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Article Aging

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Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice

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Introduction

Several autoantigens are targets of autoantibodies and T cells in type 1 diabetes. These include insulin, glutamic acid decarboxylase (GAD) 65 and 67, tyrosine phosphatase-like IA-2, heat shock protein 60, and other poorly defined antigens (1, 2). Accumulating evidence favors a role of proinsulin as a key autoantigen. In the human, insulin and proinsulin are common targets of autoantibodies (3–5) and T cells in prediabetic individuals (6). Anti-insulin antibodies are the first autoantibodies detected in children at risk for diabetes (7) and carry a high positive predictive value for diabetes in siblings of type 1 diabetes patients. In the mouse, T cell clones specific for insulin accelerate diabetes when injected in the NOD mouse (8, 9). Protection from diabetes is obtained by injecting insulin, insulin B chain (10), or insulin 2 B chain peptide 9–23 in prediabetic

mice (11, 12). In addition, a K^d-restricted CD8⁺ T cell clone that transfers diabetes into naive mice has been shown to recognize insulin B chain peptide 15–23 (13).

Proinsulin, a major β cell protein, is the only autoantigen that is almost exclusively expressed in β cells. In the human, alleles in the VNTR region flanking the *insulin* gene control both the β cell response to glucose and the expression of *proinsulin* in the thymus and are important determinants of genetic susceptibility to diabetes (14–16). In the mouse, there is no VNTR in the 5' region flanking the *insulin* gene, but two proinsulin isoforms encoded by genes located on chromosomes 7 and 19 coexist (17). They differ by two amino acid residues in B chain, three amino acid residues in C peptide, and several amino acid residues in the leader sequence. They also share most of their primary structure, especially in the A chain sequence. Proinsulin 2 is expressed in both β cells and the thymus, while expression of *proinsulin 1* in the thymus has been debated (18–21).

To evaluate the role of proinsulin 2 in diabetes, we introduced a null mutation for the *proinsulin 2* gene by crossing proinsulin 2^{-/-} 129 mice with NOD mice. Breeders were selected for carrying 16 genetic markers (including I-A^{g7} MHC gene) associated with diabetes in the NOD model. *Proinsulin 2*^{-/-} mice generated by intercrossing backcross 4 (BC4) mice developed highly accelerated insulinitis and diabetes.

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Nonstandard abbreviations used: glutamic acid decarboxylase (GAD); backcross (BC); anti-insulin autoantibody (IAA).

Methods

Animals. Proinsulin 2-deficient 129 mice (17) and NOD mice were bred in our facilities under specific pathogen-free conditions. The prevalence of diabetes in our NOD colony reaches 30% in males and 75% in females by 6 months of age. Throughout this article, *proinsulin 2*^{-/-} NOD homozygote mice are denoted as *Ins2*^{-/-}, *proinsulin 2*^{+/-} NOD heterozygote mice as *Ins2*^{+/-}, and *proinsulin 2*^{+/+} NOD heterozygote mice as *Ins2*^{+/+}.

Development of *proinsulin 2*-deficient NOD mice. A *proinsulin 2*-null allele obtained by deletion of most of the *proinsulin* coding sequence and insertion of LacZ and neomycin resistance (Neo) genes was introduced into the NOD inbred background from an original chimeric stock (17) sharing a mixed 129 and C57BL/6 genome. Proinsulin 2-deficient 129 mice were backcrossed to NOD mice for four generations. Heterozygote carriers of the *proinsulin 2*-deficient allele were genotyped for the Neo and LacZ genes using previously described primers (5'-GTG TTC CGG CTG TCA GCG CA-3' and 5'-GTC CTG ATA GCG GTC CGC CA-3' for Neo; 5'-CCA GCG ACC AGA TGA TCA CA-3' and ACG GCA CGC TGA TTG AAG CA-3' for LacZ) and were used as breeders at each backcross generation. Mice from the second to the fourth backcross generations were genotyped for microsatellite markers linked to *Idd* loci (*Idd1* to *Idd16*) as previously described (22). At BC4, *Ins2*^{+/-} mice shown to be homozygous for *Idd1* to *Idd16* NOD alleles were intercrossed. *Ins2*^{-/-} mice were distinguished from *Ins2*^{+/-} heterozygotes by amplification of the *proinsulin 2* gene using 3' and 5' primers (5'-CAG TAG TTC TCC AGC TGG TA-3' and 5'-GGC TTC TTC ACA CCC A-3') (17), providing a 675-bp fragment only in heterozygote mice. Since the *proinsulin 2* gene is located on distal chromosome 7 at 64.5 cM, we tested markers located at 3.3 cM (D7Mit191), 12.0 cM (D7Mit308), 26.2 cM (D7Mit232), 41.5 cM (D7Mit253), and 64.5 cM (D7Mit242), according to the Massachusetts Institute of Technology database.

