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Recessive tolerance to preproinsulin 2 reduces but does not abolish type 1 diabetes

Elmar Jaeckel[1], Myra A Lipes[2] & Harald von Boehmer[1]

Although autoimmune diseases can be initiated by immunization with a single antigen, it is not clear whether a single self antigen is essential for the initiation and, perhaps, the perpetuation of spontaneous autoimmunity. Some studies have suggested that insulin may represent an essential autoantigen in type 1 diabetes. Here we show that unlike tolerance to glutamic acid decarboxylase, tolerance to transgenically overexpressed preproinsulin 2 substantially reduced the onset and severity of type 1 diabetes in nonobese diabetic mice. However, some mice still developed type 1 diabetes, suggesting that insulin is a key, but not absolutely essential, autoantigen. The results are consistent with the idea that the human *IDDM2* locus controls susceptibility to type 1 diabetes by regulating intrathymic preproinsulin expression.

There are competing hypotheses concerning the initiation of spontaneous autoimmune disease such as type 1 diabetes. Many experiments have suggested that autoimmune diseases can be initiated by immune responses focused on a single crucial epitope or antigen followed by intra- and intermolecular epitope spreading. Such crucial autoantigens would necessarily be involved in the initiation and perhaps even the perpetuation of autoimmune tissue destruction. In contrast, autoimmune disease might be initiated by concomitant loss of tolerance against various antigens of the target organ. There is no doubt that autoimmune disease in rodents can be elicited by immunization with a single epitope peptide, but it remains unclear whether such a scenario is representative of the natural etiology of spontaneous autoimmune disease.

The definition of an essential autoantigen would indicate that specific induction of recessive tolerance to the respective self antigen and/or ablation of antigen expression in the target organ would prevent autoimmunity[1]. Several lines of evidence have suggested that glutamic acid decarboxylase 65 (GAD) represents such an essential autoantigen for the development of type 1 diabetes, because no diabetes has been noted in GAD-deficient mice[2–4]. In contrast, GAD is not an essential or even important autoantigen in the nonobese diabetic (NOD) mouse[1], as GAD transgenic mice tolerant to naturally produced GAD peptide epitopes develop type 1 diabetes with kinetics and incidence unchanged compared with that of nontransgenic mice.

Preproinsulin (PPIns) represents an autoantigen that may be essential in the pathogenesis of type 1 diabetes in NOD mice[5]. Insulin autoantibodies are commonly found before type 1 diabetes is established both in patients[5,6] and in NOD mice[5,7]. The development of insulin autoantibodies in parallel with other islet autoantibodies has a high predictive value for subsequent development of type 1 diabetes in humans[8] and NOD mice[7,9]. Also, susceptibility to type 1 diabetes in humans maps to a variable number of tandem repeats minisatellite sequence upstream of the insulin gene, which controls the expression of insulin transcripts in the thymus[10,11]. It was thus suggested that the abundance of thymic insulin expression may control the negative selection of insulin-specific T cells[12]. Here we have tested this hypothesis by overexpressing PPIns2 in NOD mice.

Many T cells from islets of NOD mice recognize insulin, specifically the epitope of amino acids 9–23 (p9-23), and p9-23-specific T cells can be diabetogenic when transferred into young or lymphopenic NOD mice[13,14]. In the NOD model, type 1 diabetes can be prevented by the administration of insulin[15], the insulin β-chain[16] or its p9-23 peptide by various routes[14–21]. Although reduced production of insulin by β-cells (β-cell rest) has been proposed as a mechanism for the protective effect of insulin[17], it has been suggested that most protocols prevent type 1 diabetes by inducing dominant tolerance through the generation of suppressor cells[16,18,19]. Prevention of diabetes based on dominant tolerance toward PPIns does not indicate that PPIns is an essential target antigen in the natural disease process, as regulatory cells can suppress T cells of various specificities in a bystander manner.

Mice have two PPIns genes encoding proteins that differ by 8% of their amino acids. PPIns1 might be the crucial autoantigen, as PPIns1-deficient mice are almost completely protected from type 1 diabetes[20]. In contrast, PPIns2-deficient mice have accelerated diabetes[20,21], consistent with a protective effect of this gene. The involvement of PPIns in type 1 diabetes has also been investigated by an alternative approach: by induction of PPIns-specific tolerance through transgenic overexpression of PPIns 2 with a major histocompatibility (MHC) class II antigen promoter. In one study, this

[1]Harvard Medical School, Dana Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115, USA. [2]Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 1 Joslin Place, Boston, Massachusetts 02215, USA. Correspondence should be addressed to H.v.B. (harald_von_boehmer@dfci.harvard.edu).

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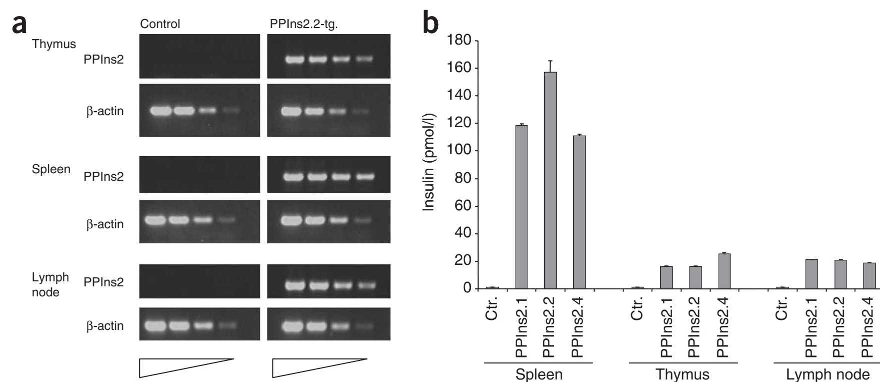

Figure 1 Characterization of PPIns2 transgenic mice. (**a**) Expression of transgenic PPIns2 mRNA in lymphoid compartments of 6-week-old female NOD mice (Control) or PPIns2 transgenic mice (PPIns2.2-tg.) by RT-PCR with fivefold serial dilutions (wedges under lanes). β-actin cDNA, amplified as an internal control. (**b**) Quantification of PPIns expression by ELISA in organ homogenates of control mice (Ctr.) and PPIns2 transgenic mice (PPIns2.1-tg., PPIns2.2-tg. and PPIns2.4-tg.) after 12 h of fasting. Data represent mean ± s.d.; $n = 4$.

