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Gene therapy with Neurogenin3, Betacellulin and SOCS-1 Reverses Diabetes in NOD Mice

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Abstract

Islet transplantation for Type 1 diabetes is limited by a shortage of donor islets and requirement for immunosuppression. We approached this problem by inducing *in vivo* islet neogenesis in NOD diabetic mice, a model of autoimmune diabetes. We demonstrate that gene therapy with helper-dependent adenovirus (HDAd) carrying neurogenin3, an islet lineage-defining transcription factor and betacellulin, an islet growth factor, leads to the induction of periportal insulin-positive cell clusters in the liver, which are rapidly destroyed. To specifically accord protection to these 'neo-islets' from cytokine-mediated destruction, we overexpressed suppressor of cytokine signaling 1 (SOCS1) gene, using a rat insulin promoter in combination with neurogenin3 and betacellulin. With this approach, about half of diabetic mice attained euglycemia sustained for over 4 months, regain glucose tolerance and appropriate glucose-stimulated insulin secretion. Histological analysis revealed periportal islet hormone-expressing 'neo-islets' in treated mouse livers. Despite evidence of persistent 'insulitis' with activated T-cells, these 'neo-islets' persist to maintain euglycemia. This therapy does not affect diabetogenicity of splenocytes, as they retain the ability to transfer diabetes. This study thus provides a proof-of-concept for engineering *in vivo* islet neogenesis with targeted resistance to cytokine-mediated destruction to provide a long-term reversal of diabetes in NOD mice.

Keywords

Gene therapy; islets; NOD; diabetes; SOCS1; Ngn3; liver

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Introduction

Type 1 diabetes (T1D) is caused by absolute insulin deficiency resulting from destruction of β-cells by auto-reactive $CD4^+$ and $CD8^+$ T-cells. This breakdown in tolerance to β-cell self-antigens is thought to be the primary factor involved in disease initiation and progression. These effector T-cells initiate β-cell apoptosis and cell-death by initiating multiple pathways including those activated by cytokines, FAS, perforin-granzyme and others[1, 2]. Suppressors Of Cytokine Signaling (SOCS) proteins are characterized by their ability to inhibit the Jak/STAT pathway and thus suppress intracellular signaling of cytokines, especially interferon γ (IFNγ)[3–5]. This regulatory mechanism was shown to robustly prevent β-cell destruction in transgenic mice over-expressing SOCS1 in β-cells of non-obese diabetic (NOD) mice and in transplant models[6–12].

Islet transplantation as a definitive treatment for type 1 diabetes is severely limited by the availability of donors, and the eventual failure of most transplanted islets because of multiple factors related to immune mediated destruction, metabolic dysfunction, and side effects from immunosuppressive medications and others[13–16]. We have demonstrated previously in streptozotocin (STZ)-diabetic mice, a non-autoimmune insulin deficient diabetes mouse model, that Helper-dependent Adenoviral (HDAd) mediated gene delivery of Neurogenin 3 (Ngn3) and betacellulin (Btc) leads to transdetermination of hepatic oval cells into insulin-secreting cells and diabetes reversal[17]. In this report we show that while this gene therapy leads to induction of insulin-expressing cell clusters in periportal areas of the liver, these ‘neo-islets’ are quickly destroyed by infiltrating T-cells with a return of hyperglycemia. Furthermore, we have developed a novel gene therapy-based strategy that protects the newly formed islets induced by the HDAd-Ngn3-Btc treatment and reverses overt diabetes in a major proportion of T1D NOD mice.

We hypothesized that these Ngn3-Btc induced ‘neo-islets’ in the liver could be protected by suppression of cytokine signaling with overexpression of SOCS1. To test this, we used HDAd-Ngn3-Btc gene delivery to induce ‘neo-islets’ in diabetic NOD mice and combined it with HDAd-RIP-SOCS1 treatment, wherein the rat insulin promoter (Rip)-driven SOCS1 is expressed only in the insulin-producing cells. We demonstrate that, with this approach, a long-term reversal of diabetes is attained in about half of all treated diabetic mice. These mice achieve euglycemia, display normal insulin levels, *in vivo* glucose-stimulated insulin secretion (GSIS) and display insulin expressing islet-like clusters in the periportal areas of the liver. Interestingly, these ‘neo-islets’ despite being surrounded by infiltrating T-cells, akin to insulitis seen in the pancreas, continue to express mature islet hormones and islet transcription factors. Thus, this study provides a proof-of-concept that induction of *in vivo* islet neogenesis and protecting them by modulating immune-mediated β-cell destruction is a viable approach to treating Type 1 diabetes.