Detection of *proinsulin* RNA in pancreas and thymus. RNAs were extracted from freshly collected thymus, kidney, and islets using guanidinium thiocyanate and treated with DNase before reverse transcription to avoid contamination with genomic DNA. Islets of Langerhans were isolated as previously described (23). *Proinsulin* transcripts were amplified using two pairs of primers, 5'-CAG CTC CAG TTG TGC CAC TTG TG-3' and 5'-CCA TCA GCA AGC AGG TTA TTG TTT C-3', that are specific for the *proinsulin 2* gene and provide a 232-bp fragment.

Assessment of diabetes, insulinitis, and sialitis. Starting at 4 weeks of age, mice were monitored weekly for glycosuria using test strips. Blood glucose was measured (EuroFlash; Lifescan, Johnson and Johnson Co., Milpitas, California, USA) when glycosuria was detected. Mice were considered diabetic after two consecutive instances of glycemia over 300 mg/dl. The onset of diabetes was dated from the first of the sequential glycemia measurements.

To score insulinitis, pancreata were fixed in 4% formaldehyde solution, dehydrated, and embedded

into paraffin; 5- μ m sections were stained with H&E and scored blindly for severity of insulinitis as previously reported (24).

Sialitis was scored on tissue sections prepared as previously reported (24). Results were expressed as numbers of mononuclear cell infiltrates per 100 squares of serous tissue, or as surface of infiltrate divided by surface of serous tissue \times 100, and expressed as mean \pm SEM.

Insulinemia and glucose tolerance tests. Glucose tolerance tests were performed in 6-month-old wild-type and proinsulin 2-deficient 129/Sv mice by intraperitoneal injection of D-glucose (2 g/kg) after overnight fasting. Blood samples were collected from the retro-orbital sinus at 0, 60, and 120 minutes, and glucose levels were determined as previously indicated. Fasting insulinemia levels were determined using an RIA kit (Linco Research Inc., St. Charles, Missouri, USA).

Adoptive transfer experiments. Erythrocyte-depleted spleen cells (2×10^7 per recipient) were injected i.v. in 8-week-old NOD-*scid* recipients. In the case of diabetic control donors, 10^7 spleen cells were transferred.

Assay for anti-insulin autoantibodies. Anti-insulin autoantibodies (IAAs) were detected as previously described (5). Briefly, 5 μ l of serum was incubated with or without human cold insulin and with ¹²⁵I-insulin (Amersham Pharmacia Biotech Europe, Orsay, France). After a 3-day incubation at 4°C, 50 μ l of protein A- and protein G-Sepharose (Amersham Pharmacia Biotech Europe) was added overnight at room temperature in a multiscreen 96-well filtration microplate (Millipore Corp., Bedford, Massachusetts, USA) precoated with buffer A (1% BSA, 0.1% sodium azide, 0.15% Tween-20). After incubation and washing, 40 μ l of scintillation liquid MicroScint-20 (Packard Instrument Co., Meriden, Connecticut, USA) was added, and radioactivity was measured in the 96-well plate in a Top Count scintillation counter (Packard Instrument Co.). Results were calculated on the basis of the difference in cpm (Δ cpm) between the well without and the well with cold insulin and expressed as an index: index = (sample Δ cpm - negative control Δ cpm) / (positive control Δ cpm - negative control Δ cpm) \times 100. Positivity was defined from values obtained in normal strains as previously described (5) and set to 0.01.

Autoantibodies to GAD. Autoantibodies to GAD were detected using ELISA (25). A GAD concentration of 10 μ g/ml in 0.1 M carbonate buffer (pH 9.6) was used to coat the plates. After blocking of the plates, diluted sera (1:5) were incubated overnight at 4°C, and alkaline phosphatase-conjugated polyclonal anti-murine IgG (Sigma-Aldrich, Saint Quentin Fallavier, France) was added. PNPP was used for detection.

Peptide synthesis. As described previously (26), the peptides were synthesized using Fmoc chemistry by the stepwise solid-phase methodology on a multichannel peptide synthesizer (27), and purified by reversed-phase HPLC (RP-HPLC) using a Perkin-Elmer preparative HPLC system (Perkin-Elmer, Courtaboeuf, France). The purity of each peptide was assessed by analytical RP-HPLC on a System gold instrument (Beckman Coulter,

Roissy, France). RP-HPLC was performed on a Nucleosil C18 5- μ m column (150 \times 4.6 mm) using a linear gradient of 0.1% trifluoroacetic acid in water and acetonitrile containing 0.08% trifluoroacetic acid at a flow rate of 1.2 ml/min. Integrity of each peptide was controlled by matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) on a Protein TOF mass spectrometer (Bruker SA, Wissembourg, France) (25). The peptide set was composed of 16 peptides of 15- to 18-mers overlapping by ten residues the preproinsulin 2 sequence and control ovalbumin peptide 323-339 (Table 1).