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completely prevented diabetes in all NOD mice[22]. However, the mice showed no tolerance toward PPIns, as an apparently insulin-specific immune response by T and B cells could be found, challenging the idea that insulin-specific tolerance was responsible for the observed protection[22,23].

Here we have analyzed how PPIns2 overexpression in a variety of hemopoietic and nonhemopoietic tissues affected the incidence of diabetes and T cell responsiveness in NOD mice. We developed a new protocol to study insulin-specific T cell responses and found that PPIns2 transgenic NOD mice showed a high degree of but incomplete protection from type 1 diabetes that correlated with T cell tolerance to PPIns1 and PPIns2. Because we found no evidence for dominant tolerance in several experimental settings, we conclude that insulin does not represent an absolutely essential autoantigen in type 1 diabetes. Nevertheless, insulin is important in the disease, as recessive tolerance to insulin was associated with delayed onset and severely diminished incidence and severity of disease.

RESULTS

Characterization of PPIns transgenic mice

We obtained three independent transgenic founder lines after injecting the PPIns2-encoding construct into fertilized NOD oocytes. All three lines had PPIns2 mRNA expression in thymus, spleen and lymph nodes (**Fig. 1a**). The recombinant protein could be detected by immunoblot (**Supplementary Fig. 1** online), and quantitative analysis of homogenates showed that the strongest expression of PPIns2 was in the spleen (**Fig. 1b**). We also found expression in thymi and lymph nodes of transgenic mice. In control experiments, we obtained no endogenous insulin signal in any organs of nontransgenic control mice after they had fasted for 12 h (**Fig. 1b**). Thus, the transgene has relatively high expression in primary and secondary lymphoid organs. Histological analysis showed that young PPIns2 transgenic mice had normal numbers of islets of average size, and glucagon or insulin staining showed no abnormalities in islet architecture (**Supplementary Fig. 2** online).

Next we investigated whether glucose metabolism or the insulin response to glucose was altered by transgenic expression of PPIns2. Basal insulin and glucose concentrations after 12 h of fasting, as well as insulin concentrations after intraperitoneal glucose injection were similar in PPIns2 transgenic mice and in nontransgenic littermates (**Fig. 2**). Thus, the transgene encoding PPIns2 did not increase insulin concentrations in the circulation. This may be because transgenically expressed proinsulin 2 (PIns2) is degraded in lysosomes before it is released and/or MHC class II–positive cells may not secrete insulin as β-cells do. In any case, even small amounts of transgenic proinsulin (PIns) should not disturb the normal glucose metabolism because of the much lower affinity of PIns versus insulin for the insulin receptor.

Insulitis and diabetes

We found that 60% of islets from 11- to 12-week-old PPIns2 transgenic mice were devoid of leukocytic infiltrates, compared with only 32% of control mice of the same age (**Fig. 3a**). Islets of the PPIns2 transgenic mice that were affected had less-severe infiltration than did control islets. However, islets of the PPIns2 transgenic mice were less well protected from insulitis than were those reported before in PPIns2 transgenic mice[22,23]. The onset of diabetes was notably delayed in PPIns2 transgenic mice compared with control mice (**Fig. 3b**). Moreover, the overall incidence of diabetes by 34 weeks was reduced from 92% in NOD control mice to 21% in the founder line 2.2 and 26% and 29% in the founder lines 2.1 and 2.4, respectively. Islets of many 30-week-old PPIns2 transgenic mice had normal architecture and normal glucagon and insulin staining (**Supplementary Fig. 3** online). This observation rules out the possibility that transgenic mice remained normoglycemic because of transgenic PPIns expression although their islets were destroyed. However, glucose tolerance tests after intraperitoneal injection of glucose into

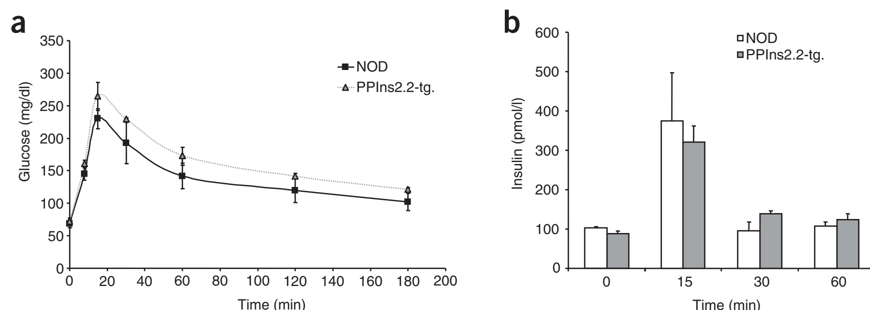

Figure 2 Metabolic characterization of PPIns2 transgenic mice. Intraperitoneal glucose tolerance tests were done in 6-week-old female NOD and PPIns2 transgenic (PPIns2.2-tg.) mice after 12 h of fasting. (**a**) Glucose concentration. (**b**) Corresponding insulin concentration. Data represent mean ± s.d.; $n = 4$.

