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Pathogenesis of NOD Diabetes is Initiated by Reactivity to the Insulin B Chain 9–23 Epitope and Involves Functional Epitope Spreading1

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Abstract

Type 1 diabetes (T1D) is mediated by destruction of pancreatic β cells by CD4 and CD8 T cells specific for epitopes on numerous diabetogenic autoantigens resulting in loss of glucose homeostasis. Employing antigen-specific tolerance induced by i.v. administration of syngeneic splenocytes ECDI cross-linked to various diabetogenic antigens/epitopes (Ag-SP), we show that epitope spreading plays a functional role in the pathogenesis of T1D in NOD mice. Specifically, Ag-SP coupled with intact insulin, Ins $B_{9–23}$ or Ins $B_{15–23}$, but not $GAD65_{509–528}$, $GAD65_{524–543}$ or $IGRP_{206–214}$, protected 4–6 week-old NOD mice from the eventual development of clinical disease; infiltration of immune cells to the pancreatic islets; and blocked the induction of DTH responses in a Treg-dependent, antigen-specific manner. However, tolerance induction in 19–21 week-old NOD mice was effectively accomplished only by Ins-SP, suggesting Ins $B_{9–23}$ is a dominant initiating epitope, but autoimmune responses to insulin epitope(s) distinct from Ins $B_{9–23}$ emerge during disease progression.

Keywords

Autoimmune disease; diabetes; T lymphocytes; epitope spreading; T cell activation; tolerance; immunoregulation; insulin; pancreatic β cells

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1. Introduction

T1D is mediated by autoreactive CD4 and CD8 T cells that accumulate and selectively target the destruction of pancreatic β cells. β cell destruction and the subsequent loss of Insulin leads to the accumulation of glucose in the blood and urine and resulting in polydispia, polyuria, weight loss, lethargy and in some cases death [1]. T1D is the most common chronic disorder found in children and the incidence of disease has been predicted to double in children under age 5 years by 2020 [2]. T1D is commonly treated with insulin replacement therapy although this does not address the underlying autoimmune disease and leaves individuals at risk for life-threatening hypoglycemia as well as end organ damage such as end-stage renal disease, blindness, cardiovascular disease and neuropathy [1].

Broad-based pharmacological targeting of T cells, B cells, costimulatory molecules or cytokines has shown some efficacy in preventing/treating T1D both in animal models and in initial human clinical trials [3]. However, these immunosuppressive approaches do not confer long-term protection and can have severe clinical side effects including deleterious cytokine production, anaphylactic shock, increased risk of infections, reactivation of latent viral infections and increased susceptibility to the development of cancer. Antigen-specific immunotherapies such as the administration of soluble islet antigens including insulin, glutamate decarboxylase 65 (GAD65) and heat shock protein 60 (HSP60) which have demonstrated to have protective effects in animal models have had limited efficacy in ongoing clinical human clinical trials [4].

Our previous work has shown that i.v. injection of antigen-pulsed splenic antigen-presenting cells (APCs) chemically fixed with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI), termed Ag-SP, is a powerful and safe method to induce antigen-specific T cell tolerance *in vivo*. Specifically, myelin peptide-coupled, ECDI-fixed syngeneic APCs effectively ablate induction and progression of experimental autoimmune encephalomyelitis (EAE), a murine Th1/17-mediated model of multiple sclerosis characterized by epitope spreading to endogenous myelin epitopes [5–8]. ECDI-fixed cells are also effective in inducing antigen-specific tolerance in Th2-mediated models of allergic airway disease and peanut allergy [9] and for inducing donor-specific tolerance for long-term survival of allogeneic islet transplants in the absence of immunosuppressive drugs. Ag-SP tolerance involves cross-presentation by host splenic marginal zone APCs of antigen acquired from apoptotic Ag-SP [10,11] and appears to lead to unresponsiveness by two distinct, but synergistic mechanisms - PD-L1/PD-1-mediated T cell anergy and activation of induced Tregs [12–15].

Development and progression of T1D in NOD mice and humans has been attributed to responses to numerous CD4 and CD8 T cell epitopes expressed on insulin (Ins), GAD65/67, islet specific glucose 6 phosphatase catalytic subunit related protein (IGRP), Islet antigen-2 (IA-2), phogrin (IA-2β), chromogranin A (ChgA), zinc transporter 8 (ZnT8), and vasostatin-1 [16–19]. However, little is known about the actual kinetics of antigen-specific T cell responses and how the autoreactive T cell repertoire evolves during T1D progression, *i.e.* the potential pattern and pathologic significance of spreading between and among epitopes on different diabetogenic antigens. To date, genetic and functional studies have suggested responses to insulin, specifically Ins $B_{9–23}$, are required for initiation of T1D in NOD mice [20–22] and treatment with insulin-coupled splenocytes has been reported to induce remission in new-onset disease [15].