T cell recognition of preproinsulin 2 peptides. Immunogenicity of preproinsulin 1 peptides was tested by subcutaneous immunization of individual NOD mice with 50 μ g of peptide emulsified in CFA (Sigma-Aldrich) as previously described (26). Spleen cells were obtained 10 days after immunization and tested for their capacity to respond to the immunizing peptide by incubation with peptide-pulsed APCs. Irradiated (20 Gy) NOD spleen cells obtained from naive 8-week-old female mice, preincubated for 2 hours with 20 μ g/ml peptide, were used as APCs. Spleen cells from each individual mouse were tested in triplicate against the 16 proinsulin peptides used for immunization. Cultures were maintained in DMEM complemented by 10% FCS, 20 mM HEPES, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 IU/ml streptomycin, 50 μ M β -mercaptoethanol, and 2 mM L-glutamine (Gibco Invitrogen Corporation, Cergy Pontoise, France). The response to peptides was assessed by IL-2 production as detected by proliferation of the IL-2-dependent cell line (CTLL-2). Proliferation was evaluated by thymidine incorporation. Results are expressed as mean cpm of triplicate wells in the presence of tested or control peptide.

Statistical analysis. Statistical analyses were performed using log-rank and χ -square tests.

Results

Characterization of *Ins2*^{+/-} and *Ins2*^{-/-} mice. Heterozygote carriers of the *proinsulin 2*-deficient allele were genotyped by PCR for diabetes susceptibility markers at each backcross generation. At BC2, four diabetes susceptibility genes were fixed, including *Idd1*. At BC4, 16 susceptibility genes were fixed (*Idd1* to *Idd16*). *Ins2*^{+/-} mice resulting from BC4 were intercrossed. Genotyping of BC4 intercross mice yielded the expected *Ins2*^{-/-}, *Ins2*^{+/-}, and *Ins2*^{+/+} progeny (Figure 1a). To control the transfer of the deficient *proinsulin 2* allele, proinsulin 1 and proinsulin 2 RNAs were examined. Proinsulin 1 RNA was detected in the pancreas of all animals studied (*Ins2*^{-/-}, *Ins2*^{+/-}, and *Ins2*^{+/+} mice) (data not shown). Proinsulin 2 RNA was detected in thymus and islets of *Ins2*^{+/+} and *Ins2*^{+/-} mice but not of *Ins2*^{-/-} mice (Figure 1b). Finally, we verified that intercross female and male mice carried the NOD D7Mit191, D7Mit308, and D7Mit232 markers, excluding a role in our model of a 22-cM region in which a C57BL/6 gene has been previously shown to accelerate diabetes (28). As previously reported in proinsulin 2-deficient 129 mice (28), there

was no difference among *Ins2*^{+/+}, *Ins2*^{+/-}, and *Ins2*^{-/-} NOD mice in weight, glycemia, and insulinemia at 4 weeks of age (data not shown).

Incidence of diabetes and insulinitis in proinsulin 2-deficient mice. Analysis of progeny resulting from BC4 intercross showed a significant acceleration of diabetes onset in both male and female mice. Seventy-seven percent of female and 28% of male *Ins2*^{+/+} mice became diabetic by 27 weeks of age. By contrast, 100% of *Ins2*^{-/-} mice became diabetic by 15 weeks of age in the case of females and by 21 weeks of age in the case of males (Figure 2). Diabetes onset occurred earlier in *Ins2*^{-/-} than in *Ins2*^{+/+} mice. The first *Ins2*^{-/-} mice that developed diabetes were 9 weeks old, as compared with 13 weeks of age in the case of *Ins2*^{+/+} mice (Figure 2). In *Ins2*^{-/-} mice, diabetes onset occurred earlier in females than in males. Onset spanned from 9 to 15 weeks of age in female mice and from 12 to 21 weeks in male mice. Finally, there was no significant increase in the incidence of diabetes or acceleration of diabetes onset in *Ins2*^{+/-} heterozygotes as compared with *Ins2*^{+/+} mice. These results suggest that the lack of proinsulin 2 expression leads to an accelerated form of diabetes, while the decrease in expression in heterozygotes has no consequence for the appearance of hyperglycemia.

Pancreata obtained from 8-week-old *Ins2*^{-/-} mice showed extensive islet infiltration, contrasting with pancreata from *Ins2*^{+/+} mice, in which only a minority of islets were infiltrated (Figure 3). Three of five female *Ins2*^{-/-} mice showed insulinitis in more than 50% of islets at 8 weeks of age. By contrast, all female *Ins2*^{+/+} mice showed more than 75% normal islets at the same age. Only 30% of islets from female *Ins2*^{-/-} mice were free of insulinitis (Figure 3). All *Ins2*^{-/-} male mice at 8 weeks of age had more than 75% normal islets. In *Ins2*^{+/-} female mice, insulinitis was comparable to that seen in *Ins2*^{-/-} mice (Figure 3), indicating a striking difference in the extent of insulinitis as compared with *Ins2*^{+/+} mice despite the absence of increased incidence of spontaneous diabetes.