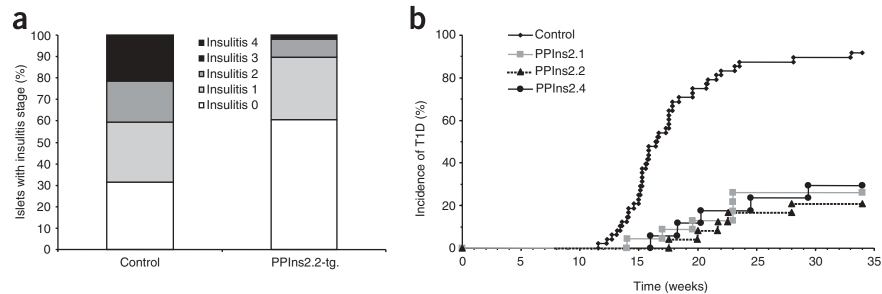

Figure 3 Insulitis and diabetes in PPIns2 transgenic versus control mice. (**a**) Histological grading of insulitis in pancreas sections of 11- to 12-week-old female mice ($n = 8$ per group). (**b**) Cumulative incidence of development of type 1 diabetes (T1D) in NOD mice ($n = 48$) and PPIns2 transgenic mice (founder lines 2.1 (PPIns2.1-tg.; $n = 23$), 2.2 (PPIns2.2-tg.; $n = 24$) and 2.4 (PPIns2.4-tg.; $n = 17$)). $P < 0.001$, NOD versus PPIns2 transgenic mice.

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these 30-week-old nondiabetic PPIns2 transgenic mice showed a slightly reduced insulin response and higher glucose concentrations compared with those of glucose-injected 4-week-old control mice, suggesting some impairment of β-cell function (data not shown). Such impairment might be expected, because we noted insulitis in some mice at week 12 (**Fig. 3a**). Thus, PPIns2 overexpression considerably reduced the incidence of diabetes in NOD mice.

Insulin-specific T cell response

Insulin-specific T cell responses are difficult to detect in humans[24,25] and NOD mice[26–28]. Like others[26,27], we were unable to detect any spontaneous T cell responses against PPIns or its peptide epitopes in pancreatic lymph nodes or spleens of 12-week-old NOD female mice regardless of whether we used proliferation assays or interferon-γ (IFN-γ) enzyme-linked immunospot assays (data not shown). This suggests that insulin-specific T cells exist at a low frequency. Likewise, we could not detect any immediate proliferative recall T cell response to insulin epitopes after subcutaneous immunization of mice with recombinant mouse proinsulin PIns1 or PIns2 in complete Freund's adjuvant (CFA)[26,27]. However, because insulin autoantibodies of the immunoglobulin G (IgG) subtype have been detected in NOD mice[5,7] and insulin-specific, diabetogenic T cell clones could be isolated from those mice[13,14,29], we attempted to amplify the insulin-specific recall T cell response *in vitro*. We immunized NOD mice with recombinant mouse PIns1 or PIns2 in CFA. After 8 d, we isolated cells from draining lymph nodes and incubated them with 10 μg/ml of the peptide antigen. On each day, we pulsed some cultures for 18 h with 1 μCi of [^{3}H]thymidine. After 4 d, all remaining cultures received fresh medium, antigen and 2×10^5 irradiated splenocytes. We first noted T cell responses against p9-23 on day 6, and they were strongest on day 8 (**Fig. 4a**). T cell responses were dependent on immunization with PIns and were not detected after immunization with GAD (**Fig. 4a**) or cultured filtrate protein of mycobacteria (data not shown). Only a few studies have investigated the specificity of PPIns-specific T cell responses[27,30] using either a limited set of peptide epitopes[30] or a few insulin-specific hybridomas[27]. To obtain a more complete picture, we analyzed the PIns-specific T cell response after immunization with properly refolded recombinant PIns by testing the recall responses against a set of overlapping insulin peptides of PIns1 and PIns2 (**Fig. 4b**) and determined cross-reactions between PIns1 and PIns2 epitopes.

After immunizing mice with PIns1, we detected recall responses against PIns1 peptides 1p9-23 and 1E and to a lower degree against 1F (**Fig. 4c**). We obtained qualitatively similar responses after immunizing mice using incomplete Freund's adjuvant (IFA; data not shown). We also obtained recall responses with peptide 2p9-23 from PIns2, which differs by the first N-terminal amino acid from the corresponding PPIns1 epitope (**Fig. 4b**), indicating cross-reactivity of the PIns1-selected T cells.

After immunizing mice with PIns2, we detected recall responses against peptides 2p9-23 and 2E (**Fig. 4c**). We obtained quantitatively similar responses after immunizing mice using IFA (data not shown). We also obtained recall responses with peptide 1p9-23 and 1E, suggesting that T cells cross-reactive for these two epitopes are selected by PPIns2. These data demonstrate that PIns1 and PIns2 can expand peptide-specific T cell populations and that expanded T cell populations show cross-reactivity to two, although not all, PIns1 and PIns2 peptides.

Insulin-specific tolerance

PPIns2 transgenic mice (founder 2.2) did not respond to any of the PIns1 and PIns2 peptides (**Fig. 4c**) in the newly developed assay, whereas founders 2.1 and 2.4 responded poorly (data not shown) against 2p9-23 (stimulation indices of 1.5 and 2.1, respectively, compared with 10.1 and 18.9 for the respective controls) in the amplified recall assay with the same immunization protocol. In contrast to cells from NOD control mice, T cells from PPIns2 transgenic mice produced neither tumor necrosis factor (TNF; **Fig. 4d**) nor IFN-γ (data not shown) in response to any of the insulin peptides when analyzed by intracellular cytokine staining (**Fig. 4d**). However, the PPIns2 transgenic mice responded vigorously to a recall with cultured filtrate protein of mycobacteria (**Fig. 4d**). Because immunization with proteins in CFA might skew the cytokine response, we did unamplified recall assays after immunization of mice with PIns1 or PIns2 in IFA as well. In these conditions, IFN-γ- and TNF-reactive T cells were detected at a very low frequency in NOD control mice but were absent from PPIns2 transgenic mice (**Supplementary Fig. 4** online). TNF production by T cells from both transgenic and control mice was strongly activated by phorbol 12-myristate 13-acetate plus ionomycin. There was no evidence for skewing toward a T helper type 2 cytokine secretion profile or for increased numbers of interleukin 10–producing regulatory T cells[31] in the transgenic mice (**Supplementary Fig. 4** online).

