induced by B16:Y islet transplants, which can destroy β cells in NOD/SCID mice with either B16:Y or B16:A pancreatic islets. Yet, B16:A islet transplants are unable to generate diabetogenic lymphocytes, for diabetes did not develop whether the recipient NOD/SCID mouse had insulin with B16:A or B16:Y. This is consistent with the importance of the native insulin B:9–23 sequence in activating diabetogenic anti-islet autoimmunity.

Immunization with insulin B:9–23 peptides induces IAs but not insulitis. To determine whether the provision of just the insulin B:9–23 peptide rather than islets with B16:A versus B16:Y insulin is sufficient to restore anti-islet autoimmunity and diabetogenicity, we immunized 4-week-old B16:A-dKO mice with 100 μg of B16:Y insulin B:9–23 peptide or B16:A insulin B:9–23 peptide in CFA (Figure 4B). More than 95% (29 of 30) of B16:A-dKO mice immunized with B16:Y insulin B:9–23 peptide developed anti-IAs (data not shown). These antibodies were not absorbed by control tetanus toxin peptide or B:9–23 peptides, but were absorbed by insulin (Figure 6A). The absorbing insulin contained the B16:Y insulin B:9–23 sequence; therefore, insulin is an autoantigen corresponding to this peptide. All B16:A-dKO mice (15 of 15) immunized with the B16:A insulin B:9–23 peptide did not rapidly produce IAs that were not absorbed by the peptides, but were absorbed by insulin (Figure 6B). As expected, B16:Y NOD mice immunized with B16:Y insulin B:9–23 peptide also produced IAs (Figure 6C).

Despite the induction of IAs, immunization with both insulin B:9–23 peptides did not induce lymphocytic infiltration of pancreatic islets analyzed 10–15 weeks after immunization (Figure 6E). This suggests that provision of the insulin B:9–23 peptide itself is insufficient to induce inflammation of endogenous pancreatic islets lacking the B16:Y insulin B:9–23 sequence. Correlating with the lack of insulitis, none of the mice progressed to diabetes.

Immunization with B16:Y insulin B:9–23 peptide–induced diabetogenic splenocytes induces diabetes in NOD/SCID recipients. As outlined in Figure 4B, immunization with insulin B:9–23 peptides did not induce insulitis in B16:A-dKO mice. Nevertheless, when we transferred splenocytes from B16:A-dKO mice immunized with B16:Y peptide, recipient B16:Y NOD/SCID mice, but not B16:A-dKO NOD/SCID mice, developed IAs and diabetes. As shown in Figure 6D and 7A, splenocytes from B16:A-dKO mice immunized with B16:Y insulin B:9–23 peptide induced high levels of IAs within 10–20 days of transfer to B16:Y NOD/SCID mice. Splenocytes from B16:A-dKO mice immunized with B16:A insulin B:9–23 peptide did not rap-

not sufficient to cause diabetes in mice whose pancreatic islets lacked the native insulin B:9–23 sequence.

Splenocytes from B16:A-dKO recipients of native B16:Y insulin–bearing islet transplants are diabetogenic. In order to evaluate whether lymphocytes from B16:A-dKO mice with B16:Y NOD/SCID islet transplants can cause diabetes if the host pancreas has the native B16:Y insulin sequence, we transferred splenocytes from B16:A-dKO mice that had received islet transplants into wild-type B16:Y NOD/SCID or B16:A-dKO NOD/SCID mice (Figure 4A). Splenocytes from B16:A-dKO mice transplanted with B16:Y NOD/SCID islets rapidly induced diabetes in B16:Y NOD/SCID mice (5 of 6; Figure 5A), which indicates that immunization with B16:Y islets generates splenocytes able to recognize and destroy B16:Y pancreatic islets (i.e., induces autoimmunity) and that B16:A-dKO recipients of B16:Y islets contain functional native insulin–autoreactive lymphocytes in the absence of disease. In contrast, when splenocytes were transferred from B16:A-dKO mice that received B16:A-dKO islets, diabetes occurred in 2 of 8 B16:Y NOD/SCID recipients, at a later time after splenocyte transfer (P < 0.01; Figure 5A). This is potentially consistent with the natural late (i.e., independent of islet transplant) priming that occurred after transfer of splenocytes into a B16:Y NOD/SCID host from unmanipulated B16:A-dKO mice (Figure 5A). In the opposing model, in which the splenocyte recipients were B16:A-dKO NOD/SCID mice, diabetes occurred in 2 of 5 mice receiving splenocytes from B16:Y islet recipients (Figure 5B). In contrast, splenocytes from B16:A-dKO mice transplanted with B16:A-dKO islets did not induce diabetes or insulitis upon transfer to B16:A-dKO NOD/SCID mice (Figure 5B).
idly induce IAAs in B16:Y NOD/SCID (Figure 7C) or B16:A-dKO NOD/SCID recipients (Figure 7D). Even splenocytes from mice immunized with B16:Y insulin B:9–23 peptide did not rapidly induce IAAs in B16:A-dKO NOD/SCID mice (Figure 7B).