We determined the efficacy of regulating T1D using NOD splenocytes coupled with intact insulin (Ins) *vs.* a variety of additional diabetogenic epitopes. Administration of Ag-SP or Ag-RBC before disease onset (4–6 wks old) coupled with intact Ins, Ins $B_{9–23}$ or Ins $B_{15–23}$,

but not $GAD65_{509–528}$, $GAD65_{524–543}$ or $IGRP_{206–214}$, protected NOD mice from the development of clinical disease; infiltration of immune cells in the pancreatic islets; and blocked the induction of DTH responses in an antigen-specific, Treg-dependent manner. In contrast, tolerance induction at late onset diabetes in 19–21 wk old NOD mice was more effectively accomplished by Ins-SP, suggesting Ins $B_{9–23}$ is a dominant initiating epitope, but autoimmune responses to insulin epitope(s) distinct from Ins $B_{9–23}$ emerge during disease progression. Thus pathogenesis of spontaneous T1D in NOD mice can be effectively regulated using antigen-specific tolerance and is driven by epitope spreading.

2. Materials and Methods

2.1. Mice

Female NOD mice, 4–5 wks old, were purchased from Taconic Farms (Germantown, MD). All mice were housed under specific pathogen-free conditions in the Northwestern University Center for Comparative Medicine and maintained according to protocols approved by the Northwestern University Animal Care and Use Committee (Chicago, IL).

2.2. Reagents

Synthetic peptides Ins $B_{9–23}$ (SHLVEALYLVCGERG), Ins $B_{15–23}$ (LYLVCGERG), $IGRP_{205–214}$ (LRNKANAFL), $GAD65_{509–528}$ (VPPSLRTLEDNEERMSRLSK), $GAD65_{524–543}$ (SRLSKVAPVIKARMMEYGTT) were purchased from Genemed Synthesis (San Francisco, CA). Bovine Insulin was purchased from Sigma (St. Louis, MO). Ethylene carbodiimide (ECDI) was purchased from Calbiochem (La Jolla, CA). Anti-CD25 (PC61) was purchased from BioXcell.

2.3. Antigen-coupled cell tolerance and PC61 treatment

Peripheral tolerance was induced via antigen-coupled splenocytes as previously described [10]. Briefly, spleens were harvested from female NOD mice. Tissue was mashed through a 100 μm cell strainer with a syringe plunger to create a single cell suspension. RBCs were lysed with Tris-NH_4Cl. The splenocytes (3.2×10^8 cells/ml) were coupled with peptide (1 mg/ml) using ECDI (150 mg/ml) on ice for 1 hour with intermittent shaking. The coupled splenocytes were washed 3X in phosphate buffered solution (PBS) and filtered to remove cell clumps. The coupled splenocytes were re-suspended at 2.5×10^8 cells/ml in PBS. NOD mice were injected i.v. with 5×10^7 Ag-SP in 200 μl PBS. For tolerance induction with peptide-coupled RBCs, donor mice were anesthetized with Nembutal (Ovation Pharmaceuticals) and blood was collected with heparin sulfate coated syringe after cardiac puncture and the buffy coat removed. RBCs were coupled with peptide as described above. A total of 1×10^9 Ag-RBCs in 200 ml PBS were injected i.v.

2.3. Assessment of diabetes

Blood glucose levels were measured in female NOD mice with One Touch® UltraSmart Blood Glucose Monitoring System weekly starting at the age of 10 wks unless otherwise indicated. Mice with two consecutive readings at or above 250 mg/dL were determined to be diabetic.

2.4. Elicitation of Ag-specific delayed-type hypersensitivity (DTH)

DTH responses were determined using a 24-hour ear-swelling assay. Pre-challenge ear thickness was determined using a Mitutoyo model 7326 engineer's micrometer (Schlesinger's Tools, Brooklyn, New York). DTH responses were elicited by injecting 10μg of peptide in 10μL PBS intradermally into the dorsal surface of the ear using a 100 μl Hamilton syringe fitted with a 30-gauge needle. Post-challenge air thickness was determined

24 hr post challenge and the pre-challenge ear thickness subtracted. Results are expressed in units of 10^{-4} inches ± SEM.

2.5. Assessment of insulitis

For histological analysis, the pancreas was removed and fixed with 2% paraformaldehyde (PFA). Multiple 10-μm sections were stained with hematoxylin and eosin and scored blindly for insulitis (Score: 0, no infiltrate; 1, peri-insulitis present; 2, <25%; 3, >25% of the islet is infiltrated). Average insulitis percentages were determined from the total number of islets counted from each treatment group. Statistical significance for islets with no infiltrate and islets with the most severe insulitis was determined by Student's *t* test for comparison of percentages between treatment groups.

2.6. Statistical Analyses

Comparisons of T1D incidence were analyzed by χ^2 using Fisher's exact probability. Two-way ANOVA with a Bonferroni post-test was used to determine statistical differences between mean DTH responses.

