PD-L1 driven Tolerance Protects Neurogenin3-induced Islet Neogenesis to Reverse Established Type 1 Diabetes in NOD Mice

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Abstract

A breakdown in self-tolerance underlies autoimmune destruction of β-cells and Type 1 diabetes. A cure by restoring β-cell mass is limited by the availability of transplantable β-cells and the need for chronic immunosuppression. Recent evidence indicates that inhibiting co-stimulation via PD-1/PD-L1 pathway is central to immune tolerance. We, therefore, tested if induction of islet neogenesis in the liver, protected by PD-L1-driven tolerance, reverses diabetes in NOD mice. We demonstrate a robust induction of neo-islets in the liver of diabetic NOD mice by gene transfer of Ngn3, the islet-defining factor, along with betacellulin, an islet growth factor. These neo-islets express all the major pancreatic hormones and transcription factors. However, an enduring restoration of glucose-stimulated insulin secretion and euglycemia occurs only when tolerance is also induced by the targeted overexpression of PD-L1 in the neo-islets, which results in inhibition of proliferation and increased apoptosis of infiltrating CD4+T-cells. Further analysis revealed an inhibition of cytokine production from lymphocytes isolated from the liver but not from the spleen of treated mice, indicating that treatment did not result in generalized immunosuppression. This treatment strategy leads to persistence of functional neo-islets that resist autoimmune destruction and consequently an enduring reversal of diabetes in NOD mice.
Restoration of functional β-cell mass to cure type 1 diabetes (T1D) has been limited by a lack of long-lasting transplantable β-cells (1). The long term success of islet transplantation is limited by the requirement for chronic immunosuppression, the limited donor availability and eventual graft failure (2). Although immunosuppressive regimens have been optimized, they lead to generalized immunosuppression, with some of the drugs themselves being β-cell toxic (3). Targeted immunomodulation, without systemic immunosuppression, to prevent islet destruction by autoimmunity still remains an elusive goal.

T-effector cells mediate the autoimmune destruction of β-cells in T1D, though mechanisms underlying this loss of self-tolerance remains poorly understood. Recent work has highlighted the central role of the Programmed Death-1/Programmed Death Ligand-1 (PD-1/PD-L1) pathway in induction and maintenance of peripheral tolerance in autoimmune diabetes (4-9). The engagement of PD-L1, expressed normally by β-cells, with PD-1 on T-effector cells leads to truncation of the TCR signal by inhibiting required co-stimulation pathways and limits cytolysis by local self-reactive T-cells (10;11), in both native and transplanted islets (12-14). In addition, NOD transgenic mice constitutively expressing PD-L1 under the human insulin promoter (RIP) were significantly protected from diabetes (15), attesting to the tolerogenic role of the PD1-PDL1 pathway.

While induction of islet neogenesis is an attractive approach to the restoration of β-cell mass, it still requires immunomodulation to prevent autoimmune destruction of the induced neo-islets. We have demonstrated previously that delivery of the islet-lineage determining gene, Neurogenin3 (Ngn3), with the islet growth factor gene, betacellulin (Btc), using helper-dependent adenoviral (HDAd) vectors, induces ectopic islet neogenesis in the periportal regions
of the liver that is sufficient to reverse insulin-deficient diabetes in streptozotocin (STZ)-induced diabetic mice (16;17). However, in NOD mice, this regimen does not lead to a diabetes reversal due to autoimmune-mediated destruction of the induced neo-islets. In this study, we demonstrate that targeted induction of tolerance by overexpression of PD-L1 in the newly induced β-cells promotes their long-term survival, leading to a reversal of diabetes in NOD mice, with restoration of glucose tolerance. We show that this tolerance is due to a local reduction in number and activation of CD4+T-cells only in the periportal regions surrounding the neo-islets. This study demonstrates that tolerance can be conferred to Ngn3-induced islet neogenesis by inhibition of co-stimulation with PD-L1 to effectively reverse T1D.

**Methods**

**Animals**

NOD/ShiLtJ and NOD.CB17-Prkdc<sup>scid</sup>/J (NOD-Scid) mice (Jackson Labs) were housed under standard conditions. All animal protocols were approved by the IACUC at Baylor College of Medicine. Non-fasting body weight and blood glucose were monitored weekly ~9AM. The vectors encoding Ngn3 (HDAd-Ngn3), Btc (HDAd-Btc) and RIP-PD-L1 (HDAd-PD-L1) were generated on serotype 5, as described previously (16). Total vector dose was maintained at 7×10<sup>11</sup> viral particles (vp) in all treatment groups: [5×10<sup>11</sup>vpNgn3+1×10<sup>11</sup>vpBtc+1×10<sup>11</sup>vp empty vector], [5×10<sup>11</sup>vpNgn3+1×10<sup>11</sup>vpBtc+1×10<sup>11</sup>vpPD-L1] or [1×10<sup>11</sup>vpPD-L1+6×10<sup>11</sup>vp empty vector]. HDAd were injected intravenously via tail vein within 48h after diagnosis of diabetes (two blood glucoses between 250-500 mg/dl). A glucose tolerance test (GTT) was performed 4 and 8 weeks after treatment, on 6hr fasted mice administered 1.5 gm/kg of D-
Glucose IP, and glucose and insulin were assayed at 0, 15, 30, 60, 120 min. BrdU (15µl/gm) was injected 2hr before sacrifice.