As shown in Figure 8, only the combination of immunization with B16:Y insulin B:9–23 peptide in B16:A-dKO mice followed by splenocyte transfer to B16:Y NOD/SCID mice caused diabetes in the recipient. In contrast, these same splenocytes did not induce diabetes in the B16:A-dKO NOD/SCID recipients, which suggests that pancreatic islet expression of native B16:Y insulin is necessary for diabetes following immunization with just the peptide in contrast to immunizing with (i.e., transplanting) whole islets. Splenocytes from mice immunized with B16:A insulin B:9–23 peptide did not rapidly transfer diabetes in B16:Y NOD/SCID mice (2 of 8 mice developed diabetes at 65 and 110 days after transfer; Figure 8; as seen in B16:Y recipients of splenocytes from nonimmunized mice; see Figure 5A).

Taken together, our results suggest the 2 requirements for efficient insulin B:9–23 peptide induction of diabetes are priming by the B16:Y insulin B:9–23 sequence and splenocytes encountering native B16:Y insulin in the pancreatic islets of the recipient. B16:Y insulin B:9–23–primed CD4+ T cells are sufficient to transfer IAA production. Immunization of B16:A-dKO splenocyte donors with native B16:Y peptide is essential for the rapid induction of IAAs after splenocyte transfer to B16:Y NOD/SCID mice. Because the spleen contains CD4+ T cells and non-CD4+ splenic cells (e.g., macrophages, B lymphocytes, and dendritic cells) that may take up B16:Y peptide, we set out to determine which of these subsets of cells were responsible for inducing IAA production. We transferred CD4+ T cells isolated from the spleens of B16:A-dKO mice immunized with B16:Y insulin B:9–23 peptide to NOD/SCID mice. Because NOD/SCID mice lack B lymphocytes, we also simultaneously transferred CD4-depleted splenocytes derived from nonimmunized B16:A-dKO mice. As shown in Figure 9A, splenic CD4+ T cells from the insulin B:9–23 peptide–immunized mice with non-CD4+ cells from nonimmunized mice upon transfer were sufficient to induce IAAs. In contrast, transfer of non-CD4+ splenocytes from immunized mice together with CD4+ splenocytes from nonimmunized mice upon transfer were sufficient to induce IAAs. In contrast, transfer of non-CD4+ splenocytes from immunized mice together with CD4+ splenocytes from nonimmunized mice failed to induce IAAs (Figure 9B), even though the immunized donor mice had IAAs. These results indicate that the induction of IAAs after splenocyte transfer is dependent on CD4+ T cells from the B16:Y insulin B:9–23 peptide–immunized mouse and non-CD4+ splenocytes do not induce anti-IAAs upon transfer.