Table 1
Preproinsulin 2 peptide library

Peptides	Sequences
2 (1-15)	MALWMRFLPLLALLF
2 (7-23)	FLPLLALLFLWESHPTQ
2 (14-30)	LFLWESHPTQAFVKQHL
2 (20-35)	HPTQAFVKQHLCCGSHL
2 (26-41)	VKQHLCCGSHLVEALYL
2 (33-47)	SHLVEALYLVCGERG
2 (40-56)	YLVCGERGFFYTPMSRR
2 (46-60)	RGFFYTPMSRREVED
2 (50-65)	YTPMSRREVEDPQVAQ
2 (56-71)	REVEDPQVAQLELGGG
2 (61-78)	PQVAQLELGGGPGAGDLQ
2 (66-83)	LELGGGPGAGDLQTLALE
2 (71-88)	GPGAGDLQTLALEVAQQK
2 (79-94)	TLALEVAQQKRGIVDQ
2 (88-103)	KRGIVDQCCTSICSLY
2 (93-110)	DQCCTSICSLYQLENYCN

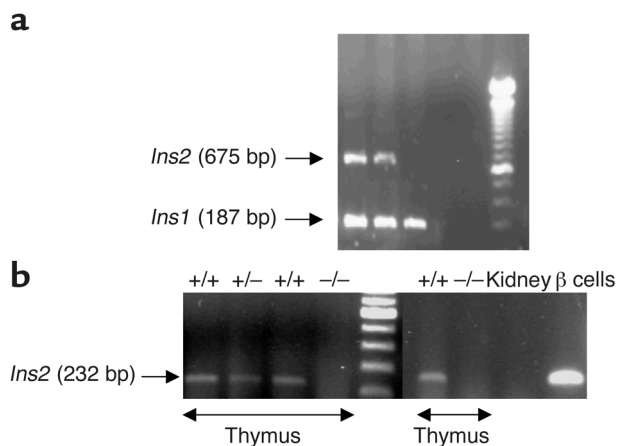


Figure 1 Genotyping of intercross mice for the gene (a) and RT-PCR analysis of proinsulin 2 gene expression (b). (a) A 675-bp PCR fragment corresponding to proinsulin 2 (*Ins2*) was observed in *Ins2*^{+/+} and *Ins2*^{+/-} mice. All mice expressed a 187-bp PCR fragment corresponding to the proinsulin 1 gene (*Ins1*). (b) Amplification of transcripts for proinsulin 2 using total thymic RNA from *Ins2*^{+/+}, *Ins2*^{+/-}, and *Ins2*^{-/-} mice. Total kidney RNA and islet RNA from *Ins2*^{+/+} mice were used, respectively, as negative and positive controls. A 232-bp fragment was obtained by PCR in *Ins2*^{+/+} and *Ins2*^{+/-} mice.

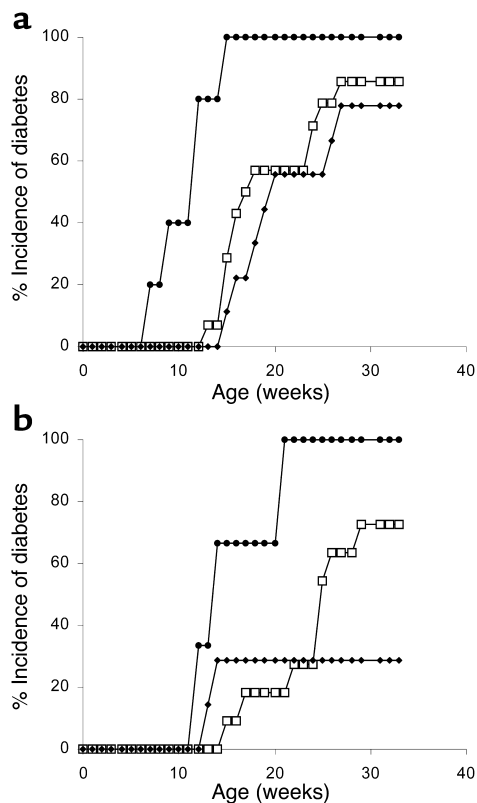
In order to eliminate the possibility that the reduced capacity to produce insulin contributed to the acceleration of disease in *Ins2*^{-/-} mice, *Ins2*^{-/-} and *Ins2*^{+/+} 129 mice were evaluated for fasting glycemia and insulinemia, and for glycemia following an intraperitoneal glucose load. No significant difference was observed between the two lines. Glycemia and insulin levels were 138 ± 30 mg/dl and 371 ± 53 pg/ml, respectively, in *Ins2*^{-/-} mice, and 120 ± 27 mg/dl and 374 ± 76 pg/ml, respectively, in *Ins2*^{+/+} mice in the fasting state. Sixty and 120 minutes after a 2-g/kg glucose load, glycemia levels were 212 ± 81 mg/dl and 160 ± 7 mg/dl, respectively, in *Ins2*^{-/-} mice, and 223 ± 47 mg/dl and 144 ± 26 mg/dl, respectively, in *Ins2*^{+/+} mice. Taken together with a previous report indicating that pancreatic insulin contents were comparable in wild-type and proinsulin 2-deficient 129 mice (28), these data suggest that accelerated development of diabetes is not dependent on β cell function.