**Immunofluorescence Microscopy and Immunohistochemistry**

Immunofluorescence and immunostaining were performed on 5µm thick formalin-fixed paraffin-embedded liver and pancreas sections, as previously described (16). Primary Antibodies used and their dilutions are available on request. For assessing the T-cell numbers and their co-localization with BrdU, TUNEL and Foxp3 in the periportal clusters, 1000-2000 cells were counted from 30-40 clusters from 3 sections 100 µm apart from 4-5 mice from each group.

**Flow Cytometry**

Mononuclear cells from the liver and spleen, 8wks after treatment (Ngn3-Btc or Ngn3-Btc+PD-L1), were isolated as described (19) and used for flow cytometry analysis immediately or after culture ex vivo. For ex vivo culture, cells were seeded in 96 well flat bottomed plate, coated with anti-CD3 (4µg/ml) overnight, and cultured for 48hrs in medium containing anti-CD28 (2µg/ml). Cells were analyzed after a 6hr incubation with 1 µl/mL Golgiplug. For in vitro stimulation and co-incubation of CD4+ cells and β cells together, CD4+ cells were purified from the spleen of non-diabetic NOD mice and stimulated with anti-CD3 and anti-CD28 as above. β-cells (Insulinoma – Ins2 β-cell line, a gift from Dr. Akio Koizumi) were infected with helper dependent empty (HDV-empty) or PD-L1 virus (HDV-PD-L1) for 2 days. Purified CD4+ were incubated with infected β-cells with ratio1:1 for 24 hours. Cells were analyzed after 6hr incubation with 1 µl/mL Golgiplug. Cells were acquired on an LSR II (BD) and analyzed with FlowJo (Tree Star, Inc.) software.
**Bioplex tissue cytokine assay**

Lysate from the livers of mice treated for 8 weeks and pancreas from diabetic and non-diabetic NOD mice were used at a final protein concentration of 0.5 mg/ml and assayed for cytokines using 23 cytokine multiplexed bead-based immunoassay kits and Bio-Plex™ Protein Array System, using the high PMT settings and at least 100 bead count per analyte, per manufacturer’s protocol (Bio-Rad).

**Adoptive transfer**

2x10⁶ splenocytes (from Ngn3-Btc-PD-L1 or Ngn3-Btc treated groups), in 250µl of serum-free media were injected IP into 6 week female NOD-Scid mice. 3-5 donors were used per treatment group with 1-2 recipients per donor. 2x10⁶ splenocytes from diabetic NOD mice were injected into 6 week old female NOD-Scid mice or Ngn3-Btc-PD-L1 treated mice. Weekly blood glucose was measured in the recipients to determine the diabetes transfer rate.

**Skin Transplantation**

Skin Transplantation was performed as described previously (20). Briefly, ear skin (1.0 cm²) from the donor mice (8-12 weeks old, C57/BL6) was grafted onto the flank of recipient NOD mice (Ngn3-Btc-PD-L1 or Ngn3-Btc treated mice). Grafts were scored daily until rejection (defined as 80% of grafted tissue becoming necrotic and reduced in size).
Virus clearance

Helper virus, Ad2LC8cCARM – serotype 2 serotype, $1 \times 10^{11}$ vp/mouse) was injected IV (21), to Ngn3-Btc-PD-L1 or Ngn3-Btc mice. Mice were euthanized at day 3 or 3 weeks after injection and liver tissue was harvested. DNA was extracted using Dneasy Blood and Tissue Kit (Qiagen) and viral copy number analyzed by qPCR, using serotype specific primers.

Statistical Methods

Student’s t-testing or ANOVA were used for significance testing. For adoptive transfer experiment, Kaplan-Meier survival analysis using log-rank test was used in Sigma Plot. p<0.05 was considered significant.

Results

Ngn3-Btc+PD-L1 gene transfer reverses diabetes in NOD mice

We have shown before that Ngn3-Btc gene transfer using HDAd vectors leads to induction of insulin-expressing neo-islets in the liver and reverses diabetes in streptozotocin-induced diabetic mice (16;22). However, this did not ameliorate the hyperglycemia in overtly diabetic NOD mice, which continued to lose weight with time (Fig. 1A-B). We, therefore, combined Ngn3-Btc with Rat Insulin Promoter (RIP) -driven PD-L1, so that the expression of PD-L1 is limited only to insulin-expressing cells induced in the liver of treated mice. In contrast to Ngn3-Btc or PDL-1 gene transfer alone, the combination therapy led to a rapid reversal of hyperglycemia and promoted weight gain in over 75% of treated mice. This was sustained for at least 14 weeks, the duration of the study (Fig. 1A-B). The treatment restored normal circulating plasma insulin (Fig.
1C) with no deleterious effects on liver function (Fig. 1D-E). A glucose tolerance test (GTT) demonstrated restoration of normal glucose tolerance and in vivo glucose-stimulated insulin secretion (GSIS) to the levels seen in non-diabetic mice, by 4 weeks (Fig. 1F-G) and sustained at 8 weeks after treatment (Fig. 1H-I) only when treatment with Ngn3-Btc was combined with PD-L1.