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Results

Ngn3-Btc gene delivery leads to an evanescent partial amelioration of hyperglycemia in NOD mice

We have previously demonstrated that gene delivery of Ngn3 and Btc, in combination, induces the formation of new islet-like insulin-expressing cell clusters ('neo-islets') in the periportal regions of the liver that effectively reverse STZ-induced insulin deficient diabetes[17]. We, now, set out to test if this approach is similarly effective in the autoimmune diabetes setting using the NOD mouse model. We followed the NOD mice with weekly blood glucose (9–10AM) and body weight measurements from 8 weeks of age. After the onset of diabetes, defined as two consecutive blood glucose measurements >250 mg/dl, separated by 24 hours, we injected the mice with either HDAd carrying Ngn3 (HDAd-Ngn3 – 5×10^{11} vp) and Btc (HDAd-Btc – 1×10^{11} vp) or empty vector intravenously (IV), within a 48 hr window. Serial blood glucose and body weight determinations revealed that there was only a partial and transient amelioration of the hyperglycemia in the HDAd-Ngn3+Btc mice for the first 1–3 weeks followed by a rapid return to profound hyperglycemia, decreasing body weight and subsequently death within 6–8 weeks (Fig. 1A–B) after the onset of diabetes.

Ngn3-Btc gene delivery induces ectopic islet neogenesis in the liver that is rapidly destroyed by autoimmunity

The initial response, though transient, correlated to that seen in the non-autoimmune STZ-diabetes setting, as previously reported[17, 18]. This led us to reason that the transient response may be the destruction of any induced 'neo-islets' by a resurgent autoimmunity. To test this hypothesis, we sacrificed mice at 4 weeks after treatment and assessed the liver for the presence of insulin expressing cells. Insulin expressing cell clusters were seen in the periportal regions of the liver only in the mice treated with HDAd-Ngn3-Btc, but not in the untreated diabetic mice (Fig. 2A–B). On further examination, there was a significant infiltration of these insulin-expressing cell clusters with lymphocytes (CD3+ cells in Fig. 2C–D), akin to insulitis seen in the diabetic NOD pancreas (Fig. S1).

Ngn3-Btc-RipSOCS1 gene delivery leads to reversal of hyperglycemia

Since, Ngn3-Btc gene delivery was able to successfully induce insulin-expressing cells in the liver, at least transiently, even in the autoimmune setting of diabetic NOD mice, we reasoned that engineering these neo-islets to resist autoimmunity-driven cytokine-mediated cell death would be an effective approach to accord these 'neo-islets' immune-protection to reverse diabetes. Suppressor of Cytokine signaling (SOCS) proteins are induced by cytokines and inhibit the signaling to limit their downstream effects of these cytokines as a negative-feedback loop[4]. However, SOCS-1 may have possible undesirable effects when it is expressed in hepatocytes, as it inhibits insulin-induced IRS-1/2 phosphorylation leading to insulin resistance[19]. To circumvent this possible adverse action of SOCS-1 in hepatocytes, we transferred the SOCS-1 cDNA that is driven by the rat insulin promoter using HDAd, so that SOCS-1 expression would be limited to the insulin-expressing neo-islets induced in the liver, protecting them against cytokine mediated cell death, whereas the non-insulin-

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expressing hepatocytes would not express the SOCS-1 transgene and be spared of its possible undesirable action.

We administered HDAd-Ngn3-Btc with either empty HDAd or with HDAd-RipSOCS-1 (1×10^{11} vp) to diabetic NOD mice and monitored their weekly blood glucose and body weight. As shown in Fig. 3A–B, there was a significant response, in which about half of diabetic mice (13 out of 28) injected with combination HDAd-Ngn3-Btc-RipSOCS1 demonstrated a complete reversal of hyperglycemia (Fig. 3A), and an increase of body weight (Fig. 3B), in contrast to the two control groups that received only HDAd-Ngn3+Btc (+ empty HDAd) or only HDAd-Rip-SOCS1. The return to euglycemia was accompanied by a normalization of fasting plasma insulin (Fig. 3C). Interestingly, even the mice treated with HDAd-Ngn3-Btc-RipSOCS1 that did not have a sustained response and remained hyperglycemic maintained better body weight (non-responders, Fig. 3B) and survived a few weeks longer than the other two control groups not treated with HDAdRipSOCS1. This was probably secondary to some low level insulin production that was restored, as these mice still had detectable fasting plasma insulin at a level that was higher than the untreated diabetic mice (Fig. 3C). The restoration of euglycemia and plasma insulin in the mice treated with HDAd-Ngn3-Btc-RipSOCS1 was not due to insulin secretion from the pancreas as there were no residual islets detectable in the pancreas of these mice (Fig. S1). There was no evidence of hepatotoxicity as both the morphology of hepatocytes and serum AST levels were normal in the treated mice (Fig. S2).