Tolerance was induced by bone marrow–derived cells as well as by radioresistant thymic epithelial cells from the transgenic mice. This was evident after transfer of transgenic bone marrow into nontransgenic recipients and after transplantation of embryonic transgenic thymi into nontransgenic thymectomized mice that had received nontransgenic NOD bone marrow cells after X-irradiation (data not shown).

At present, insulin autoantibodies represent one of the most reliable parameters of an adaptive immune response against insulin in non-immunized mice[5,7,9]. When analyzing various strains of mice, we found that whereas BALB/c and C57BL/6 mice had no insulin autoantibodies, a few 4-week-old and 50% of 12-week-old NOD female mice had positive insulin autoantibodies titers. In contrast, we detected no insulin autoantibodies at any time in any of our founder lines that expressed PPIns2 transgenes (**Fig. 5a**), suggesting complete tolerance to insulin. The tolerance was specific for insulin, as

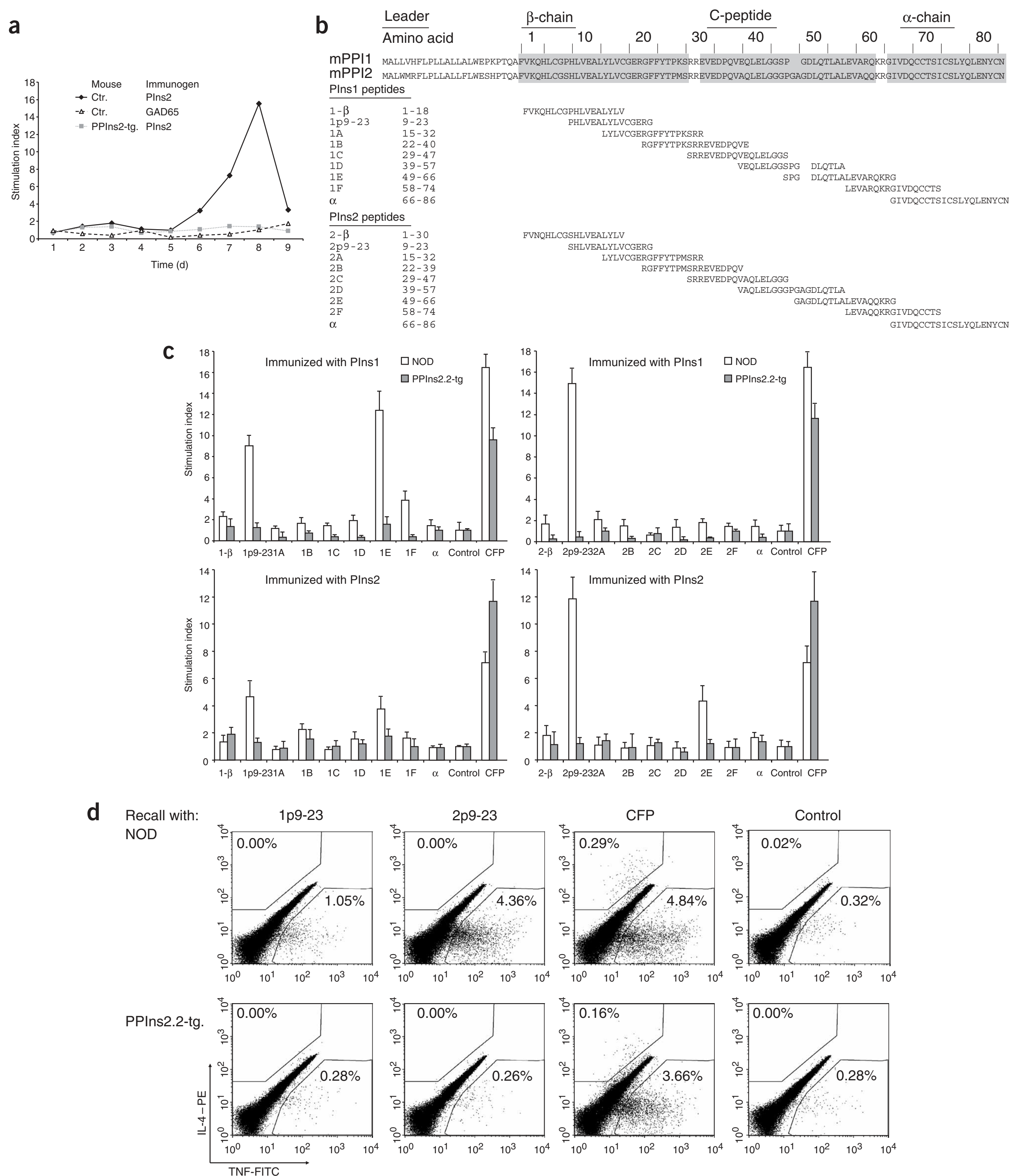

Figure 4 Insulin-specific T cell response. (**a**) Proliferation of T cells against peptide 2p9-23 after immunization with recombinant PIns2 or GAD65 in CFA. Cultures were supplemented after 4 d with irradiated antigen-presenting cells and peptide. Proliferation was assessed by an 18-hour [^{3}H]thymidine pulse. Immune responses of NOD and PPIns2 transgenic mice. (**b**) Set of overlapping peptides used. (**c**) Proliferative T cell responses in 10-week-old female NOD and PPIns2 transgenic (founder 2.2) mice after immunization with either PIns1 or PIns2 in CFA with the assay described in **a**. Recall assays were done with the isoform-specific and with the cross-reactive peptide set (horizontal axes). Responses against cultured filtrate protein were used as positive controls. Data represent mean ± s.d.; $n = 4$. (**d**) Intracellular cytokine staining (interleukin 4 (IL-4) versus TNF) of CD4^{+} T cells after immunization with PIns2 in CFA. Cultures were pulsed with antigen-presenting cells and antigen on day 4. Cells were stained on day 8. Numbers in outlined areas indicate percent in that area. CFP, culture filtrate protein; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