Transfer of diabetes by spleen cells from proinsulin 2-deficient mice. Increased incidence of diabetes in *Ins2*^{-/-} mice and increased insulinitis in *Ins2*^{-/-} and *Ins2*^{+/-} mice show that introduction of a null proinsulin 2 gene mutation on the NOD background leads to an accelerated form of

Figure 2 Incidence of diabetes in BC4 intercross progeny. The number of *Ins2*^{+/+} (filled diamonds), *Ins2*^{+/-} (open squares), and *Ins2*^{-/-} (filled circles) mice was respectively 9, 14, and 5 among female mice (a) and 7, 14, and 3 among male mice (b). Significant differences were seen between *Ins2*^{+/+} and *Ins2*^{-/-} mice ($P < 0.001$ and $P < 0.05$, respectively, in female and male mice) and between *Ins2*^{-/-} and *Ins2*^{+/-} mice ($P < 0.001$ and $P < 0.01$, respectively, in female and male mice), but not between *Ins2*^{+/-} and *Ins2*^{+/+} mice.

diabetes. In order to determine whether this relates to increased diabetogenic potential of T cells in proinsulin 2-deficient mice, we performed transfer experiments by injecting spleen cells from control or proinsulin-deficient mice into NOD-*scid* recipients. Spleen cells from 8-week-old female conventional NOD mice show a low capacity to transfer diabetes into NOD-*scid* recipients. In the present experiments, spleen cells from 8-week-old *Ins2*^{+/+} mice transferred diabetes in only 20% of NOD-*scid* recipients (Figure 4). By contrast, spleen cells from female *Ins2*^{-/-} mice transferred diabetes in 82% of NOD-*scid* recipients by 100 days after transfer ($P < 0.04$, *Ins2*^{+/-} vs. *Ins2*^{+/+} female donors). Transfer efficacy was 100% when spleen cells from diabetic donors were injected into the NOD-*scid* recipients. Spleen cells from 8-week-old *Ins2*^{-/-} male or *Ins2*^{+/-} female donors showed no increase in their capacity to transfer diabetes as compared with *Ins2*^{+/+} female donors.

Anti-insulin and anti-GAD autoantibodies in proinsulin 2-deficient mice. Acceleration of diabetes in *Ins2*^{-/-} mice was hypothesized to relate to silencing of a key autoantigen in the development of anti- β cell autoimmunity. IAAs detected at 8 weeks of age in prediabetic NOD mice have been suggested to correlate with diabetes susceptibility (5). To evaluate whether specific recognition of insulin was altered in *Ins2*^{-/-} mice, sera were analyzed for the presence of IAAs. Striking differences in the prevalence of IAAs were seen among *Ins2*^{-/-}, *Ins2*^{+/-}, and *Ins2*^{-/-} NOD mice. Altered recognition of insulin was an early defect in proinsulin 2-deficient mice. At 4 weeks of age, no female *Ins2*^{+/+} NOD



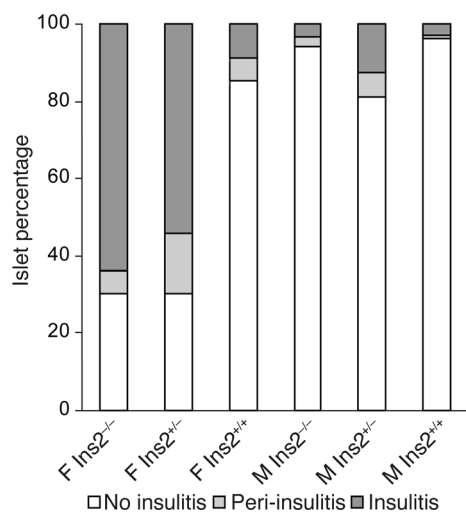


Figure 3 Insulinitis in 8-week-old *Ins2*^{+/+}, *Ins2*^{+/-}, and *Ins2*^{-/-} mice. Three pancreatic sections were obtained from each individual mouse (five mice per group). A mean of 32 islets for each group was scored. F, female; M, male.