Ngn3-Btc-RipSOCS1 gene delivery leads to reversal of glucose intolerance and restoration of *in vivo* glucose-stimulated insulin secretion (GSIS)

Since, GSIS is the hallmark of a mature and fully functional β-cell; we assessed glucose tolerance and GSIS during a glucose tolerance test (GTT) on the treated mice. The treated mice had a normal glucose tolerance that was accompanied by a normal *in vivo* GSIS as measured by plasma insulin during the GTT (Fig. 4A–B). This was in contrast to untreated diabetic mice that had no significant increase in their plasma insulin during the GTT. This normalization of glucose intolerance seen in diabetic NOD mice strongly indicated that there were normally functioning insulin secreting β-cells that were induced by treatment with HDAd-Ngn3-Btc-RipSOCS1.

Ngn3-Btc-SOCS1 gene delivery leads to neo-islet formation in the periportal areas of the liver

Our previous work in non-autoimmune STZ-diabetes model indicated that HDAd-Ngn3-Btc induced neo-islets from oval cells in the periportal areas of the liver without any induction of islet regeneration in the pancreas[17]. We, therefore, tested if indeed there were insulin positive ‘neo-islets’ in the portal triads of the treated diabetic NOD mice. A similar induction of A6 positive oval cell clusters are prominently seen in all Ngn3-Btc treated NOD mice (Fig S3). As shown in Fig. 5A, there were insulin-producing clusters of cells in these periportal areas in the mice that received Ngn3-Btc-RipSOCS1 but not in untreated diabetic mice and only rarely in the mice that received Ngn-Btc but not Rip-SOCS1. This strongly suggested that the HDAd-Ngn3-Btc gene delivery induced insulin positive cells in the liver and that Rip-SOCS1 was instrumental in the reversal of hyperglycemia with

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restoration of plasma insulin by allowing the persistence of these ‘neo-islets’. In addition, these neo-islets also express other islet hormones, such as glucagon, SST and pancreatic polypeptide (Fig. 5B and Fig. 5F) along with islet specific transcription factors in the liver, including Nkx6.1 and Pdx1 (Fig. 5D–F). These cells persistently expressed high levels of Ngn3 (Fig. S4). Interestingly, the mice that had received HDAd-Btc-RipSOCS1 but did not have a sustained reversal of hyperglycemia (non-responders) still exhibited the other islet hormone expressing cells (data not shown) surrounded by ‘insulitis’ in the periportal regions. This suggested that even in the non-responders the ‘neo-islets’ were induced, but unlike in the responder mice, in these mice the insulin-expressing cells were likely destroyed by the infiltrating lymphocytes.

Ngn3-Btc-SOCS1 gene delivery leads to persistence of ectopic neo-islets in the liver despite surrounding insulitis

Our strategy of induced overexpression of SOCS1 only in the insulin positive cells in the liver would be expected to impair the intracellular cytokine signaling in these cells but not affect the activation of and migration of lymphocytes to the periportal areas harboring the ‘neo-islets’. Hence, not surprisingly, there is a significant collection of lymphocytes, as detected by their CD3 positive expression (Fig. 6) in the periportal areas, suggestive of ‘insulitis’, of all mice that were treated with Ngn3-Btc, whether or not they received RipSOCS1 in addition. Furthermore, this was also accompanied by a significant expression TNF-α, suggesting activation of these lymphocytes invading the periportal areas of the liver (Fig. 6). These results demonstrate that the NOD mice did mount an autoimmune response against the induced ‘neo-islets’ in the liver, but were unable to destroy the ‘neo-islets’ that received Ngn3-Btc-RipSOCS1, as indicated by a persistence of the insulin positive cell clusters.