3. Results and Discussion

3.1. Ag-SP tolerance with intact insulin or the dominant insulin epitopes protects young NOD mice from development of T1D

Reactivity to multiple different islet autoantigens has been purported to be important for the initiation and/or progression of T1D in both animal models and human disease [16]. Autoantibodies against Insulin, IA-2, GAD65 and IGRP has been associated with increased risk of developing autoimmune diabetes [23,24]. Adoptive transfer of CD4 T cells specific for Ins $B_{9–23}$, $GAD65_{524–543}$ and CD8 T cells specific for $IGRP_{206–214}$ can induce or accelerate the development of T1D in NOD mice [25–28]. Insulin, particularly Ins $B_{9–23}$, has been shown to required for the development of autoimmunity [15,20,22]. We employed Ag-SP tolerance to intact insulin as well as immunodominant epitopes on insulin, GAD65 and IGRP at various stages of T1D development in NOD mice to determine the efficacy of antigen-specific immunotherapy for prevention and treatment of T1D and to determine if disease progression is associated with epitope spreading.

NOD mice treated before onset of disease at 4–5 wks of age with Ins $B_{9–23}$-SP, containing both a dominant CD4 and CD8 epitope, or Ins $B_{15–23}$-SP, containing the dominant CD8 epitope alone, had significantly reduced onset and incidence of T1D (20–30% disease incidence vs. 80% in Sham-SP treated controls, $p < 0.05$) (Fig 1A). In contrast, tolerization of young NOD mice with either of two different GAD65 CD4 T cells epitopes, $GAD65_{509–524}$ and $GAD65_{524–543}$, or with a dominant IGRP CD8 epitope, $IGRP_{206–214}$, failed to protect young NOD mice from eventual disease development. DTH responses, an *in vivo* measure of IFN-γ secretion, to Ins $B_{9–23}$, but not to $IGRP_{206–214}$, in NOD mice tolerized with either Ins $B_{9–23}$-SP or Ins $B_{15–23}$-SP were significantly reduced when compared to responses in Sham-SP tolerized mice at 5 wks after treatment (Fig 1B) and remained reduced for as long as 15 wks post tolerance induction (Fig 1C). Interestingly, we also found that tolerization of young NOD mice with syngeneic splenocytes coupled with intact insulin led to total protection from T1D development (Fig. 1D).

IGRP-reactive $CD8^+$ T cells comprise a significant population of infiltrating T cells in the pancreatic islets of diabetic mice and transfer of T cells specific for $IGRP_{206–214}$ accelerates onset of clinical disease [27]. However we found that tolerance induction with $IGRP_{206–214}$-SP failed to protect NOD from onset of T1D (Fig 1A) even though DTH to this epitope was specifically inhibited (data not shown). These data support the results of a previous study

showing tolerance via transgenic overexpression of proinsulin, but not IGRP, in NOD APCs reduces the incidence of T1D [21]. In addition, it has been reported that $IGRP_{206–214}$ reactive T cells fail to adoptively transfer disease in the absence of insulin reactive T cells suggesting that $IGRP_{206–214}$ reactive T cells are neither required or sufficient for pathogenesis of T1D [22]. Although NOD mice have elevated levels of GAD65 serum autoantibodies, the failure of splenocytes coupled with the GAD65 CD4 T cells epitopes to regulate disease is supported by previous findings that NOD mice tolerant to GAD are still susceptible to T1D [29] and considered with the observation that NOD mice have reduced expression of GAD65 in the pancreatic islets compared to humans and Biobreeding rats [30]. This suggests that GAD65 responses may not be critical for T1D pathogenesis.

Taken together these results demonstrate that i.v. administration of diabetogenic antigen-coupled splenocytes induces long lasting antigen-specific tolerance induction that protects against the onset of clinical disease as we have previously demonstrated in the EAE model of MS and other TH1/17 autoimmune disease models [14]; confirm previous findings showing the importance of Ins $B_{9–23}$ for initiating autoimmunity in NOD T1D [15,20,22]; and confirm that Ag-SP tolerance is due to cross-presentation of autoantigens by host APCs since tolerance could be induced by splenocytes coupled with intact insulin [10].

3.2. Ag-SP tolerance with Ins $B_{9–23}$-SP and Insulin-SP reduce infiltration of immune cells in the pancreatic islets

T1D is mediated by T cell and macrophage infiltration and targeted destruction of pancreatic β-cells [31]. We thus asked if tolerance to either intact insulin or Ins$B_{9–23}$ blocked the infiltration of inflammatory cells in the pancreatic islets which accompanies the development of T1D. Young NOD mice tolerized prior to disease were sacrificed 2 or 15 wks after Ag-SP tolerance to quantify the degree of inflammatory mononuclear cell infiltration (Fig 2A–C. Ins $B_{9–23}$-SP and Ins-SP treated NOD mice had significantly larger populations of un-infiltrated islets when compared to the Sham-SP tolerized group as early as two weeks after treatment well before the appearance of any clinical symptoms (Fig 2D). Un-infiltrated islets were maintained 15 weeks after treatment and mice had a significantly reduced severity of insulitis (Fig 2E). Thus treatment with Ag-SP reduces accumulation of immune cells in the target organ and maintains long-term protection from insulitis.