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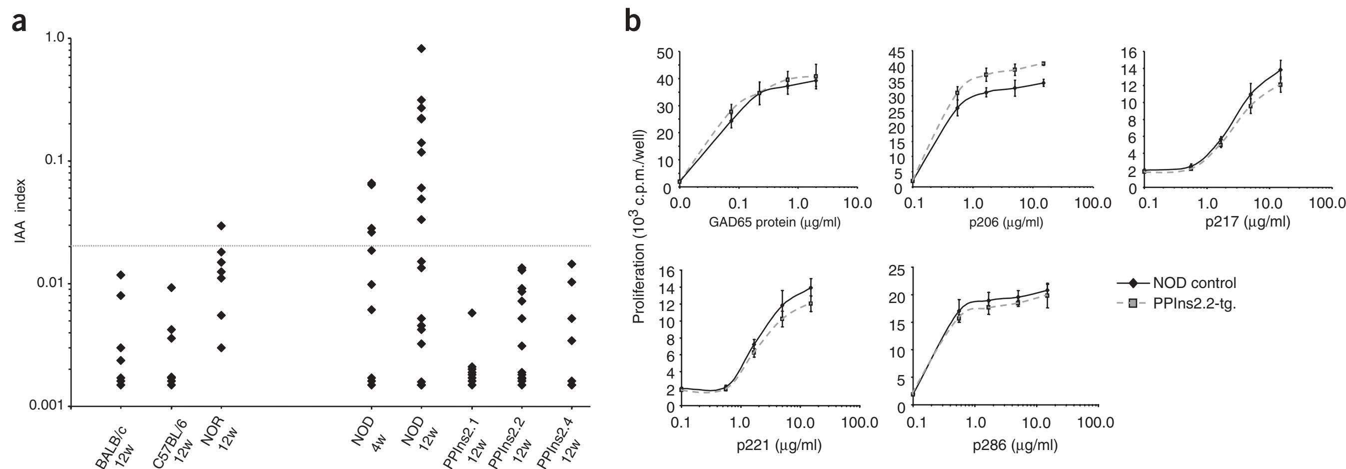

Figure 5 Spontaneous anti-insulin immune response and response to unrelated autoantigen. (**a**) Insulin autoantibodies (IAA). in female PPIns2 transgenic and NOD mice as well as in control mice (nonobese diabetes-resistant (NOR), C57BL/6 and BALB/c mice), measured by radiobinding assay. 12w, 12-week-old; 4w, 4-week-old. $P < 0.003$, PPIns2 transgenic versus NOD. Dotted horizontal line indicates upper normal for IAA. (**b**) Proliferative responses against the immunodominant epitopes of GAD65 after immunization with GAD65 in CFA in NOD and PPIns2 transgenic mice. Data represent mean ± s.d.; $n = 4$.

PPIns2 transgenic mice had GAD-specific T cell responses that in magnitude and epitope specificity were indistinguishable from those of control mice (**Fig. 5b**). Tolerance as manifest by the absence of insulin autoantibodies has not been noted in NOD mice expressing PPIns2 transgenes different from ours[22,23] or in PPIns1-deficient mice[20] or PPIns2-deficient mice[20,21]. The results indicate, therefore, that the PPIns2 overexpression in the mice reported here was associated with a previously unreported degree of insulin-specific tolerance.

Recessive or dominant tolerance?

The severity of type 1 diabetes can be reduced in NOD mice by induction of dominant tolerance after insulin application[16,18,19]. Dominant tolerance after subcutaneous[16], oral[19,32] or plasmid[18,33] delivery of insulin can usually be transferred by T lymphocytes injected into nontolerized mice. In contrast, transfer of splenocytes from 16-week-old PPIns2 transgenic mice together with diabetogenic splenocytes neither prevented nor delayed the development of diabetes (**Fig. 6a**). In contrast, diabetes was prevented by transfer of $CD4^+$ thymocytes from 4-week-old NOD mice together with diabetogenic splenocytes as described[34], showing that the experimental system used in our studies was suited for demonstrating dominant tolerance.

As thymic expression of self antigens can induce antigen-specific $CD4^+CD25^+$ regulatory T cells[35,36], we tested whether $CD4^+CD25^+$ cells specific for insulin were induced in PPIns2 transgenic mice. Sorted $CD4^+CD25^+$ splenocytes from NOD mice and PPIns2 transgenic mice were anergic to stimulation with antibody to CD3 and in coculture assays could suppress the proliferation of a naive, polyclonal $CD4^+CD25^-$ responder population to a similar extent (**Fig. 6b**). To detect suppression by insulin-specific regulatory cells, we established coculture assays in which we used $CD4^+CD25^-$ T cell receptor–transgenic T cells recognizing the GAD65 epitope p206 (ref. 37) in the context of I-A^{g7} as indicator cells for suppression. $CD25^+$ T cells from PPIns2 transgenic mice stimulated *in vitro* with PIns2 protein were unable to suppress proliferation of $CD4^+CD25^-$ indicator cells in coculture (**Fig. 6c**). A small number of antigen-specific regulatory cells