mice were positive for IAAs as compared with 47% of *Ins2*^{-/-} ($P = 0.0007$) and 60% of *Ins2*^{+/-} mice ($P = 0.0001$). Among males, 25% of *Ins2*^{-/-} and 20% of *Ins2*^{+/-} mice, but only 7% of *Ins2*^{+/+} mice, were positive (Figure 5a). At 8 weeks of age, 82% of female *Ins2*^{-/-} NOD mice possessed IAAs as compared with 50% of *Ins2*^{+/-} ($P < 0.055$) and 40% of *Ins2*^{+/+} NOD mice ($P < 0.007$) (Figure 5b). Among male mice, the prevalence of IAAs was higher in *Ins2*^{-/-} than in *Ins2*^{+/+} mice, although the difference was not statistically significant (data not shown).

The possibility that increased prevalence of IAAs was secondary to accelerated destruction of β cells rather than directly related to altered recognition of insulin in *Ins2*^{+/+} mice had not, however, been excluded. To address this issue, we evaluated proinsulin 2-deficient mice for the presence of autoantibodies to a control β cell autoantigen and for the prevalence of sialitis. As shown in Figure 5c, all mice were positive for anti-GAD autoantibodies. No difference in the prevalence of anti-GAD autoantibodies among *Ins2*^{-/-}, *Ins2*^{+/-}, and *Ins2*^{+/+} mice was seen at 4 and 8 weeks of age. Likewise, extent of sialitis at 12 weeks of age was comparable in *Ins2*^{-/-} and *Ins2*^{+/+} mice. The number of mice with sialitis was four of four, eight of nine, and five of six in *Ins2*^{+/+}, *Ins2*^{+/-}, and *Ins2*^{-/-} mice, respectively. The mean infiltrate surface was $2.63\% \pm 0.80\%$, $2.2\% \pm 0.79\%$, and $2.07\% \pm 0.82\%$ and the mean number of mononuclear cell infiltrates was 1.31 ± 0.18 , 0.78 ± 0.17 , and 0.90 ± 0.25 in *Ins2*^{+/+}, *Ins2*^{+/-}, and *Ins2*^{-/-} mice, respectively.

The proinsulin-specific T cell repertoire in Ins2^{-/-} and Ins2^{+/-} mice. Since proinsulin 2-deficient mice showed accelerated insulinitis and diabetes, increased capacity to transfer diabetes, and increased immune response to insulin, we asked whether the absence of proinsulin expression in the thymus could imprint an altered T cell repertoire in the periphery. In order to characterize the repertoire of

T cells specific for proinsulin 2 in proinsulin 2-deficient and wild-type mice, we immunized *Ins2*^{-/-} and *Ins2*^{+/-} mice against each of the 16 proinsulin 2 peptides defined in Table 1. Both *Ins2*^{-/-} and *Ins2*^{+/-} mice showed a significant T cell response, with IL-2 secretion, to proinsulin 2 peptides 14-30, 20-35, 33-47, and 71-88 (Figure 6). Most interestingly, however, *Ins2*^{-/-} mice further showed a significant IL-2 response against proinsulin 2 peptide 88-103. As previously reported in the conventional NOD strain (26), no response to peptide 88-103 was observed in *Ins2*^{+/+} mice, suggesting that expression of proinsulin 2 in wild-type mice imprinted the silencing of peptide 88-103-specific T cells. Interestingly, proinsulin 2 peptide 88-103 corresponds to a shared sequence between proinsulin 2 and proinsulin 1.

Discussion

Proinsulin 2 has been reported as the major proinsulin isoform that is recognized during the autoimmune reaction to β cells in the NOD mouse (8-12). A majority of CD4⁺ T cell clones generated in vitro from islet infiltrates recognize insulin 2 peptide B9-23 (8) and accelerate diabetes development when injected into young NOD recipients (9). The expression of two proinsulin isoforms in the mouse allowed us to introduce a null proinsulin 2 mutation on the NOD background and evaluate its consequences for the development of autoimmunity in the absence of significant metabolic disturbance. An increase in proinsulin 1 gene expression has been shown to compensate for proinsulin 2 knockout in 129/Sv mice (17) and is expected to maintain normoglycemia and normal insulin levels prior to the development of autoimmunity to β cells in the NOD mouse (29). We observed accelerated diabetes development in *Ins2*^{-/-} NOD mice. Extensive insulinitis developed in both *Ins2*^{-/-} and *Ins2*^{+/-} mice. Importantly, all phenotyped