Provision of the B16:Y insulin B:9–23 sequence by transgenesis induces IAAs and insulitis. In order to explore whether induction of IAAs following provision of B16:Y insulin B:9–23 sequences to a B16:A-dKO mouse is dependent upon postnatal B:9–23 exposure, we evaluated an additional model for induction of IAAs. We created double Ins1 and Ins2 knockout NOD mice with a mutated B16:Y preproinsulin transgene.
(B16:Y-dKO mice) driven off the same promoter as our mutated B16:
A preproinsulin transgene. Introducing this transgene with the B16:Y
insulin B:9–23 sequence into B16:Y-dKO mice resulted in expression
of IAAs (Figure 10A). More than half of B16:Y-dKO mice (5 of 10)
developed high levels of IAAs, whereas 2 of 31 B16:A-dKO mice devel-
oped insulitis as severe as that of age-matched wild-
type NOD mice, whereas age-matched B16:A-dKO mice did not (P < 0.01; Figure 10, B–D). We evalu-
ated mice for islet infiltration between 10 and 22
weeks of age. Most B16:A-dKO mice did not have
any lymphocytic infiltration, and 2 of 9 mice had
peri-islet infiltration, which was observed in less
than 20% of the islets in individual mice. In con-
trast, all of the 4 B16:Y-dKO mice developed severe
insulitis, and 50%–70% of the islets demonstrated
intraislet infiltration. These results indicate that
even genetically induced expression of B16:Y pro-
insulin restores anti-islet autoimmunity as well as
postnatal provision via islet transplant.

Discussion
We have evaluated 3 phenotypes (anti-IAA, insu-
litis, and development of diabetes) in multiple
NOD-derived animal models by using varying
combinations of islet transplants with different insulin B:9–23
sequences and corresponding insulin B:9–23 sequences in the recipi-
ent pancreatic islets (Tables 1 and 2). Our results highlight a dramat-
ic dependence on the presence or absence of the native B16:Y insu-
ulin sequence, consistent with the hypothesis that the insulin B:9–23
peptide is a key determinant of NOD autoimmune diabetes and that

Figure 5
Rapid induction of diabetes with splenocytes from mice transplanted with B16:Y islets
transferred into NOD/SCID mice with B16:Y. Splenocytes from B16:A-dKO mice that
received B16:Y NOD/SCID islets, B16:A-dKO islets, or no transplant (unmanipulated)
were transferred into wild-type B16:Y NOD/SCID mice (A) or B16:A-dKO NOD/SCID mice
(B). P < 0.01, B16:Y islets versus B16:A-dKO islets in B16:Y NOD/SCID recipients.

Figure 6
Development of IAAs but not insulitis by immunization with insulin B:9–23 peptide. (A–D) Serum from B16:A-dKO mice immunized with native
B16:Y insulin B:9–23 peptide (A), B16:A-dKO mice immunized with altered B16:A insulin B:9–23 peptide (B), wild-type B16:Y NOD mice
immunized with native B16:Y insulin B:9–23 peptide (C), and wild-type B16:Y NOD/SCID mice that received splenocytes from insulin B:9–23
peptide–immunized B16:A-dKO mice (D) was incubated with I125-insulin in the presence of tetanus toxin peptide (TT), native B16:Y insulin B:9–23
peptide, B16:A insulin B:9–23 peptide, or human insulin. Each line represents an individual mouse. (E) More than 10 pancreatic islets from each
B16:A-dKO mouse immunized with B16:Y insulin B:9–23 peptide (n = 10), B16:A insulin B:9–23 peptide (n = 4), or PBS (n = 10) in CFA were
evaluated for lymphocytic infiltration. The y axis represents the mean ± SD of the insulitis score.
essential for inducing anti-islet autoimmunity, although it is possible that the native B16:Y proinsulin sequence in B16:A-dKO mice was not sufficient to initiate anti-islet autoimmunity for disease transfer to NOD/SCID, and diabetes induction was specific to the B16:Y insulin B:9–23 sequence in pancreatic islets. We believe it is likely that in addition to CD4+ T cells, CD8+ T cells participate in the generation of diabetes as shown through the previously described insulin B15–23 CD8 epitope (37).