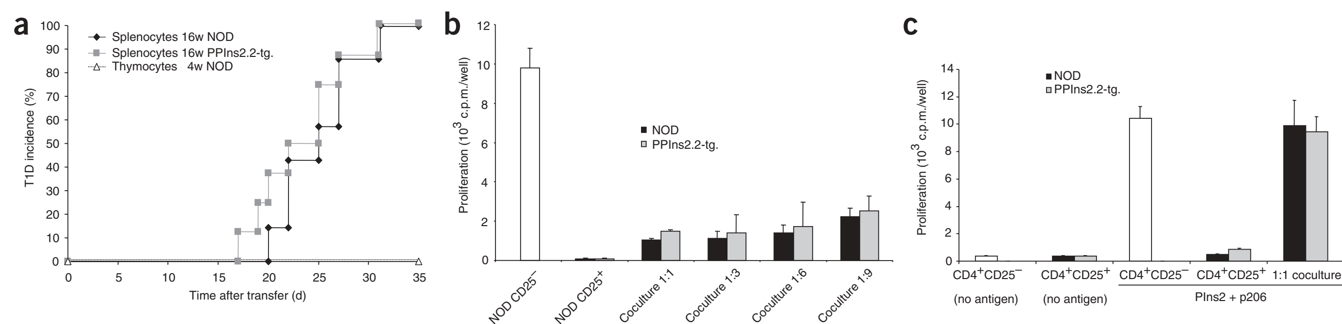

Figure 6 Dominant versus recessive tolerance. (**a**) Splenocytes (1.5×10^7) of diabetic NOD mice were transferred together with splenocytes (1.5×10^7) of 16-week-old (16w) PPIns2 transgenic mice ($n = 8$) or NOD mice ($n = 7$) into lethally irradiated 12-week-old NOD mice. As positive control, diabetogenic splenocytes (1.5×10^7) were transferred together with $CD4^+$ thymocytes (1.5×10^7) from 4-week-old (4w) NOD mice ($n = 4$). (**b**) Inhibition assay. Sorted $CD4^+CD25^-$ splenocytes were activated with antibody to CD3 (open bar). These responder cells were cocultured with titrated numbers (horizontal axis, ratio, regulators:responders) of sorted $CD4^+CD25^+$ splenocytes from NOD and PPIns2 transgenic mice. (**c**) Inhibition assay as described in **b**. Responder cells were $CD4^+CD25^-$ splenocytes from TCR transgenic mice recognizing the p206 peptide of GAD65 I-A^{g7} restricted. The responder cells were stimulated with the corresponding peptide p206. These responder cells were cocultured with equal numbers of sorted $CD4^+CD25^+$ splenocytes from NOD and PPIns2 transgenic mice stimulated with recombinant PIns2. Data represent mean ± s.d.; $n = 4$.

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Table 1 Diabetes in thymic chimeras

Recipient organ:	Transplanted organ:			
Right kidney	Thy-Ctr.	Thy-PPI 2	Thy-Ctr.	Thy-PPI 2
Left kidney	–	–	Thy-Ctr.	Thy-Ctr.
Type 1 diabetes	4/4	0/4	4/5	5/5
Type 1 diabetes (%)	100	0	80	100
Time until Type 1 diabetes (weeks)	13.2 ± 1.2	–	14.1 ± 2.3	13.7 ± 1.8

Thy, embryonic thymic of control (Ctr.) or PPIns2 transgenic (PPI2) mice.

could still be sufficient to prevent an autoimmune response *in vivo*. To address this possibility, we generated thymic chimeras with non-transgenic or PPIns2 transgenic thymi transplanted under the kidney capsule of thymectomized mice. After X-irradiation and reconstitution with NOD marrow, four of four mice receiving control thymi became diabetic after an average of 13.2 weeks, whereas none of the mice receiving PPIns2 transgenic thymi developed diabetes (**Table 1**). Four of five mice receiving a nontransgenic control thymus under the left and the right kidney capsule became diabetic. Diabetes was not prevented or delayed in mice receiving a PPIns2 transgenic thymus under one kidney capsule and a control thymus under the other, suggesting that the PPIns2 transgenic thymus did not induce dominant tolerance in the developing T cells (**Table 1**).

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DISCUSSION

Our results have shown that PPIns2 transgenic mice, like nontransgenic littermates, initially have normal islet function, islet architecture and glucose metabolism. At 12 weeks, some transgenic mice had insulitis that was less severe than that of nontransgenic control mice and had a considerable delay in the development of type 1 diabetes with a much lower overall incidence. In addition, we identified the immune response to and cross-reactivity between PPIns1 and PPIns2 epitopes by *in vitro*–amplified recall responses against overlapping peptides of both proteins. With the aid of these assays, we have shown that transgenic mice expressing PPIns2 under the control of an MHC class II invariant chain promoter were tolerant to all epitopes of PIns1 and PIns2, whereas immune responses to unrelated antigens were unperturbed. Overall, our results have shown that tolerance to PPIns1 and PPIns2 reduced but did not entirely prevent the incidence of insulitis and diabetes. Therefore, our data suggest that insulin is a key but not absolutely essential autoantigen in the development of type 1 diabetes in NOD mice. Some remaining reactivity to insulin that was not apparent in the *in vitro* assays and did not result in the formation of insulin-specific autoantibodies may be involved in type 1 diabetes in the 21% of PPIns2 transgenic mice still developing type 1 diabetes. However, the observed tolerance in our assays is more complete than that reported in PPIns transgenic mice that are completely protected[22,23]. The fact that PPIns2 is a key antigen is consistent with the hypothesis that the control of type 1 diabetes susceptibility by the *IDDM2* locus in humans is correlated with the extent of tolerance to intrathymically expressed PPIns2. In fact, thymi from PPIns2 transgenic mice are sufficient to induce tolerance and protect from diabetes.

The mechanism of tolerance is likely to represent an important parameter in considering the apparently discordant findings in PPIns2 transgenic mice that have been produced in different labs. In published reports[22,23], no insulitis or diabetes was detected in mice expressing transgene-encoded PPIns2 under control of an MHC class II promoter. In contrast, although we found a strong reduction in the incidence of disease in the mice here, some older transgenic mice became diabetic despite showing complete tolerance to insulin. Because neither recessive insulin-specific tolerance at the T or B cell level nor dominant tolerance could be demonstrated in the earlier studies[22,23], the full protection from type 1 diabetes may have had no immunological basis. Thus, the absence of a PPIns-specific T cell response may not have been the reason for the observed protection. Alternatively, some undetected dominant tolerance may have been functional in these mice.