3.3. Insulin epitopes outside of Ins $B_{9–23}$ are important for pathogenesis at later stages of disease

While we demonstrate the efficacy of Ins $B_{9–23}$-SP for disease prevention, T1D is usually not diagnosed until the clinical presentation of hyperglycemia. Although an earlier report demonstrated that treatment with Ins-SP can induce remission in up to 50% of NOD mice with recent onset T1D [15], the majority of β-cells have already been destroyed at clinical onset of T1D which may concomitantly require β-cell regenerative therapy or necessitate islet cell transplantation to recover sufficient insulin production. Although increasing efforts are underway using a variety of criteria (including identification of genetic and environmental risks factors, and detection of T1D specific biomarkers, islet specific antibodies and autoreactive T cells in peripheral blood) to identify at risk patients before significant loss of pancreatic islets [32–34], in most cases therapy can only be initiated after disease onset. To test the efficacy of Ag-SP tolerance at later stages of disease development, non-diabetic NOD mice were tolerized at 19–21 wks of age just prior to disease onset in our colony. Remarkably, as with NOD mice treated with Ins-SP at 4–5 wks of age (Fig. 1D), older mice treated immediately prior to disease onset were totally protected from the eventual development of T1D (Fig. 3A). However unlike 4–5 wk old NOD mice, treatment of older NODs with Ins $B_{9–23}$-SP did not lead to significant protection from disease development. DTH analysis shows that tolerization with Ins $B_{9–23}$ inhibited the T cell

response to the specific epitope, but not to challenge with intact insulin, while responses to both intact insulin and Ins $B_{9–23}$ was inhibited in mice treated with Ins-SP (Fig. 3B). Collectively this analysis shows that insulin-specific tolerance before presentation of hyperglycemia in both young and older NOD mice is considerably more effective in averting disease onset than in previous studies in which treatment was initiated after onset of overt hyperglycemia [15] and suggests that insulin-encoded spread epitope(s) outside of the $B_{9–23}$ region are important for the pathogenesis of T1D during the period just prior to disease onset. A role for epitope spreading in the pathogenesis of T1D is also suggested by the different temporal appearance of DTH responses in $InsB_{9–23}$, $InsB_{15–23}$ and $IGRP_{206–214}$ noted in NOD mice at various ages (Fig. 3C).

The exact epitopes involved in older NOD mice are currently under investigation and may include other sequences on the B chain outside of Ins $B_{9–23}$ or sequences on the Insulin A chain. Studies have shown that antigenic determinants derived from the Insulin C peptide, which is cleaved from the preproinsulin precursor protein before the release of mature insulin protein may also play a pathogenic role [35]. Specifically, CD4 T cells specific for Ins $A_{7–21}$ and Ins $C_{15–30}$ have been isolated from NOD mice that spontaneously developed T1D [35–37]. Additionally, as autoimmunity progresses chronic inflammation and altered presentation of islet antigens by newly generated β-cells can give rise to new (*e.g.* GAD65 and/or IGRP) and altered spread epitopes (*e.g.* oxidized proteins) which are critical for propagating autoimmunity and may need to be targeted in combination by antigen-specific therapies [38]. The identification of spread epitopes is not only important for the development of a more targeted antigen specific therapy but would also provide insight on underlying mechanism for autoimmunity.

3.4. Regulatory T cells are required for effective therapy using Ag-SP during the induction tolerance

Tregs are critical for the maintenance of self tolerance to prevent autoimmune disease [39,40] and have been shown to suppress T effector functions through a variety of mechanisms including (but not limited to) production of inhibitory cytokines (IL-10, TGF-β), IL-2 sequestration and blockade of costimulatory molecules [41]. Our recent studies using anti-CD25 depletion/inactivation [42] in Ag-SP tolerance in the SJL/J mouse R-EAE model in mice have indicated that Tregs are not required for Ag-SP tolerance induction, but are required for long-term tolerance maintenance [11]. Similarly, Tregs were initially shown to be dispensable for Ag-SP tolerance therapy in an adoptive transfer model of T1D [15]. NOD.SCID mice reconstituted $CD4^+CD25^-$ BDC2.5 TCR Tg T cells that were treated with splenocytes coupled with the BDC2.5 mimotope were protected from the onset of T1D suggesting the efficacy of Ag-SP tolerance in the absence of Tregs [15]. However our published studies using ECDI-fixed allogeneic splenocytes to induce tolerance for protection of islet allografts indicated that both PD-L1/PD-1 induced T cell-intrinsic anergy and alloantigen-specific Tregs were required for tolerance induction [13]. We thus wished to assess the requirement for Tregs for tolerance in the NOD T1D model using the spontaneous NOD T1D model. We find that administration of anti-CD25 during tolerance induction with Ins-SP in both young (4–5 wks old) (Fig. 4A) and adult (19–21 wks old) (Fig. 4B) NOD mice both before and at late onset of disease not only abolished the protective effects of the treatment but resulted in earlier onset and increased incidence when compared to anti-CD25 treatment in Sham tolerized NOD (Fig 4A, B). This data suggests that not only are T regulatory cells are required during tolerance induction, but that the absence of Tregs renders Ag-SP immunogenic.