Our studies indicate that it is CD4+ T cells from the splenocytes of immunized donor mice that transfer production of IAAs to NOD/SCID recipients. CD4+ T cells from a nonimmunized splenocyte donor were unable to induce IAAs in NOD/SCID recipients. Anti-IAAs appeared within 20 days, but only when the recipient mouse islets had the B16:Y insulin B:9–23 sequence. The same was true for the rapid induction of diabetes with insulin B:9–23 peptide immunization and splenocyte transfer. The speed with which IAAs appeared suggests that B lymphocytes of even the nonimmunized B16:A donor mice exist, awaiting non tolerant CD4+ T lymphocytes (i.e., B16Y primed) to help rapidly produce IAAs. Alternatively, the transferred B lymphocytes may rapidly acquire insulin with native insulin B:9–23 sequences from the B16Y NOD/SCID host (38). Of note, the IAAs were not simply antibodies to the insulin B:9–23 peptide and were not absorbed with the peptide, but were absorbed with intact insulin, similar to the IAAs of regular NOD mice or NOD and Balb/c mice directly immunized with B:9–23 peptide (39). The ability of CD4+ T cells from B16Y insulin B:9–23–immunized splenocyte donors alone to induce IAAs and diabetes should provide an assay to further define these pathogenic helper T lymphocytes.
A potentially unique feature of the insulin B\textsubscript{9}–23 peptide is that TCR recognition of this epitope appears to be primarily determined by specific conserved V\textsubscript{o} and J\textsubscript{0} sequences without defined conservation of the N region of the \( \alpha \) chain or TCR \( \beta \) chain (40). Recognition with such a simplified TCR motif raises the possibility that there may be a high precursor frequency of thymic T cells reacting with insulin B\textsubscript{9}–23. Insulin is a molecule that circulates at low concentrations but is expressed at extremely high concentrations within pancreatic \( \beta \) cells (41). The general hypothesis we are pursuing is that insulin B\textsubscript{9}–23 peptide is recognized by a low-stringency common TCR, which increases the ease of breaking CD4\textsuperscript{+} T cell tolerance to insulin, and that such loss of tolerance leads to production of IAAs and specific \( \beta \) cell destruction.

Our present results indicate that loss of tolerance to the B16/Y insulin B\textsubscript{9}–23 sequence at sites distinct from the pancreas and pancreatic lymph node (i.e., renal capsule or subcutaneous sites) can rapidly engender diabetogenic T lymphocytes and anti-IAAs. This raises the possibility that environmental mimotopes of such a peptide may contribute to diabetes by activating CD4\textsuperscript{+} T cell reactivity to a primary autoantigen. Once such tolerance to insulin or other islet molecules is lost, additional autoantigens are likely to be targeted. Whether there are additional epitopes of insulin or other molecules as central to the development of diabetes in the NOD mouse warrants further study. At present, it is unknown whether there exists a similar dominant epitope of insulin or another islet molecule for human type 1 diabetes. Our findings suggest that it may be fruitful to search for such a determinant, especially in individuals with a fixed HLA, such as DR3/DR4 heterozygous diabetics, which comprise 30%–50% of patients with type 1 diabetes.

Methods

Mice. B16:A-dKO mice were established as previously described (42). Briefly, Ins1 and Ins2 knockout NOD mice were separately established by breeding the original insulin knockouts (kindly provided by J. Jami, INSERM, Paris, France; ref. 43) onto NOD/Bdc mice using speed congeneric techniques (44). Mutated B16:A NOD mice were produced by microinjection of mutated B16:A Ins2 cDNA constructs ligated to rat insulin 7 promoter (pRIP7) directly into NOD fertilized eggs (42). Ins1 knockout, Ins2 knockouts, and B16:A NOD mice were combined and genotyped for the native insulin genes and the mutated insulin transgene (42). Native B16/Y Ins2 cDNA constructs ligated to pRIP7 were created by directly replacing nucleotides (GCC for alanine) in the vector carrying B16:A transgene with TAC for tyrosine using site-directed mutagenesis (Stratagene) and were microinjected into NOD fertilized eggs. B16:Y-dKO mice were established in the same manner as B16:A-dKO mice (42). NOD/SCID mice (NOD.CB17-Prkdc\textsuperscript{scid}) were purchased from The Jackson Laboratory (stock no. 001303). B16:A-dKO mice were crossed with NOD/SCID mice, and (B16:A-dKO \times NOD/SCID) F1 mice were intercrossed to obtain B16:A-dKO NOD/SCID mice. The NOD/SCID mutation was confirmed by PCR amplification of extracted genomic DNA (forward primer, GACTAGAAAGCTAGAGAGCT; reverse primer, AGTTTAACAGCTGGGTTGGC) followed by incubation with Alu I (Invitrogen) at 37°C for 2 hours. A final product of 239 bp was considered wild type; the NOD/SCID mutation was 209 bp.