**Neo-islets induced in the liver of Ngn3-Btc treated NOD mice persist only when PD-L1 is also expressed.**

Ngn3-Btc transiently induced insulin-expressing cells in the periportal regions of the liver in overtly diabetic NOD mice (Fig. 2A), which were quickly destroyed by an immune infiltrate. In contrast, the periportal neo-islets induced by Ngn3-Btc+PD-L1 combination therapy in the liver are similar to pancreatic islets, in that they express the four major islet hormones that were clearly identified by 4 weeks, and sustained when re-examined at 8 and 12 weeks, after treatment (Fig. 2A-E and Fig. S1A). These neo-islets stained robustly both for insulin and C-peptide (Fig. 2B), indicating they produced proinsulin that was processed to mature insulin. The induction and persistence of neo-islets was limited to the liver, as the pancreas did not display any recovery of islets (Fig. S1B-C). Strong staining for PD-L1 was limited only to the insulin expressing neo-islets (Fig. S2A-B).

The periportal neo-islets also expressed transcripts of the different islet hormones (Fig. 3A). At 4 weeks, consistent with the data shown in Fig. 2, Ngn3-Btc, either alone or combined with PD-L1 induced many of these hormones. However, by 8 weeks after treatment, the level of expression of these hormones was significantly higher in the mice that were treated with Ngn3-Btc+PD-L1,
in parallel with the persistence and expansion of the neo-islets when PD-L1 was added to Ngn3-Btc (Fig. 3A and S3A). A similar pattern of gene expression was observed with the islet transcription factors (Fig. 3B, Fig. S3B) and in the progressive increase of the protein levels of Pdx-1 and Nkx6.1, transcription factors critical to the normal development and function of β-cells (Fig. 3C-D). Interestingly, expression of Ngn3 itself and many of its immediate downstream target genes, such as Neurod1, persisted in mice treated only with Ngn3-Btc, despite the autoimmune mediated destruction of insulin-expressing cells. This observation supports the hypothesis that the Ngn3-Btc is effective in inducing the transcription factors required for neo-islet development but PD-L1 is required, only to allow their persistence in the face of an autoimmune attack. These neo-islets also expressed hepatic oval cell markers OC2-1D11 (23) and A6 (Fig. S4A-B), consistent with our previous study that these neo-islets arose from reprogramming of hepatic oval cells (16).

**Targeted PD-L1 expression in ‘neo-islets’ leads to a decrease in CD4+ T-cells locally in and around neo-islets**

To elucidate mechanisms underlying PD-L1 induced tolerance of these neo-islets in the liver, we quantified the number of T-cells and their subsets in the liver sections from mice treated with Ngn3-Btc, with and without PD-L1. Immunostaining revealed that CD3+ T-cells were significantly reduced in and around the induced neo-islets, in the periportal regions of the livers of Ngn3-Btc+PD-L1 treated mice (Fig. 4A-B). Interestingly, this was due to a decrease in the CD4+T-cells, but not CD8+ T-cells. We then tested if neo-islet-restricted expression of PD-L1 had an effect on T-cells beyond the periportal areas. When single cell suspensions of the whole
liver were assessed by flow cytometry, the total number of CD4+ and CD8+ T-cells was not different in the two groups treated with Ngn3-Btc with or without PD-L1 (Fig. S5A-D), in contrast to that observed only in the periportal areas surrounding the neo-islets (Fig. 4A-B). This suggests that expression of insulin promoter-driven PD-L1 resulted only in local immunomodulation in the periportal regions.

**Targeted PD-L1 expression in ‘neo-islets’ inhibits proliferation and promotes apoptosis of local CD4+ T-cells by a Foxp3-independent mechanism**

To determine the mechanism of the decrease in local CD4+ T-cells in Ngn3-Btc+PD-L1 treated mice, we tested the rate of proliferation and apoptosis of the infiltrating T-cells in periportal regions of the liver. BrdU incorporation, indicative of active proliferation, was significantly lower in CD3+ T-cells (Fig. 4C-D) but not in CD8+ T-cells (Fig. 4C-D), consistent with the reduction only in numbers of CD4+ T-cells. Furthermore, an increase in apoptosis was detected by TUNEL staining in CD3+ T-cells infiltrating the neo-islets in Ngn3-Btc+PD-L1 treated mice, as compared to the controls that received only Ngn3-Btc (Fig. 4E-F).

Since, Foxp3+ T-regulatory (Treg) cells have been invoked as mediating tolerance induction by PD-L1 (24;25), we measured the number and expression of Foxp3+ cells in these mice. Surprisingly, the number of Foxp3+ cells in the whole liver, assessed by flow cytometry, was not different between the mice that received PD-L1 and those that did not (Fig. S6A-B), a finding that was corroborated by western blotting of whole liver lysate for Foxp3 protein (Fig. S6C-D). Furthermore, the number of Foxp3+ cells among the infiltrating periportal T-cells also was not different between the two treatment groups (Fig. S6E). This data excluded Foxp3+ Tregs as
playing a major role in the induction and maintenance of peripheral tolerance to the neo-islets with Ngn3-Btc+PD-L1 treatment.

**Inactivation of infiltrating T-cells in the liver results from targeted PD-L1 expression in neo-islets**

We then investigated if PD-L1 expressed on the neo-islets was also leading to local T-cells inactivation. To test this, we assessed, by flow cytometry, the expression of cytokines in T-cells isolated from mice 8 weeks after treatment and stimulated ex vivo using anti-CD3 and anti-CD28. CD4+T-cells expressing IFN-γ (7.38%) or TNF-α (6.63%) were significantly decreased in the livers of mice that received Ngn3-Btc+PD-L1 (Fig. 5A-B), as compared to mice that received Ngn3-Btc but no PD-L1 (16.4% and 13.1% respectively). This led us to conclude that addition of PD-L1 to the neo-islet inducing Ngn3-Btc regimen is sufficient to decrease the activation of the liver T-cells in the periportal ‘fighting’ zone. In parallel experiments we also show that this degree of CD4+ activation is similar to that seen in the liver of untreated diabetic NOD mice (Fig. 5C-D). This was also consistent with a significantly lower TNF-α protein on western blotting of Ngn3-Btc+PD-L1 treated mouse liver lysates, as compared controls that received only Ngn3+Btc (Fig. 5E).