Ngn3-Btc-SOCS1 does not lead to peripheral immunosuppression

This approach to protect the ‘neo-islets’ from immune-mediated cell death by limiting the transgene expression of SOCS1 to the neo-islets in the liver would not be expected to lead to peripheral immunosuppression or immune tolerance. However, to definitively test this we performed an adoptive transfer of splenic lymphocytes harvested from HDAd-Ngn3-Btc-RipSOCS1 treated mice that became euglycemic and HDAd-Ngn3-Btc treated mice that remained hyperglycemic, into 6 week old female NOD-Scid mice. On follow-up of weekly blood glucose there was no difference in the development of diabetes between the two groups (Fig. 7). This indicated that the splenocytes from the Ngn3-Btc-RipSOCS1 treated mice were equally diabetogenic as those mice that were hyperglycemic and had not received RipSOCS1. In other words, Rip-SOCS1 did not induce any peripheral immunosuppression and this was not the cause of the observed response of diabetes reversal.

In summary these results demonstrate that HDAd-Ngn3-Btc could be used to induce ‘neo-islets’ in the liver of diabetic NOD mice and that these ‘neo-islets’ could be protected from autoimmune destruction by overexpression of SOCS-1 without peripheral immunosuppression.

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Discussion

With increasing evidence that current immunosuppressive protocols for islet transplantation have fallen short of enabling a long term cure of type 1 diabetes[16, 20, 21], the need for alternate therapeutic strategies becomes more urgent. To circumvent the need for donors and associated immunosuppression for alloimmunity, we have adopted a gene therapy approach to induce islet neogenesis *in vivo*. We have previously shown that Ngn3-Btc gene therapy induces hepatic oval cells to transdetermine into insulin and other islet hormone-expressing 'neo-islets' that reverse insulin deficient STZ-diabetes[17]. In this study we tested this approach in an autoimmune setting to determine if these 'neo-islets' will trigger autoimmunity and if so, could these be engineered to acquire target cell-specific resistance to immune destruction to induce long-term reversal of diabetes in NOD mice.

In this study we first provide evidence that gene delivery of Ngn3 in combination with betacellulin is sufficient to induce 'neo-islets' that express insulin, in the liver of diabetic NOD mice, but these are targeted by autoreactive T-cells culminating in their destruction. This implies that the 'neo-islets' induced by Ngn3-Btc appear to behave, immunologically, in a very similar fashion as mature native or transplanted islets in that they trigger an identical autoimmune response that targets pancreatic islets. This, of in itself, is very different from earlier gene therapy studies that had attempted in a reversal of diabetes in NOD mice by either producing insulin in the hepatocytes (NOD-PEPCK-Ins Tg)[22] or converting the hepatocytes into insulin producing cells using PDX1 gene therapy[23], as in both of these studies the insulin producing cells behave differently as compared to native mature β-cells and appear not to be targeted indicating that these were not recognized as β-cells by the immune system.

We then demonstrate that an overexpression of SOCS1, a suppressor of cytokine signaling, in these neo-islets is sufficient to accord protection from autoimmune destruction in about half of diabetic mice and render them euglycemic for an extended period of time. As expected, the SOCS1 overexpression does not prevent 'insulitis' in the liver or the pancreas. However, the 'neo-islets' in the liver are protected from destruction as evidenced by their persistence despite the insulitis. This is consistent with previous reports indicating persistent insulitis in the pancreas of NOD-transgenic mice expressing SOCS1 in the β-cells[8, 24]. It has also been suggested that in addition to the direct effects of cytokine signaling, SOCS-1 may protect β-cells by affecting target cell recognition[24]. However, in this study, the overexpression of SOCS1 did not induce a suppression of the diabetogenicity of peripheral T-cells, as splenocytes from these mice induce diabetes in adoptive transfer experiments. Interestingly, these results are in contrast to those from Ad-Rip-PDX1 gene therapy treated NOD mice, wherein the authors found that the mice treated with Ad-Rip-PDX1 developed immunotolerance and had reduced diabetogenicity on adoptive transfer experiments though the mechanisms remain unexplained[23]. Furthermore, it has been reported that the lymphocytes infiltrating the islets from NOD-SOCS1Tg mice did have a reduced diabetogenicity and this was thought to be resulting from SOCS1 in the target β-cell preventing apoptosis-driven antigen release and thus reducing the presentation of antigens and subsequent activation of more T-cells[8]. It remains to be determined if this was also the case with the T-cells surrounding the 'neo-islets' in the liver.