Only female mice were used for experiments. All mice were bred and housed in a pathogen-free animal colony at the UCHSC Center for Comparative Medicine. All experiments were approved by the University of
Colorado at Denver Health Sciences Center (UCDHCSC), (Denver, Colorado, USA) Animal Care and Use Committee.

**Bone marrow transplantation.** Bone marrow was harvested from femurs and tibias of 4-week-old NOD mice, and red blood cells were removed using RBC lysis buffer (Sigma-Aldrich). To deplete mature T and B cells, harvested bone marrow was incubated with CD4+, CD8+, and B220+ MicroBeads (Miltenyi Biotec), and the labeled cells were magnetically depleted by AutoMACS (Miltenyi Biotec). After cell sorting, we confirmed that the contamination of CD4+ cells was below 5% by flow cytometric analysis using anti-CD8 (clone 53-6.7; BD Biosciences—Pharmingen), anti–guinea pig IgG antibody (Kierkegaard & Perry Laboratories Inc.). To evaluate insulitis, more than 10 pancreatic islets from an individual mouse were randomly selected, and each islet was scored as 0 (no insulitis), 0.25 (peri-islet insulitis), and 1 (intra-islet insulitis) by the same reader blinded to the group of mice. The insulitis score was calculated as follows: ([0.25 × no. islets with peri-islet insulitis] + no. islets with intra-islet insulitis)/total no. estimated islets.

**Diabetes.** The blood glucose levels of B16:A-dKO mice were measured every 2 weeks, and recipient NOD/SCID mice that received splenocytes were measured twice per week with the FreeStyle blood glucose monitoring system (TheraSense). Mice were considered diabetic after 2 consecutive blood glucose values greater than 250 mg/dl.

**Statistics.** The incidence of IAAs and insulitis scores were analyzed with the χ² test. Survival curves were analyzed with the log-rank test. Statistical tests used PRISM software (version 3.02, GraphPad Software). A P value less than 0.05 was considered significant.

### Table 1

Transplantation of islets and immunization with insulin B:9–23 peptide in B16:A-dKO mice

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Pancreatic β cell</th>
<th>IAA</th>
<th>Insulitis</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Islet transplant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16:Y (0/11)</td>
<td>85% (11/13)</td>
<td>0.38 ± 0.15</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>B16:A</td>
<td>13% (1/8)</td>
<td>0.09 ± 0.21</td>
<td>0%</td>
<td>0% (0/7)</td>
</tr>
<tr>
<td><strong>Peptide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16:Y</td>
<td>97% (29/30)</td>
<td>0.11 ± 0.12</td>
<td>0%</td>
<td>0% (0/6)</td>
</tr>
<tr>
<td>B16:A</td>
<td>100% (15/15)</td>
<td>0.14 ± 0.13</td>
<td>0%</td>
<td>0% (0/6)</td>
</tr>
</tbody>
</table>

**Measurement of micro-IAA.** B16:A-dKO mice were bled before and every 2 weeks after transplantation or immunization to measure anti-IAAs. Recipient B16:Y NOD/SCID or B16:A-dKO NOD/SCID mice were bled before and weekly after splenocyte transfer. To measure spontaneous development of IAAs, B16:A-dKO and B16:Y-dKO mice were bled every 2–3 weeks between 4 and 30 weeks of age. IAA levels were measured with the 96-well filtration plate micro-IAA assay previously described (10) and expressed as an index. A value of 0.01 or greater was considered positive.

To investigate the absorption by insulin B:9–23, 20,000 cpn of human 125I-insulin (GE Healthcare) was incubated with 5 μl of serum overnight along with 100 μg/ml of B16:A insulin B:9–23 or B16:A insulin B:9–23 peptide, precipitated with protein A/G sepharose (GE Healthcare), and counted in a TopCount beta counter (Packard).

**Histology.** The pancreata and islet grafts transplanted under kidney capsules were fixed in 10% formalin and embedded in paraffin. Paraffin-embedded tissue sections were stained with H&E, and sections from islet grafts were also stained with polyclonal guinea pig anti-insulin antibodies (Millipore) followed by incubation with a peroxidase-labeled anti-guinea pig IgG antibody (Kierkegaard & Perry Laboratories Inc.). To evaluate insulitis, more than 10 pancreatic islets from an individual mouse were randomly selected, and each islet was scored as 0 (no insulitis), 0.25 (peri-islet insulitis), and 1 (intra-islet insulitis) by the same reader blinded to the group of mice. The insulitis score was calculated as follows: ([0.25 × no. islets with peri-islet insulitis] + no. islets with intra-islet insulitis)/total no. estimated islets.