3.5. Ag-RBC tolerance with insulin confers protection from both the onset and incidence of type 1 diabetes

Previous studies have demonstrated the therapeutic efficiency of Ag-SP tolerance for treatment of a variety of autoimmune disease models [14] and this methodology is currently being tested in an MRI-controlled Phase I/IIa clinical trial in new-onset relapsing-remitting MS in a collaboration between the Miller lab and Dr. Roland Martin at the Univ. of Hamburg, Germany. However, due to the complexity of manufacture antigen-coupled cells, the ideal treatment would utilize a more clinically viable cellular vehicle. We thus evaluated the possibility of using RBC as carriers of ECDI-linked diabetogenic proteins and peptides for tolerance induction. RBC can be easily matched to recipients and are readily available from blood centers or can be easily sourced directly from patients. For unknown reasons, initial experiments showed coupling intact insulin protein, but not the $InsB_{9–23}$ peptide to RBCs resulted in cell lysis, release of hemoglobin and toxicity upon i.v. injection. To overcome the toxicity, Ins-RBCs were sonicated before injection. Treatment with Insulin-RBC was just as effective as preventing T1D onset in young NOD mice as Ins-SP (Fig 1D) supporting the potential use RBC as a viable donor vehicle in peripheral induction using antigen-coupled cells. Interestingly, Ins $B_{9–23}$ –RBC did not protect NOD mice from onset of T1D even when given 2 doses first at 4–5 wks and again at 6–7 wks. The efficacy of sonicated Ins-RBC for protection from development of T1D indicates that intact cells are not required for peripheral tolerance induction and the tolerance is induced via cross presentation of autoepitopes

4. Conclusions

In summary, our data indicate that Ag-SP tolerance, which avoids the potential side effects associated with generalized immunosuppression [43] and potential anaphylactic shock associated with tolerance induced by soluble antigens [44], is an effective therapy leading to long-lasting protection from the onset and late progression of T1D in NOD mice. Moreover, these results indicate that the chronic pathogenesis of T1D is associated with functional epitope spreading wherein disease onset is associated with responses to epitopes within the insulin B chain 9–23 region, where progression to overt disease is associated with responses to epitopes on insulin distinct from the initiating $B_{9–23}$ region. Relevant to antigen-specific therapy of long-standing T1D, combining Ag-SP tolerance with β cell regeneration therapies or islet transplantation may enhance the efficacy of Ag-SP tolerance after the onset of hyperglycemia. We are thus currently determining if combining autoantigen-specific tolerance induced with leukocytes ECDI-fixed with diabetogenic antigens with allo/xenoantigen-specific tolerance induced with ECDI-fixed donor leukocytes can reverse diabetes in fully diabetic NOD mice following islet transplantation.

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Abbreviations used in this paper

EAE	experimental autoimmune encephalomyelitis
GAD65	glutamate decarboxylase 65kDa
IGRP	islet-specific glucose-6-phosphatase catalytic subunit-related protein
Ins	insulin
NOD	non-obese diabetic

T1D type 1 diabetes

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1. T1D in the NOD is driven by epitope spreading in that progression of disease in young 4–5 week old NOD mice can be induced by tolerance using antigen-coupled syngeneic splenocytes (Ag-SP) to either intact insulin or the insulin B chain 9–23 epitope (Ins $B_{9–23}$), but not $GAD65_{509–528}$, $GAD65_{524–543}$ or $IGRP_{206–214}$.
2. Ag-SP tolerance in young NOD mice is shown to regulate not only development of clinical diabetes, but infiltration of immune cells to the pancreatic islets; and to block the induction of DTH responses in a Treg-dependent, antigen-specific manner.
3. Ag-RBC were also shown to regulate disease induction.
4. Most interestingly, regulation of disease in 19–21 week old NOD mice can only be induced by tolerance to intact insulin suggesting that Ins $B_{9–23}$ is a dominant initiating epitope, but autoimmune responses to insulin epitope(s) distinct from Ins $B_{9–23}$ emerge during disease progression.