We then explored the molecular mechanisms underlying this local T-cell inactivation by PD-L1 expression on neo-islets. PD-L1 mediated co-stimulation inhibition occurs by inhibiting the TCR-MHC-II/co-stimulation pathways. However, the neo-islets do not express MHC-classII antigens (Fig. S7A). This raised the possibility that a simultaneous spatio-temporal TCR-engagement may not be required for PD-L1 mediated inhibition of co-stimulation. To test this,
we stimulated isolated CD4+T-cells, with anti-CD3 and anti-CD28 antibodies to activate the TCR/co-stimulation pathways. When these stimulated CD4+T-cells were subsequently co-incubated with PD-L1 overexpressing mouse insulinoma cells, there was a significant decrease in IFN-γ and TNF-α positive cells as compared to controls (Fig. 5E-G). This demonstrated that PD-L1 mediated T-cell inactivation can be spatio-temporally separated from TCR-MHC-II engagement. We also identified MHC-II expressing B-cells infiltrating the neo-islet that may be providing the stimulus to activate the TCR signaling cascade in these infiltrating CD4+ T-cells (Fig. S7B-C).

To comprehensively test the effect of the targeted expression of PD-L1 in the neo-islet on the liver cytokine profile, we assessed 23 cytokines in whole liver lysate, using Bio-Plex suspension array system. Many cytokines involved in the pathogenesis of diabetes in NOD mice, including IL-1β, TNF-α, IL-10 and IL-17, were down regulated in the liver, when PD-L1 was added to Ngn3-Btc (Fig. 6A). Interestingly, when compared with the results from that of diabetic NOD mouse pancreas (Fig. 6B), there was a remarkable inverse correlation, in that most of the cytokines that were increased in the NOD diabetic pancreas were decreased in the liver of mice that were treated with Ngn3-Btc+PD-L1, indicating that mechanisms that destroy β-cells in the pancreas have been specifically attenuated by PD-L1 and supports this as a mechanism for the tolerance induction by PD-L1 in this setting.

**Ngn3-Btc-PD-L1 treatment is not associated with systemic immunosuppression**

We then assessed if systemic immunosuppression could have contributed to the observed tolerance in Ngn3-Btc+PD-L1 treated mice. To assess this we isolated lymphocytes from the
spleen of mice treated with Ngn3-Btc, with and without PD-L1 and compared the expression of TNF-α and IFN-γ upon TCR-dependent stimulation with anti-CD3 and anti-CD28. Splenocytes from mice treated with Ngn3-Btc with or without PD-L1 exhibited similar response upon stimulation, in terms of IFN-γ and TNF-α expression (Fig. 7A-C). Other controls for this experiment performed are shown in Fig. S8A-D. We also performed skin allografts from C57Bl6 mice into NOD-diabetic mice that had received Ngn3-Btc with or without PD-L1. Ngn3-Btc+PD-L1 mice rejected the skin graft at the same rate and time course as the controls (data not shown) that did not receive PD-L1, confirming that generalized immunosuppression was not induced by Ngn3-Btc+PD-L1 therapy. Having, thus, excluded systemic immunosuppression as a mechanism for the tolerance to neo-islets in Ngn3-Btc+PD-L1, we then tested to see if the immune system in the liver could clear other antigens. We injected mice treated with Ngn3-Btc with or without PD-L1 with an adenovirus (of a serotype that was different from the original vectors) and assessed viral clearance rate after 3 weeks. This experiment indicated that both groups had similar viral clearance rates (Fig. S9) indicating that PD-L1 expression limited to the neo-islets in the liver did not impair the ability of the liver to clear other antigens.

**Ngn3-Btc+PD-L1 induced neo-islets resist destruction by adoptively transferred diabetogenic splenocytes**

To exclude the possibility that a loss of diabetogenicity of the T-cells in mice that received PD-L1 underlies the tolerance, we performed adoptive transfer of splenocytes from the Ngn3-Btc+PD-L1 and Ngn3-Btc treated mice into NOD-Scid recipients. Splenocytes from Ngn3-Btc+PD-L1 treated donor mice not only were able to robustly transfer diabetes (Fig. 7D),
indicating that there was no reduction in the diabetogenicity of the splenocytes and no peripheral tolerance to islet antigens in the donor Ngn3-Btc+PD-L1 mice. Indeed, they actually led to a significantly earlier onset of diabetes in the recipients as compared to control NOD-Scid mice that received splenocytes from Ngn3-Btc treated donors, which may be a reflection of the persistent islet antigenic presence in the Ngn3-Btc+PD-L1. We then did the converse experiment wherein we performed adoptive transfer using diabetogenic splenocytes from newly diabetic NOD mice and transferred them to NOD-Scid mice (controls) and to euglycemic Ngn3-Btc+PD-L1 treated NOD-diabetic mice. The Ngn3-Btc+PD-L1 mice remained completely resistant to diabetes up to 15 weeks of the study, while all the NOD-Scid recipients became diabetic by 7 weeks (Fig. 7E). These experiments demonstrate that Ngn3-Btc+PD-L1 treatment induced a local tolerance in the periportal regions despite the presence of diabetogenic T-cells in the body leading to the persistence of the neo-islets and a long-term reversal of diabetes in NOD mice.