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We achieved a reversal of hyperglycemia in about half (13/28 mice) of the treated mice with the overexpression of SOCS1 in the 'neo-islets'. The remaining mice did display 'neo-islets' with other hormone expressing cells but appear to have lost only the insulin-expressing cells leading to their lack of sustained reversal of hyperglycemia. The lack of sustained response likely results from destruction of the induced insulin-expressing cells by immune mechanisms that are independent of SOCS-1 mediated inhibition or by those that can overcome it. Since there are multiple pathways that mediate the ultimate destruction of β-cells in autoimmune diabetes in NOD mice[1], it is not entirely unexpected that there was a significant fraction of diabetic mice that did not achieve euglycemia by induced overexpression of SOCS1. This is in keeping with the data from SOCS-1 transgenic NOD mice wherein a protection was accorded to 30–80% of mice depending on the transgenic line[6, 9]. Interestingly, most of the mice that did not have sustained response were mice that became diabetic at a much earlier age than the responders. This could imply that the mice that became diabetic at an earlier age had a more aggressive autoimmunity and hence could not be protected from by the overexpression of SOCS1. This interesting observation opens the window to future studies to address if there are differences in mechanisms that determine the aggressive nature of the diabetes that may require unique targeting.

In summary, this study demonstrates that using Ngn3-Btc to induce 'neo-islets' in the liver of NOD mice and then subsequently accord target-cell protection from cytokine-mediated cell death by overexpressing SOCS1 is a viable approach to reverse autoimmune diabetes in NOD mice. SOCS1 independent pathways may also need to be targeted in to allow a more universal response to the Ngn3-Btc therapy in the future.

Materials and Methods

Animals

All animal protocols were approved by the IACUC at Baylor College of Medicine. NOD female mice were purchased from Jackson Labs (Bar Harbor, ME) at 8 weeks of age and followed weekly with non-fasting blood glucose (One touch glucometer, LifeScan) and body weight measurements between 9–10AM on regular chow (2020x Teklad). If the blood glucose was greater than 250 mg/dl, it was retested the following day. Diabetes was defined as two consecutive blood glucose measurements >250 mg/dl. Diabetic mice were treated with HDAd vectors by tail vein injection within 48 hours after the diagnosis of diabetes. Glucose tolerance testing was performed, as described before[17], at 4 weeks after treatment in 6 hour fasted mice with an intra-peritoneal injection of 1.5 gm/kg body weight of D-glucose. Insulin was assayed using the Mercodia Ultrasensitive ELISA kits.

Vectors

Construction of the mouse Ngn3, Btc viruses has been described before. HDAd-RIP-SOCS-1 was also constructed similarly. In brief, mouse SOCS1 cDNA were cloned from the spleen by RT-PCR and the fully sequenced cDNA were then sub-cloned into pLPBL1 shuttle plasmid with a rat insulin promoter and rabbit β-globin polyadenylation signal. We used p-delta28 plasmids as backbone for the HDAds and these were amplified as described previously[25]. Diabetic mice were administered a single IV injection of HDAd vectors via

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tail vein. To exclude any non-specific effect related to vector dose, the total vector dose was maintained at 7×10^{11} vp in all treatment groups: (5×10^{11} vp Ngn3 + 1×10^{11} vp Btc+ 1×10^{11} vp empty vector), (5×10^{11} vp Ngn3 + 1×10^{11} vp Btc + 1×10^{11} vp RIP-SOCS1), (1×10^{11} vp RIP-SOCS1 + 6×10^{11} vp empty vector) and (7×10^{11} vp empty vector).

Gene Expression

Liver and pancreas were removed at stated time points from anesthetized mice, snap frozen in liquid nitrogen and stored at −80C. They were homogenized in Trizol Reagent (Invitrogen, CA) and total RNA extracted as per manufacturer's instructions. This RNA was then subjected to an on-column purification and DNaseI digestion (RNeasy mini, Qiagen) to remove any genomic DNA contamination. The eluted RNA was quantitated and stored at −80C. RT was performed by using 20μg of RNA with Superscript RT III cDNA synthesis kit (Invitrogen, CA). We performed quantitative real-time PCR using SYBR Green reagent (BioRad). The Ct values were obtained after normalizing to the non-reactive ROX dye which served as a reference control. Using the delta-delta-Ct method, after normalizing to housekeeping genes (GAPDH), we quantified various transcripts using appropriate primers (Supplemental Table 1) and analyzed melting curves to confirm accurate readings.