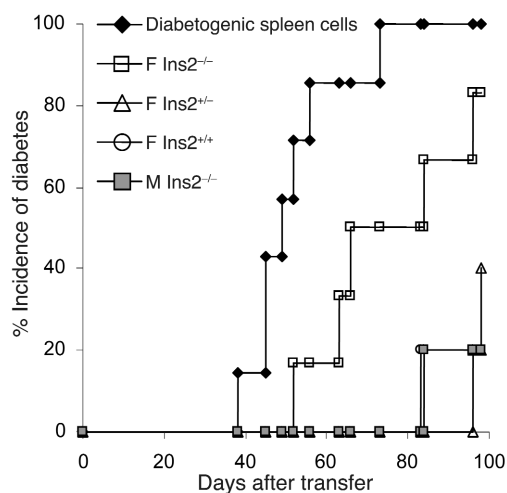


Figure 4 Transfer of spleen cells from 8-week-old proinsulin 2-deficient and control NOD mice in NOD-*scid* recipients. Spleen cells were pooled from five donors in each group. Diabetogenic spleen cells were used as positive control. Five recipient mice were used in each group. F, female NOD donors; M, male NOD donors.

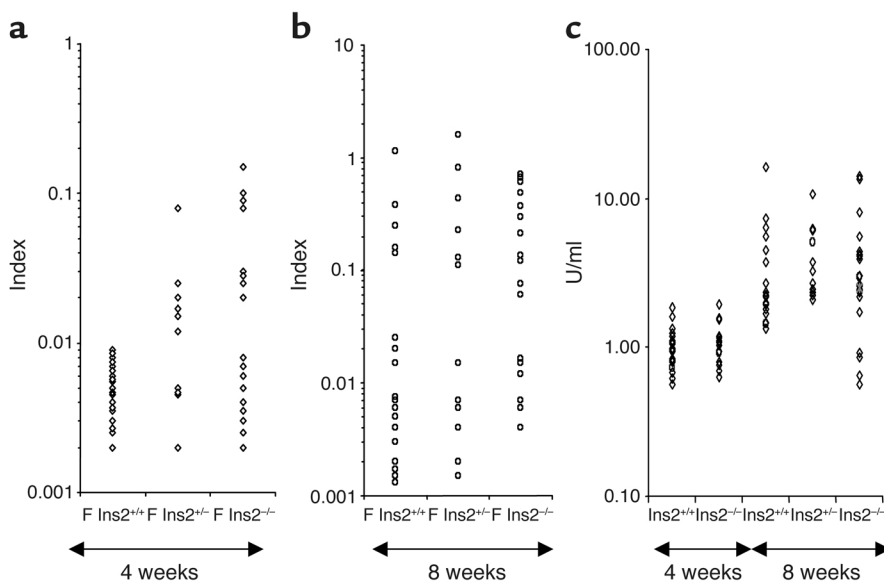


Figure 5 Autoantibodies in proinsulin 2-deficient NOD mice. Serum samples from 4-week-old (a and c) and 8-week-old (b and c) female (F) *Ins2*^{+/+}, *Ins2*^{+/-}, and *Ins2*^{-/-} mice were analyzed. (a and b) IAAs. (c) GAD autoantibodies.

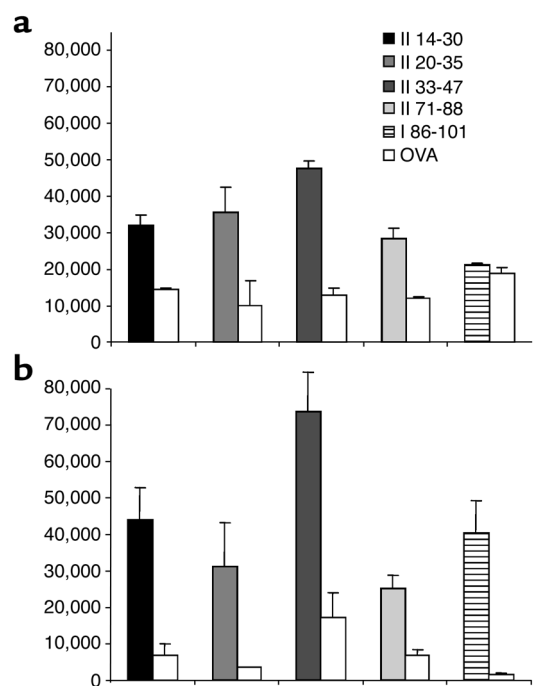
mice carried NOD alleles within a 22-cM chromosome 7 plateau in which a C57BL/6 gene has been reported to accelerate diabetes (28), indicating that the role of this non-NOD gene was excluded in *Ins2*^{-/-} mice. There is no evidence that insulinitis extent was increased at 8 weeks of age, even though diabetes development was accelerated in male *Ins2*^{-/-} mice. This does not exclude, however, the possibility that proinsulin 2-deficient males developed accelerated insulinitis at a later age. Accelerated activation of T cells against β cells was evidenced by increased transfer of diabetes by spleen cells from young *Ins2*^{-/-} mice. Acceleration of diabetes in *Ins2*^{-/-} mice was hypothesized to relate to either altered immune response to proinsulin or nonspecific acceleration of autoimmunity independently of proinsulin. Our data favor the first hypothesis. Anti-GAD autoantibodies and IAAs have been detected during the disease process in the NOD mouse (2). IAAs detected at 8 weeks of age have further been shown to strongly associate with the development of diabetes (5). IAA levels were increased in *Ins2*^{-/-} mice, while the prevalence of autoantibodies to GAD was comparable in *Ins2*^{-/-} and *Ins2*^{+/-} mice. The prevalence of sialitis was also comparable in *Ins2*^{-/-} and *Ins2*^{+/-} mice. It is thus likely that deficient expression of *proinsulin 2* led to proinsulin-specific acceleration