**Discussion**

While there have been innumerable studies that have been reported to decrease the incidence of diabetes in NOD mice (26), there have been only a few successful interventions that reverse diabetes after disease onset. Most notable success has been achieved by targeting the TCR complex by anti-CD3 antibodies (27-30) and anti-thymocyte globulin (31-34), though generalized immunosuppression limits the translational applicability of these approaches. Other targeted therapies including antigen-specific approaches by using DC-expanded islet specific Tregs (35), IL-2 therapy expanded Tregs (36), have had some success in restoring euglycemia, though an enduring response was rarely seen and never with a high efficacy. Recently, mixed hematopoietic chimerism achieved by anti-CD3/anti-CD8 conditioning regimen with bone
marrow transplantation to inhibit autoimmunity reversed diabetes in 60% of NOD mice when combined with 2 months of daily injections of EGF and Gastrin to induce β-cell formation (37).

In this study, we tested if engineered neo-islets that specifically resist T-cell targeted destruction offer a cure for diabetic NOD mice. With targeted expression of PD-L1 in the Ngn3-Btc induced insulin-expressing cells, we demonstrate that this approach is sufficient to reverse diabetes by persistent functional neo-islets in the liver. In data not shown, these treated mice remain euglycemic for prolonged periods up to a year without any untoward effects such as liver dysfunction, tumors or hypoglycemia. We show that this occurs due to PD-L1 mediated reduction in activated T-cells locally around the neo-islets, without systemic immunosuppression. Our data is consistent with previous studies demonstrating that the expression of PD-L1 induces tolerance by a reduction in the number of infiltrating T-cells, both by inducing apoptosis while inhibiting proliferation of T-cells (38).

While Foxp3+ Tregs have also been shown to mediate the tolerance induction by PD-L1 in some settings (25), our data indicates that the locally induced tolerance to the neo-islets is Foxp3-independent. This is also consistent with other studies that reveal that systemic development of Tregs may not occur when manipulation of immune responses is done in an organ-specific or limited to a microenvironment (39-41). While the liver microenvironment has been suggested to be more tolerogenic than other tissues (42), it may have a contributory but not a determinant role in this study as the neo-islets induced in the control mice that received Ngn3-Btc were rapidly destroyed and the expression of PD-L1 on the neo-islets was required for their continued survival. The persistent diabetogenicity of splenocytes in the mice that expressed PD-L1 on the neo-islets and the resistance of these neo-islets to destruction by extraneous diabetogenic splenocytes demonstrate the critical tolerogenic role played by modulation of the
microenvironment surrounding the neo-islets without perturbing the systemic immune system. Our experiments also demonstrate that it is not required for the PD-L1 expression to be on the same cell that carries the MHC-II to engage the TCR on the CD4+ cells.

There have been some paradoxical observations in transgenic models wherein PD-L1 was overexpressed in β-cells in different genetic backgrounds – overexpression in a B6 background led to an increase in allograft rejection and induction of autoimmune diabetes (43), whereas in the NOD background there has been a clear protection from diabetes (15). However, the preponderance of evidence in NOD mice points to a significant inhibition of T-cell response and protection with the activation of the PD/PD-L1 pathway in both transgenic and transplant models (12;15;44;45). These studies are consistent with our observation that expression of PD-L1 in target cells leads to a decrease in activation of T-cells, and an inhibition of their proliferation.

One other study, using a first-generation adenoviral vector-mediated gene transfer of Pdx-1 to the liver, reported induction of insulin expression in hepatocytes with a decrease in hyperglycemia in less than half of the mice treated (46). A decreased diabetogenicity of the splenocytes from treated mice was reported as the reason for the tolerance, though the mechanism of this decreased diabetogenicity was not defined. In contrast to this, splenocytes from the treated mice in our study displayed increased diabetogenicity in destroying recipient pancreatic islets in adoptive transfer experiments. This is likely to be secondary to the persistent antigen exposure from the neo-islets that would perpetuate diabetogenic lymphocytes in the body.

A recent study demonstrated the cell-intrinsic requirement of PD-1 expression on islet-reactive CD4+ T-cells in diabetes induction (45;47). This is consistent with a model wherein the PD-L1
expressed on the neo-islets engages the PD-1 on the infiltrating T-cells to inactivate them for tolerance induction. However, other studies have identified the important role of PD-L1 binding to B7-1 mediating T-cell inhibition (48;49), specifically in diabetogenesis in NOD mice (50). The current study was not designed to address this question and future experiments will have to address whether the binding of PD-L1 to PD-1 or B7-1 mediates the tolerance induction seen in this study.

With the regimen of Ngn3-Btc+PD-L1, 24% of the mice did not respond to tolerance induction and remained diabetic and displayed significant ‘insulitis’ and loss of insulin-positive ‘neo-islets’, similar to mice that did not receive PD-L1. It is likely that other pathways that are not regulated by the inhibition of co-stimulation by PD-L1 exist in these mice and unraveling the mechanisms that underlie the resistance to tolerance induction will require further study.