How can these results in the NOD model be compared with the failure to prevent diabetes in humans by insulin injection[38]? Obviously, the mode of insulin application was very different in the human trial[38] (low-dose injection of insulin in humans versus ubiquitous expression in PPIns2 transgenic NOD mice). Also, in the PPIns2 transgenic mice, insulin was overexpressed from the earliest stages of development, whereas in humans it was administered well after the immune system had matured. Finally, tolerance to insulin was not assessed in the human study[38] (although some arms of the trial are being reevaluated). Because of these differences, it is impossible to determine whether the human disease differs from the mouse disease model in the function of insulin as a key autoantigen.

In contrast to previous studies[27,30], we have shown cross-reactivity of T cells for the p9-23 epitope, as T cells reacted with either 1p9-23 or 2p9-23 after priming with PIns1 or PIns2. The cross-reactivity seems plausible, given that both peptides differ only by their first N-terminal amino acid. In one previous study, only two T cell hybridomas were analyzed[30], whereas we analyzed a polyclonal population of insulin-reactive T cells, which could explain the different results obtained. The fact that there is a strong cross-reactivity of PIns1 and PIns2 epitopes is not easily compatible with the hypothesis proposing that PPIns1, but not PPIns2, represents the main autoantigen of the insulin-specific immune response in the NOD mouse[20]. This hypothesis was based on the finding that PPIns1-deficient mice are substantially protected from type 1 diabetes[20]. The observations in PPIns1-deficient mice, however, are difficult to explain by the hypothesis above, given the observation that in PPIns2-deficient mice[21,28], diabetes develops more rapidly. The last results are in accordance with the findings that PPIns2 is expressed more strongly in the thymus than is PPIns1 (refs. 30,39) and that the amount of thymic insulin expression controls the negative selection of insulin-reactive T cells[12]. The different results could be explained by the postulation that the targeted deletion of PPIns1 causes β-cell hyperplasia, which makes these cells less susceptible to an autoimmune attack[40]. Alternatively, it could be argued that PPIns1 may be the main PPIns present in the pancreas, whereas PPIns2 is involved in tolerizing the PPIns1-PPIns2 cross-reactive T cells in the thymus. The last scenario would suggest that normal amounts of intrathymic PPIns2 expression help establish partial tolerance to shared PPIns1 and PPIns2 epitopes and thus the absence of PPIns2 accelerates the development of diabetes by allowing greater autoimmune responses to PPIns1. This hypothesis is supported by our finding that much of the response to PPIns2 is cross-reactive to PPIns1 (ref. 21) and by the finding that PPIns2-deficient mice respond better to insulin than do wild-type mice[28]. Our hypothesis is also consistent with the observation that diabetic humans have an increased proinflammatory response to insulin[24].

Overall, our data suggest that insulin is a key autoantigen in type 1 diabetes but is not essentially required for the development of type 1 diabetes in NOD mice, because apparently complete tolerance to PPIns1 and PPIns2 considerably reduces but does not abolish the incidence of diabetes. Thus, by this criterion it is possible to

distinguish between disease-relevant (insulin) and mostly disease-irrelevant (GAD) autoantigens in type 1 diabetes. The identification of disease-relevant antigens is of considerable interest, because attempts to prevent or ameliorate disease by recessive or perhaps more effectively via dominant antigen-specific tolerance require knowledge of antigens that are recognized by autoimmune as well as suppressor T cells in pancreatic tissue and draining lymph nodes. GAD-specific suppressor cells fail to ameliorate disease[37], whereas suppressor cells expressing a transgenic T cell receptor specific for an as-yet-unidentified islet antigen prevent disease in a transgenic model of type 1 diabetes[37,41]. Thus, the generation of insulin-specific suppressor cells by various means[14,42] and/or their amplification[40,41] may represent a useful strategy for combating type 1 diabetes.

METHODS

Mice. NOD/Ltj mice were purchased from Jackson Laboratories or were bred in our facility. Diabetes incidence in females was 92%. NOR, C57BL/6 and BALB/c mice were bred and maintained in the Dana Farber Cancer Institute animal facility. Diabetes development was monitored by obtaining blood from mouse tail veins and analyzing it with an Accu-Chek Advantage device (Roche Diagnostics). Two subsequent glucose measurements over 200 mg/dl were considered indicative of type 1 diabetes. All animal experiments were done according to National Institutes of Health guidelines and experimental protocols were approved by the Dana Farber Cancer Institute animal care and use committee.

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Generation of PPIns2 transgenic mice. The cDNA for mouse PPIns2 was cloned from NOD pancreas total RNA by RT-PCR with proof reading Turbo pfu polymerase (Stratagene) and primers Insfw (5′-ATGGCCCTGTGGATGCGC-3′) and InsRv (5′-CTAGTTGCAGTAGTTCTCCAGCTGG-3′). The C-terminal end was fused to the LAMP1 cytoplasmic tail containing a 'Tyr-x-x-hydrophobic' motif for routing the protein into late endosomes and lysosomes[43]. The fragment was cloned in through *Hin*dIII and *Bam*HI restriction sites. The coding sequence was followed by an β-globin polyadenylation signal. Expression of the construct was driven by a hybrid invariant chain promotor[44]. The purified DNA was injected into fertilized NOD oocytes, which were reimplanted into foster mothers. Offspring were screened for the presence of the PPIns2 transgene by PCR of genomic DNA with primers Insfw and LAMPrv (5′-TGCAAAGCTTATCGATGGATCCGTTAACGCTATCTCTGGTGCACCTGCCCAC-3′) for transgenic mice.

npg

Expression analysis. Total RNA was prepared from frozen tissue samples with TRIzol (Life Technologies), and 2 μg of total TNA was reverse-transcribed with oligo($dT_{12–15}$) priming. Fivefold serial dilutions of cDNA were amplified with primers Insfw and LAMPrv. Primers for the fusion construct were spanning a 489–base pair intron. β-actin cDNA was amplified as an internal control with intron-spanning primers 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′.