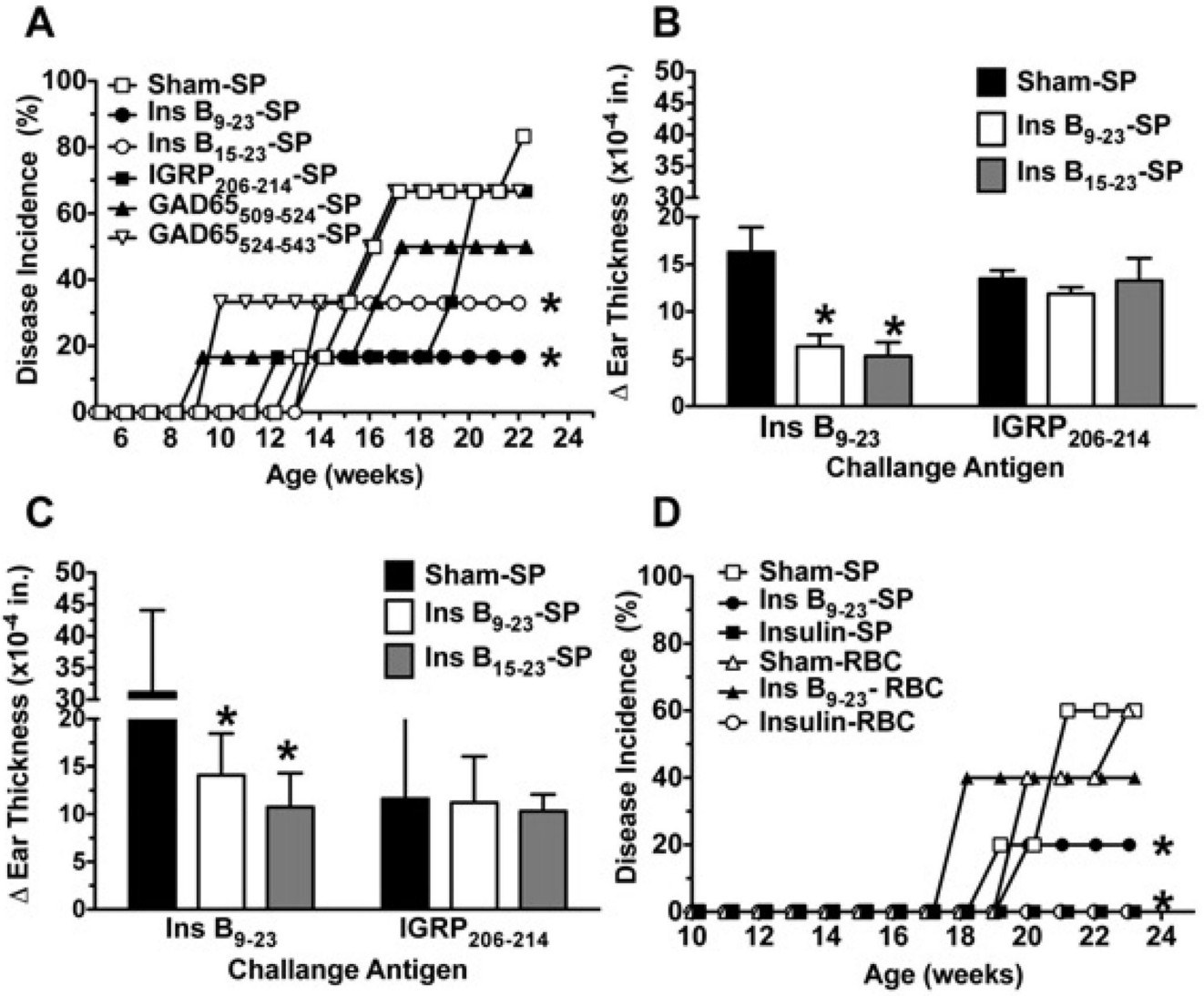

Figure 1. Female NOD mice tolerized with Ins$B_{9–23}$-SP, InsB15–23-SP, Insulin-SP and Insulin-RBC are protected from the onset of T1D

4–5 week old non-diabetic NOD mice (10–14 per group) were injected i.v. with a various diabetogenic peptides or intact bovine insulin ECDI cross-linked to either 5×10^7 syngeneic NOD splenocytes (**A & D**); or 10^9 syngeneic NOD RBCs at 4–5 wks and again at 6–7 wks of age (**D**). Control mice (Sham-SP) received cells fixed with ECDI in the absence of peptide or with $OVA_{323–339}$. Blood glucose levels were monitored weekly and data are plotted as mean percent incidence of T1D (sustained BG >250 mg/dL). DTH responses to Ins $B_{9–23}$ and $IGRP_{206–214}$ were determined at 5 wks (n = 5/ group) (**C**) and 15 wks (n = 3/ group; Sham-SP, n = 2) (**D**) post-tolerance induction in the Sham-SP, Ins $B_{9–23}$-SP and Ins $B_{15–23}$-SP treated mice. Asterisks indicate a significant difference in disease incidence or DTH responses as compared with Sham-SP controls (*$p<0.05$). Data are representative of 2–3 separate experiments.