In summary, we demonstrate that Ngn3-Btc gene transfer is adequate to induce ectopic islet neogenesis in the liver of diabetic NOD-mice and reverse diabetes when combined with targeted PD-L1 expression to inhibit the co-stimulatory pathway selectively in T-cells infiltrating the newly induced β-cells. To our knowledge, this is the first report of a successful enduring reversal of diabetes that was achieved in a majority of overtly diabetic NOD mice by inducing islet neogenesis with concurrent β-cell specific tolerance without systemic immunosuppression. This treatment strategy may be an attractive and viable approach to β-cell replacement therapy of autoimmune type 1 diabetes.
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**Figure Legends**

**Fig. 1:** Ngn3-Btc+PD-L1 reverses hyperglycemia and restores glucose tolerance. (A, B) Blood glucose and body weight in diabetic NOD mice. Ngn3-Btc+PD-L1 group (n=16), n=4-7 for the other groups. (C) Non-fasting plasma insulin at indicated time points. (n=4-8). (D) Plasma aspartate aminotransferase (AST) and (E) alanine aminotransferase (ALT) at the indicated time points. n=4-6. (F-I) Plasma glucose and insulin during an IP-GTT at 4 weeks (F, G) and 8 weeks (H, I). Non-diabetic (green); PD-L1 only (blue); Ngn3-Btc (purple); Ngn3-Btc+PD-L1 (orange). All values are mean±SEM; * p≤0.05.

**Fig. 2:** Ngn3-Btc+PD-L1 induces neo-islets in the periportal regions of the liver. (A-E) Representative sections of Ngn3-Btc+PD-L1 treated diabetic NOD mouse liver stained by immunohistochemistry for (A) insulin; (B) C-peptide; (C) glucagon; (D) somatostatin (SST); (E) pancreatic polypeptide (PP) at indicated time points after diabetes onset or treatment. Scale bar represents 20µm. PV-Portal vein. Of note, periportal cluster of cells are only occasionally seen in PD-L1 group and are shown in panels A&B to contrast their staining with that of the Ngn3-Btc and Ngn3-Btc+PD-L1 groups. Most of the periporal areas in the PD-L1 group are similar to those shown in panels C-E.

**Fig. 3:** Neo-islets express islet hormones and transcription factors. RT-qPCR showing the expression of (A) islet hormones and (B) transcription factors involved in islet development, at 8 weeks. n=4-5, all values are mean±SEM; * p≤0.05. (C-D) Representative sections of liver stained for Pdx-1 and Nkx 6.1 in different groups. Scale bar represents 20µm. PV-Portal vein.
**Fig. 4:** Ngn3-Btc+PD-L1 treatment reduces the local number of CD4+ T-cells infiltrating the peri-portal neo-islet clusters. (A) Representative sections in control and Ngn3-Btc+PD-L1 treated NOD mice liver stained for CD3, CD4 and CD8. (B) CD3, CD4 and CD8 positive cells represented as a percent of all cells only in periportal clusters. (C) Representative sections in the liver of control and Ngn3-Btc+PD-L1 treated NOD mice co-stained for Brdu with CD3, CD4 or CD8. (D) Percent of BrdU positive T-cells in the peri-portal clusters. (E) Representative sections in the liver of control and Ngn3-Btc+PD-L1 treated NOD mice co-stained for TUNEL and CD3. (F) Percent of TUNEL positive T-cells in peri-portal clusters. All values are mean±SEM; *p≤0.05. n=4-5 for each group. Scale bar represents 50µm.

**Fig. 5:** PD-L1 protects neo-islets by inactivating T-cells. (A-B) IFN-γ and TNF-α positive CD4+ T-cells isolated from livers of treated mice after ex vivo stimulation with anti CD3 and anti CD28 on FACS analysis. Representative dot plot from one set of mice is shown in (A) and quantification from 3-5 separate mice in (B). (C) IFN-γ and TNF-α positive CD4+ T-cells isolated from liver or spleen of non-diabetic or diabetic mice after ex vivo stimulation with anti-CD3 and anti-CD28 on FACS analysis. Representative dot plot from one set of mice is shown in (C) and quantification from 3-4 separate mice in (D). Control shown is unstimulated CD4+ T-cells from the spleen of Ngn3-Btc treated (A) or untreated diabetic (C) mice. (E) Western blotting of whole liver lysate for TNF-α at 8 weeks after treatment. All values are mean ± SEM; * p≤0.05, # p≤0.06.

**Fig. 6:** (A-C) IFN-γ and TNF-α protein was assessed in isolated CD4+ T-cells purified from the spleen of non-diabetic NOD mice that were first stimulated with anti-CD3 and anti-CD28 for 2
days and then co-incubated with HDAd-empty or HDAd-PD-L1 virus infected β-cells
(Insulinoma - Ins2 cells) for 24 hrs. Representative dot plot of one set of experiments is shown in
(A-B) and quantification from 4 separate experiments in (C). CD4-empty: stimulated CD4+ cells
co-incubated with β-cells infected with HDAd-empty; CD4-PD-L1: stimulated CD4+ cells co-
incubated with β-cells infected with HDAd-empty PD-L1. Control indicates unstimulated CD4+
cells. All values are mean ± SEM; * p≤0.05.

Fig. 7: The levels of 23 cytokines in the livers (A) of Ngn3-Btc and Ngn3-Btc+PD-L1 treated
NOD mice or in the pancreas (B) of diabetic NOD mice (5-6 weeks after diabetes onset) and
non-diabetic NOD mice (27 weeks old) analyzed by Bioplex tissue cytokine assay. N=3-5/group.
All values are mean±SEM. * p≤0.05; #p value between 0.05-0.06.