Insulin enzyme-linked immunosorbent assay (ELISA). Mice were made to fast for 12 h to prevent disturbance by endogenous insulin. Quantitative analysis of insulin expression of organ extracts was done with the ultrasensitive Mouse Insulin ELISA according to manufacturer's instructions (ALPCO Diagnostics).

Generation of recombinant PIns1 and PIns2. Mouse PIns1 and PIns2 cDNA was cloned by RT-PCR from pancreas RNA. It was fused N-terminally to a six-histidine tag and cloned into the pET3a expression vector (Novagen) and was expressed and purified as described[1]. As the tertiary conformation of insulin might determine the epitope hierarchy of insulin[45], the protein was completely reduced and denatured in 0.5 M dithiothreitol and 6 M urea followed by a subsequent coordinated refolding in an alkaline cystine–cysteine redox system. Such a protocol results in 60–70% correct refolding of the protein[46]. The protein was then extensively dialyzed against PBS. Endotoxin was removed with a polymyxin-B–Affi-Prep matrix (Bio-Rad). Finally the solution was filtered and tested for endotoxin content with LAL-lysate (Associates Of Cape Cod). The endotoxin concentration was below 0.3 U/ml. The quality and quantity of protein was tested by SDS-PAGE, Coomassie staining and immunoblots with polyclonal guinea pig antibody to insulin (Linco Research).

Histology. Histology and immunohistochemistry for insulin and glucagon were done as described[1]. Samples were assigned scores for infiltration according to the following: grade 0, normal islets; grade 1, mononuclear infiltrate mostly in the periphery in less than 25% of the islet; grade 2, 25–50% of the islet; grade 3, more than 50% of the islet; grade 4, small retracted islet with minor infiltrate. For each mouse, 32–64 islets were assigned scores.

Intraperitoneal glucose tolerance test. After 12 h of fasting, mice were injected with glucose (1.5 g per kg body weight). Glucose and insulin concentrations were monitored serially. Insulin concentration was determined with an ultrasensitive Mouse Insulin ELISA (ALPCO Diagnostics).

Anti-PPIns T cell responses. First, 10- and 24-week-old NOD mice were immunized with PIns1 or PIns2 in CFA or IFA injected into the foot pad. Then, 8 d later, cells of draining popliteal and inguinal lymph nodes (5×10^5 cells) were obtained and were incubated for 96 h with PIns1 or PIns2 peptides (10 μg/ml; **Fig. 3a**) in HL1 medium (BioWhittaker) containing 100 U/ml of penicillin, 100 g/ml of streptomycin, 55 μM 2-mercaptoethanol and 0.3 mg/ml of glutamine. Culture filtrate protein was used as positive control in recall assays. Cultures were pulsed for final 18 h with [^{3}H]thymidine and incorporated radioactivity was measured with scintillation fluid in a β-counter. Measurements were made in triplicate. Stimulation indices were calculated as counts per minute of the sample divided by counts per minute of the control. In some assays, 2×10^5 irradiated splenocytes and fresh antigen were added after 96 h and incubation was continued for another 24–96 h without the addition of cytokines. Insulin autoantibodies were measured by radiobinding assay as described[1,9]. Intracellular cytokine staining was done as described[47].

Antibodies and flow cytometry. Biotin-conjugated monoclonal antibodies (mAbs) to CD4 (H129.19) and Thy1.1 (HIS51); phycoerythrin-conjugated mAbs to CD4 (GK1.5), CD25 (PC61), interleukin 2 (JES6–5H4), interleukin 10 (JES5-16E3), IFN-γ (XMG.1.2), TNF (MP6-XT22), rat IgG_1 isotype control (R3-34) and rat IgG_{2b} isotype control (A95-1); CyChrome-conjugated streptavidin; mAb to CD8 (53-6.7); and allophycocyanin-conjugated mAbs to CD4 (RM4-5), CD25 (PC61) and Thy1.2 (53-2.1) were purchased from Becton Dickinson. Fc receptor–blocking mAb 2.4G2 was used from a hybridoma culture supernatant. Surface staining was done as described[1,47]. A FACSCalibur with CellQuest software (Becton Dickinson) and FlowJo software (Treestar) was used for flow cytometry.

Transfer experiments. Bone marrow transplants and generation of thymic chimeras were done as described[1]. For adoptive transfer of diabetogenic splenocytes, 1.5×10^7 splenocytes of diabetic NOD mice were transferred with 1.5×10^7 splenocytes from 16-week-old PPIns2 transgenic (founder 2.2) or NOD control mice into 12-week-old NOD mice lethally irradiated twice with 600 rads. As positive control for diabetes prevention, 1.5×10^7 diabetogenic splenocytes were transferred with 1.5×10^7 $CD4^+$ thymocytes from 4-week-old NOD mice[34].

Inhibition assay. Sorted $CD4^+CD25^-$ T cells (2×10^4) from the spleens of NOD mice 6–8 weeks of age were cultured with varying numbers of sorted $CD4^+CD25^+$ T cells from the spleens of PPIns2 transgenic or NOD mice in the presence 2×10^5 T cell depleted, irradiated splenocytes (3,000 rads) and antibody to CD3 (clone 2C11; 5 μg/ml). After 72 h, cultures were pulsed with [^{3}H]thymidine for 24 h. Incorporated radioactivity was measured with scintillation fluid in a β-counter. In other assays, 2×10^4 sorted $CD4^+CD25^-$ T cells from the spleens of T cell receptor–transgenic NOD mice recognizing the I-A^{g7}-restricted p206 peptide (TYEIAPVFVLLEYVT)[48] of GAD65 were used as responder cells.

Statistical analyses. All results of proliferation assays, intraperitoneal glucose tolerance tests, insulin autoantibody tests and inhibition assays were analyzed by Student's *t*-test. A *P* value of less than 0.05 was considered significant. Differences in diabetes incidence between thymic chimeras were analyzed by the Chi quadrate test. The diabetes onset between transgenic and nontransgenic mice was compared by Kaplan-Meier analysis.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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