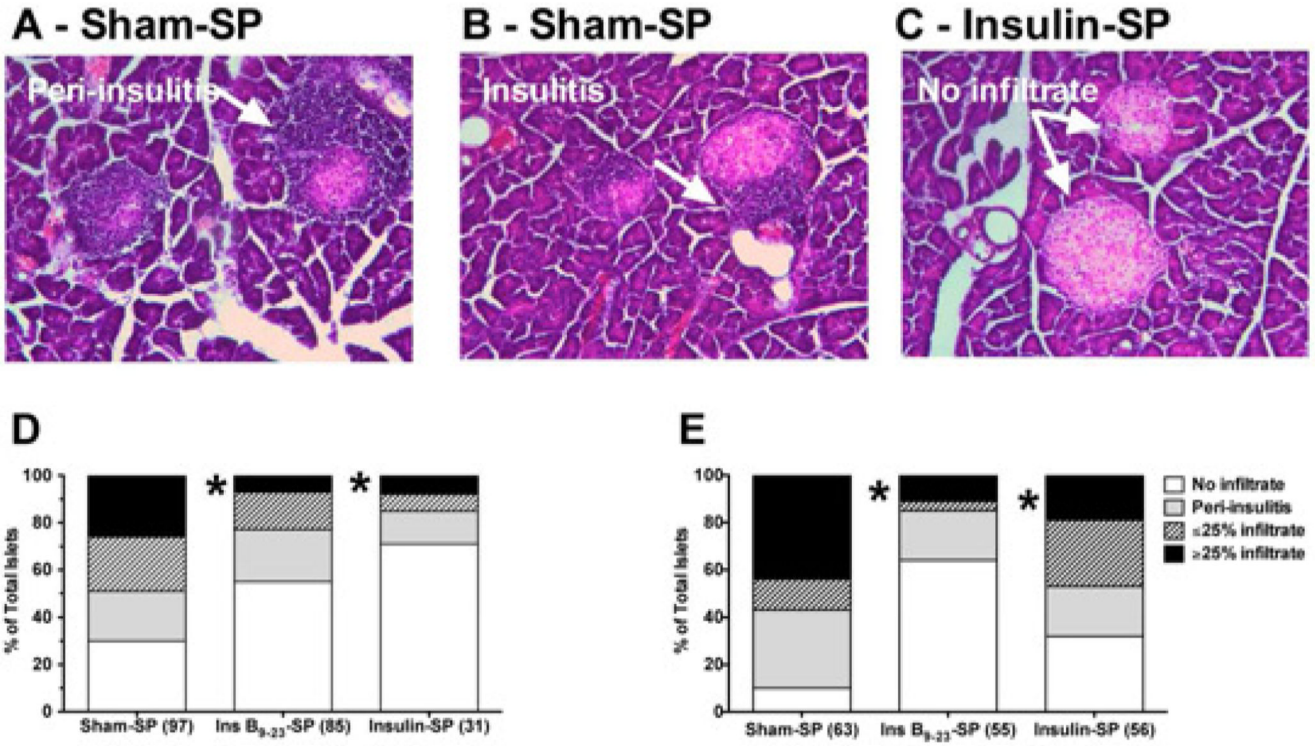

Figure 2. NOD mice tolerized with InsB$_{9–23}$-SP or Insulin-SP exhibit reduced pancreatic inflammatory infiltrates

Pancreata from InsB$_{9–23}$-SP, Ins-SP and Sham-SP treated controls were stained with hematoxylin and eosin. Representative pictures from pancreata isolated 2 wk post treatment with Sham-SP (**A, B**) showing representative stages of peri-insulitis and insulitis, and from mice treated with Ins-SP (**C**) displaying lack of inflammatory infiltrates are shown. Clinical severity was quantitated by blinded scoring of individual islets for the presence of mononuclear infiltrates at 2 wks (n = 5/ per group) (**D**) and 15 wks (n = 5/group) (**E**) after tolerance induction. Total numbers of islets scored are indicated in parenthesis. Number of islets exhibiting inflammatory cell infiltration significantly less than Sham-SP control (*$p<0.05$).