Fig. 8: No peripheral immunosuppression in the mice treated with Ngn3-Btc+PD-L1. (A-C)
IFN-γ and TNF-α positive CD4+ T-cells isolated from spleens of treated mice after ex vivo
stimulation with anti CD3 and anti CD28 on FACS analysis. Representative dot plot from one set
of mice is shown in (A-B) and quantification from 3-5 separate mice in (C). (D) Diabetes
induction in NOD-Scid mice after adoptive transfer of splenocytes from Ngn3-Btc-PD-L1 or
controls. (E) Diabetes induction in Ngn3-Btc-PD-L1 or control mice after adoptive transfer of
splenocytes from newly diabetic NOD mice. n=3-5 group. All values are mean±SEM; * p≤0.05.
Fig. 1

A. Blood Glucose (mg/dl) over time with different treatments.

B. Body Weight (gm) over time with different treatments.

C. Plasma Insulin (ng/ml) over time with different treatments.

D. Plasma AST (U/L) over time with different treatments.

E. Plasma ALT (U/L) over time with different treatments.

F. Plasma Glucose (mg/dl) with different treatments and time points.

G. Plasma Insulin (ng/ml) with different treatments and time points.

H. Plasma Glucose (mg/dl) with different treatments and time points.

I. Plasma Insulin (ng/ml) with different treatments and time points.
Fig. 2

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**Insulin**

**C-peptide**

**Glucagon**

**SST**

**PP**
Fig. 3

A

B

C

D

Relative mRNA Level

Relative mRNA Level

0 4 8

0 5 10 15 20

Ins-1, Ins-2, Glucagon, PP, SST, Ghrelin

Pdx-1, Ngn3, Isl-1, Neurod1, Pax4, Pax6, Nkx6.1

4w 8w 12w

Non-diabetic, PD-L1, Ngn3+Btc, Ngn3+Btc+PD-L1

Nkx6.1, Pdx-1

PV

PV

PV

PV

PV

PV

PV

PV

PV

PV
Fig. 4

A. CD3, CD4, and CD8 expression in periportal clusters. Ngn3-Btc and Ngn3-Btc+PD-L1 conditions shown.


C. CD3, CD4, and CD8 expression with BrdU among T cells in periportal clusters. Ngn3-Btc and Ngn3-Btc+PD-L1 conditions shown.


E. CD3, CD4, and CD8 expression with Tunel among CD3+ in periportal clusters. Ngn3-Btc and Ngn3-Btc+PD-L1 conditions shown.

F. Percentage of Tunel+ among CD3+ in periportal clusters. Ngn3-Btc and Ngn3-Btc+PD-L1 compared.
Fig. 5

A

B

C

D

E

Diabetes
**Fig. 6**

### A

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<td>TNF-α</td>
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### B

Bar chart showing the comparison of IFN-γ and TNF-α expression between CD4-Empty and CD4-PD-L1 conditions.
Fig. 7

![Graph showing relative protein levels of various cytokines and chemokines in non-diabetic and diabetic pancreas. The graph compares Ngn3+Btc liver and Ngn3+Btc+PD-L1 liver.](image-url)
Fig. 8

A

IFN-γ

Ngn3+Btc

Ngn3+Btc+PD-L1

Control

10^4

10^6

10^8

10^10

10^12

Ngn3+Btc

Ngn3+Btc+PD-L1

Control

10^4

10^6

10^8

10^10

10^12

B

TNF-α

CD4

10^4

10^6

10^8

10^10

10^12

Ngn3+Btc

Ngn3+Btc+PD-L1

Control

10^4

10^6

10^8

10^10

10^12

C

IFN-γ + or TNF-α + among CD4 (+%)

Ngn3+Btc

Ngn3+Btc+PD-L1

0

2

4

6

8

10

12

14

16

IFN-γ

TNF-α

p=0.022

D

Fraction Diabetes free

Ngn3+Btc+PD-L1 (Donor)

Ngn3+Btc (Donor)

p=0.039

E

Fraction Diabetes free

Ngn3+Btc+PD-L1 (Recipient)

NOD-Scid (Recipient)

p=0.022

Weeks
Supporting Figure Legends

**Fig. S1**: Ngn3-Btc+PD-L1 induces neo-islets in the periportal regions of the liver. (A) Representative sections of Ngn3-Btc+PD-L1 treated diabetic NOD mouse liver stained by immunohistochemistry for insulin; C-peptide; glucagon; somatostatin (SST); pancreatic polypeptide (PP) at indicated time points after treatment. Periportal neo-islets are shown encircled by the red line. (B-C) Insulin staining in the pancreas with lower (C) and higher (D) magnifications of a neo-islet is indicated by the red arrow in (B). Scale bar represents 20μm in (A), 100μm in (B) and 50μm in (C). PV-Portal vein.

**Fig. S2**: PD-L1 and PD-L2 expression level in the liver or pancreas of Ngn3-Btc and Ngn3-Btc+PD-L1 treated NOD mice. (A) PD-L1 staining in the liver or pancreas of Ngn3-Btc and Ngn3-Btc+PD-L1 treated NOD mice. (B) PD-L1 and PD-L2 RNA level in the liver of Ngn3-Btc and Ngn3-Btc+PD-L1 treated NOD mice. Scale bar represents 20μm.