**Diabetes.** The blood glucose levels of B16:A-dKO mice were measured every 2 weeks, and recipient NOD/SCID mice that received splenocytes were measured twice per week with the FreeStyle blood glucose monitoring system (TheraSense). Mice were considered diabetic after 2 consecutive blood glucose values greater than 250 mg/dl.

**Statistics.** The incidence of IAAs and insulitis scores were analyzed with the χ² test. Survival curves were analyzed with the log-rank test. Statistical tests used PRISM software (version 3.02, GraphPad Software). A P value less than 0.05 was considered significant.

### Table 2

Transfer of splenocytes from immunized B16:A-dKO mice to NOD/SCID mice

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Immunized splenocyte donor</th>
<th>Splenocyte recipient</th>
<th>IAA</th>
<th>Insulitis</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Islet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16:Y</td>
<td>B16:A</td>
<td>B16:Y</td>
<td>ND</td>
<td>0.69 ± 0.28</td>
<td>83% (5/6)</td>
</tr>
<tr>
<td>B16:A</td>
<td>B16:A</td>
<td>B16:Y</td>
<td>ND</td>
<td>0.07 ± 0.06</td>
<td>0% (0/8)²</td>
</tr>
<tr>
<td>B16:Y</td>
<td>B16:A</td>
<td>B16:A</td>
<td>ND</td>
<td>0.26 ± 0.24</td>
<td>40% (2/5)</td>
</tr>
<tr>
<td>B16:A</td>
<td>B16:A</td>
<td>B16:A</td>
<td>ND</td>
<td>0</td>
<td>0% (0/6)</td>
</tr>
<tr>
<td><strong>Peptide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16:Y</td>
<td>B16:A</td>
<td>B16:Y</td>
<td>86% (6/7)</td>
<td>0.55 ± 0.28</td>
<td>86% (6/7)</td>
</tr>
<tr>
<td>B16:A</td>
<td>B16:A</td>
<td>B16:Y</td>
<td>0% (0/8)²</td>
<td>0.11 ± 0.09</td>
<td>0% (0/8)²</td>
</tr>
<tr>
<td>B16:Y</td>
<td>B16:A</td>
<td>B16:A</td>
<td>0% (0/8)</td>
<td>0</td>
<td>0% (0/5)</td>
</tr>
<tr>
<td>B16:A</td>
<td>B16:A</td>
<td>B16:A</td>
<td>0% (0/8)</td>
<td>0</td>
<td>0% (0/6)</td>
</tr>
</tbody>
</table>

ND, not determined. ²Within the group, 25% (2 of 8) were followed up for 35 weeks. ³Within the group, 37% (3 of 8) were followed up for 35 weeks.
Acknowledgments

This work was supported by grants from the NIH to G.S. Eisenbarth (DK32083, DK55969, and DK62718) and to E. Liu (DK064605); by a Diabetes Endocrine Research Center grant from the National Institute of Diabetes and Digestive and Kidney Diseases, NIH to G.S. Eisenbarth (P30 DK57516); and by a mentor-based fellowship from the American Diabetes Association to M. Kobayashi; by a grant to the Juvenile Diabetes Foundation to G.S. Eisenbarth (JDRF10-2006-51) and an advanced postdoctoral fellowship to M. Nakayama (JDRF10-2006-51); and by program funds to G.S. Eisenbarth from the Children’s Diabetes Foundation.

Received for publication December 29, 2006, and accepted in revised form March 20, 2007.

Address correspondence to: George S. Eisenbarth, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, 1775 North Ursula Street, Mail Stop B140, PO Box 6511, Aurora, Colorado 80045-6511, USA. Phone: (303) 724-6847, Fax: (303) 724-6839; E-mail: george.eisenbarth@uchsc.edu.

J.N. Beilke’s present address is: Department of Microbiology and Immunology, UCSF, San Francisco, California, USA.