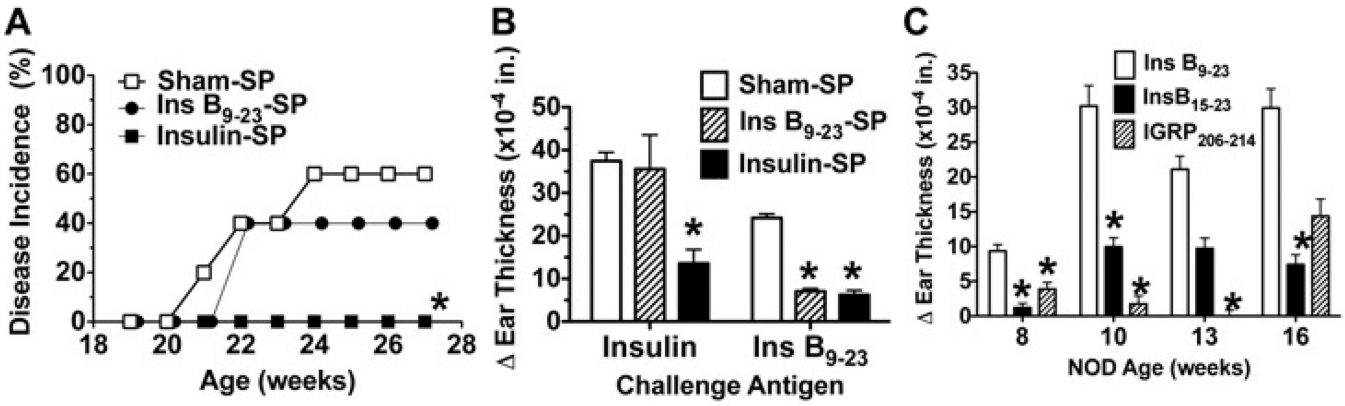

Figure 3. Tolerization with Insulin-SP, but not $InsB_{9–23}$-SP, prevents disease progression in NOD mice treated just prior to onset of overt T1D

19–21 wk old non-diabetic NOD mice (10–12 per group) were tolerized with Sham-SP, $InsB_{9–23}$-SP, Ins-SP. Blood glucose levels were monitored weekly and data are plotted as mean percent incidence of T1D (sustained BG >250 mg/dL) (**A**). DTH responses to Insulin and $InsB_{9–23}$ were determined at 7 wks after tolerance induction (n = 5/group) (**B**). Asterisks indicate a significant difference in percent disease incidence or DTH responses as compared with Sham-SP controls (*$p<0.05$). Data are representative of 2–3 separate experiments. A time course of DTH responses to challenge with $InsB_{9–23}$, $InsB_{15–23}$ and $IGRP_{206–214}$ was determined in NOD mice at 8, 10, 13, and 16 weeks of age (**C**). Asterisks indicate a DTH response significantly above responses to challenge with the irrelevant $OVA_{323–339}$ peptide (*$p<0.05$).

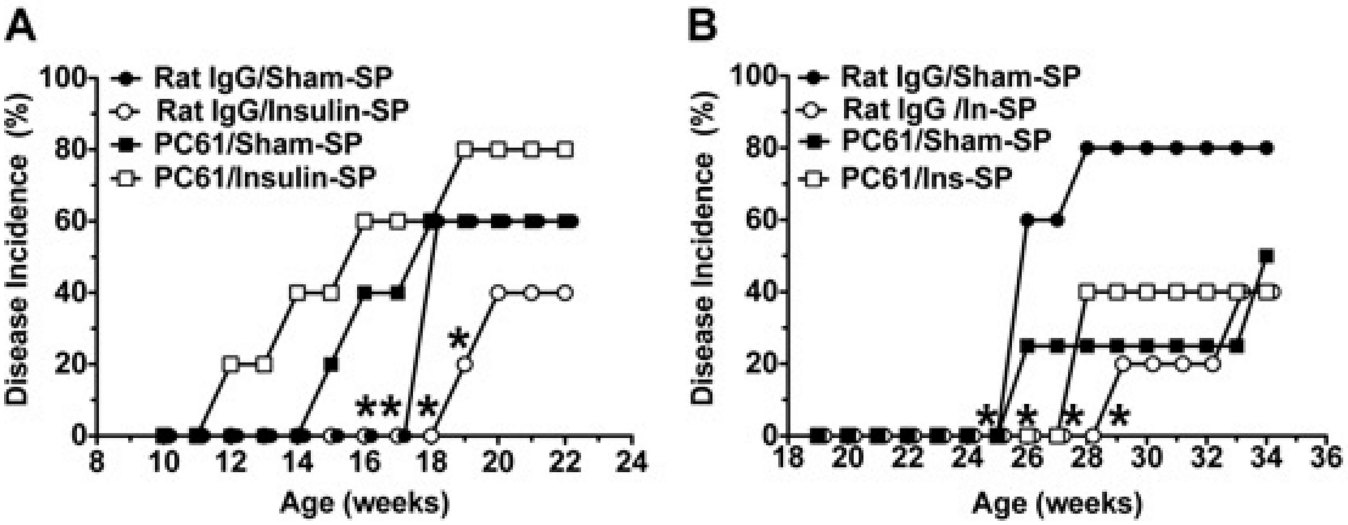

Figure 4. Regulatory T cells are required for efficient induction of Ag-SP tolerance for the prevention of T1D

Non-diabetic NOD mice were injected with two doses of anti-CD25 mAb (clone PC61) or Rat IgG control (500 μg/injection i.p.) on days -2 and 0 relative to tolerization with Sham-SP or Ins-SP at 4–5 wks of age (**A**) or at 19–20 wks of age (**B**) (n = 10–12). Blood glucose levels were monitored weekly and data are plotted as mean percent incidence of T1D (sustained BG >250 mg/dL). Asterisks indicate a significant difference in clinical disease as compared with Rat IgG/Sham-SP controls (*$p<0.05$). Data are representative of 2 separate experiments.