**Fig. S3**: Neo-islets express islet hormones and transcription factors. RT-qPCR showing the expression of (A) islet hormones and (B-C) transcription factors involved in islet development at 4 weeks and non diabetic and diabetic NOD control mice. n=4-5, all values are mean±SEM; * p≤0.05.

**Fig. S4**: Neo-islets induced by Ngn3-Btc+PD-L1 in the liver expresses the hepatic oval cell marker. (A) A6 staining. (B) OC2-1D11 and CD3 staining in different groups. Scale bar represents 20μm.
**Fig. S5**: There is no difference in the number of CD4 + and CD8+ cells in the whole liver of Ngn3-Btc and Ngn3-Btc+PD-L1 treated mice. Representative dot plot of CD4+ (A) and CD8+ (C) T cells among CD3+ T cells in whole liver treated with Ngn3-Btc or Ngn3-Btc+PD-L1. (B, D) Percentage of CD4+ (D) and CD8+ (F) among CD3+ T cells in whole liver. All values are mean±SEM; * p≤0.05. n=4-5 for each group. Scale bar represents 50μm.

**Fig. S6**: There is no difference in the number of Foxp3+ in the whole liver or in the periportal regions of the liver of Ngn3-Btc and Ngn3-Btc+PD-L1 treated mice. (A) Representative dot plot of Foxp3+ among CD4+ T cells in whole liver treated with Ngn3-Btc or Ngn3-Btc+PD-L1. (B) Percentage of Foxp3+ among CD4+ T cells in whole liver. (C-D) Western Blot from the whole liver also shows no change of Foxp3 expression in the liver of different treated groups. (E) The quantitation for counting the number of Foxp3+ among CD4+ T-cells in the periportal clusters in different treated groups N=4-5/group. All values are mean±SEM.

**Fig. S7**: Neo-islets do not express MHCII antigens. (A) MHCII staining does not co-localize with c-peptide staining in neo-islets. (B) B220 (B cell marker) positive cells and CD4 positive cells are both present infiltrating the periportal neo-islet. (C) MHCII is expressed in the B220 positive B lymphocytes in the periportal area. Scale bar represents 50μm.

**Fig. S8**: TNF-α and IFN-γ production in CD4 lymphocytes in different tissues of recent onset diabetic NOD mice. Representative dot plot and quantitation of IFN-γ production (A,C) and TNF-α (B,D) in the spleen, pancreas, pancreas lymph node and mesentery lymph node of
diabetic NOD mice. Control shown is unstimulated CD4+ T-cells from the spleen of untreated NOD mice. C & D represent the quantification from 3 separate mice in the group.

**Fig. S9:** Virus clearance shows no immunosuppression in the mice treated with Ngn3-Btc+PD-L1. (A) 1*10^11 helper virus was iv injected to Ngn3-Btc and Ngn3-Btc+PD-L1 treated mice for 3d and 3w. Q-PCR shows relative virus clearance in different mice. N=2-3/group.
**Fig. S1**

**Ngn3-Btc+PD-L1**

**A**
- Insulin
  - 8 weeks
  - 12 weeks
- C-peptide
- Glucagon
- SST
- PP

**B**
- Insulin
  - Non-diabetic
  - PD-L1

**C**
- Insulin
  - Ngn3-Btc+PD-L1

Liver

Pancreas

4 weeks

4 weeks

Diabetes
Fig. S2

A

Liver

Pancreas

Ngn3-Btc

Ngn3-Btc+PD-L1

B

Relative mRNA Level

*  

Ngn3+Btc

Ngn3+Btc+PD-L1

Relative mRNA Level

PD-L1  

PD-L2

Diabetes
Fig S3

A

Relative mRNA Level

Ins-1  Ins-2  Glucagon  PP  SST  Ghrelin

B

Relative mRNA Level

Pdx-1  Isl-1  Neurod1  Pax4  Nkx2.2  Pax6  Nkx6.1

C

Relative mRNA Level

Ngn3  Ngn3+Btc  Ngn3+Btc+PD-L1

Fig S3
Fig. S4

A

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B

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Fig. S5

In the whole liver

A

B

C

D

Ngn3-Btc

Ngn3-Btc+PD-L1

Ngn3-Btc

Ngn3-Btc+PD-L1

CD4+ among CD3+ in whole liver (%)

CD8+ among CD3+ in whole liver (%)

CD4

CD3

CD8

CD3
Fig. S6

A

Ngn3-Btc

Ngn3-Btc+PD-L1

CD4

FOXP3

0.00% 14.1%

0.00% 16.3%

foxp3 among CD4+ in the whole liver (%)

B

In the whole liver

FOXP3 among CD4+ in whole liver (%)

Ngn3-Btc

Ngn3-Btc+PD-L1

C

FOXP3

GAPDH

Ngn3+Btc

Ngn3-Btc+PD-L1

D

FOXP3 Relative Level

Ngn3-Btc

Ngn3-Btc+PD-L1

E

In the periportal cluster

FOXP3 among CD4+ in periportal clusters (%)
Fig. S8

A

Spleen
Pancreas
PLN
MLN
Control

B

Spleen
Pancreas
PLN
MLN
Control

C

1
0.8
0.6
0.4
0.2

IFN-γ among CD4

0
Spleen
Pancreas
PLN
MLN

D

1.6
1.2
0.8
0.4

TNF-α among CD4

0
Spleen
Pancreas
PLN
MLN
Fig. S9

Relative virus clear rate in 3 weeks (%)

Ngn3-Btc  Ngn3-Btc-PD-L1

3d  3w