Figure 6 T cell response of wild-type (a) and *Ins2*^{-/-} (b) NOD mice to proinsulin 2 peptides. Each individual mouse was immunized against one single peptide of the proinsulin 2 peptide library described in Table 1, in CFA. Spleen cells were tested against the peptide used for immunization, and ovalbumin peptide 323-339 as control. IL-2 was evaluated in supernatants as described in Methods. Each histogram represents the mean \pm SD of triplicate wells, expressed in cpm.

of autoimmunity to β cells rather than a nonspecific defect of immune tolerance.

Our present data give evidence that expression of *proinsulin 2* is not a prerequisite for development of autoimmunity to β cells in the NOD mouse. The detection of IAAs in *Ins2*^{-/-} NOD mice also confirms that proinsulin 1 is an autoantigen in this model. Using a series of overlapping preproinsulin peptides in a systematic approach to characterize proinsulin epitopes recognized by CD4⁺ T cells in the NOD mouse, we previously identified T cells specific for epitopes located on both preproinsulin 1 and preproinsulin 2 (26). In the human, proinsulin epitopes distinct from B chain peptides are recognized by CD4⁺ T cells, including epitopes located within the C peptide region (6, 30).

Increased immune recognition of proinsulin and acceleration of diabetes in *Ins2*^{-/-} mice possibly relate to several mechanisms. A first possibility is that proinsulin 2 epitopes specifically activate regulatory T cells. This hypothesis fits with previous reports pointing to the protection induced by administration of insulin B chain peptide 9-23 in nondiabetic NOD mice (10-12). However, the demonstration that regulatory T cells specifically



recognize peptide 9-23 is missing, and antigen specificity of regulatory T cells remains elusive in most models. An alternative hypothesis is that proinsulin 2 operates by controlling the peripheral T cell repertoire, as in experimental allergic encephalomyelitis (31). *Proinsulin 2* is expressed within the thymus (18–21). The importance of *proinsulin* expression in the thymus is strengthened by data showing that allelic variation of *proinsulin* expression influences the risk for type 1 diabetes in the human (14–16) and that *insulin* expression in the thymus modulates T cell tolerance to insulin in the mouse (20). The role of thymic expression of viral antigens in the mouse has further been shown to control both the frequency of specific T cell precursors and the severity of diabetes in transgenic mice expressing lymphochoriomeningitis proteins in β cells (32). To address whether expression of *proinsulin 2* modulates the repertoire of insulin-specific T cells in our model, we used a series of overlapping preproinsulin 2 peptides in a systematic approach to characterize epitopes recognized by CD4⁺ T cells in wild-type and *Ins2*^{-/-} mice. In addition to four peptides that were recognized by peripheral T cells from both wild-type and *Ins2*^{-/-} mice, *Ins2*^{-/-} mice showed a response to peptide 88-103. Wild-type mice showed no response to peptide 88-103, giving evidence that T cell precursors specific for peptide 88-103 are eliminated or silenced in mice expressing *proinsulin 2*. Interestingly, peptide 88-103 is mostly located in insulin A chain and is common to proinsulin 2 and proinsulin 1, which is the only isoform expressed in *Ins2*^{-/-} mice. Peripheral 88-103-specific T cells are thus likely to contribute to acceleration of diabetes development in *Ins2*^{-/-} mice.

In conclusion, the observation that NOD mice that are deficient for proinsulin 2 expression both within β cells and within the thymus develop accelerated diabetes points to the importance of proinsulin as an autoantigen in the NOD mouse. There is strong evidence in the human that VNTR alleles flanking the insulin gene are key determinants of diabetes susceptibility. They may control the β cell response to glucose within the islets of Langerhans and the expression of proinsulin in the thymus (14–16). In the mouse, there is no allelic variability in the 5' VNTR flanking the insulin gene. *Ins2*^{-/-} NOD mice may thus represent a new model that will allow study of the role of thymic expression of insulin in susceptibility to type 1 diabetes.

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