Immunostaining

Immunostaning was performed as described before[17]. Primary Antibodies used were insulin (1:100 guinea pig polyclonal, Abcam), Glucagon (1:200 rabbit anti-human, Dako), pancreatic polypeptide (1:200 rabbit anti-human, Dako), somatostatin (1:200 rabbit anti-human, Dako) CD3 (1:150, rabbit anti-mouse, Abcam), TNF-α (1:200 mouse anti-mouse, Abcam), proinsulin (1:300 guinea pig polyclonal, Progen biotechnik), A6 (1:200 rat anti-mouse, gift from Dr. Valentina M. Factor, NIH), Pdx1 (1:5000 goat anti-mouse, a gift from Dr. Christopher Wright), Nkx6.1 (1:300, Beta Cell Biology Consortium), CD3 (1:150 rabbit anti-mouse, Abcam), Ngn3 (1:200 Beta Cell Biology Consortium). For immunohistochemistry, a biotin labeled secondary antibody was used and the Elite ABC kit and Novared substrate (Vector labs) kit was used for visualization. Microscopy was performed using an Axiovert (Zeiss) microscope with Axiovision imaging software 4.0. All the images with the same staining within the same experiment were acquired at the same settings and processed identically.

Adoptive transfer

Splenocytes were isolated by collagenase digestion and 2×10^{6} cells in 250μl serum free media were injected IP into 6 week old female NOD-Scid mice (purchased from Jackson Laboratories). Weekly blood glucose was measured in these NOD-Scid mice to determine the diabetes transfer rate.

Statistical Methods

All statistical testing was performed either by two-tailed student's T-Testing, assuming unequal variance for 2 groups or ANOVA for multiple group testing (with Tukeys for post-hoc analysis) $p\leq0.05$ considered significant between the groups. For adoptive transfer experiment, Kaplan-Meier survival analysis using log-rank test was used to determine

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significant with $p<0.05$ being considered significant. Statement regarding sample sizes: Sample sizes were chosen based on previous pilot studies to obtain reproducible results in physiological testing. Mice were chosen for different treatments randomly but to ensure that the final groups were similar at baseline before treatment. Where possible for analysis the analysis was blinded during data collection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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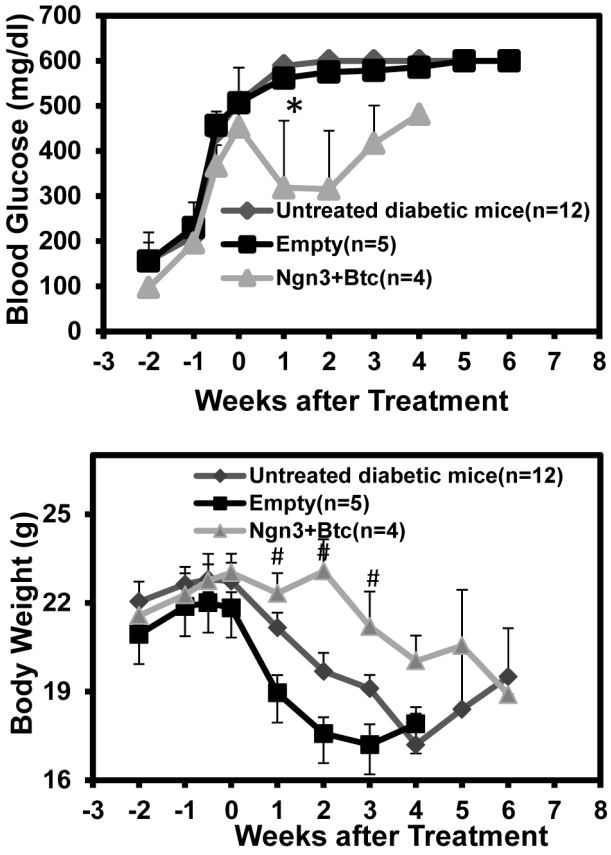

Fig. 1. Ngn3-Btc gene therapy leads to only a transient improvement of hyperglycemia in diabetic NOD mice

Non-fasting 9–10AM blood glucose (A) and body weight (B) in diabetic NOD mice are shown. A decrease in blood glucose and maintenance of body weight is seen with Ngn3-Btc treatment, which is lost after 2 weeks after treatment. Values represent mean±SEM. * $p \leq 0.05$ as compared to empty virus.

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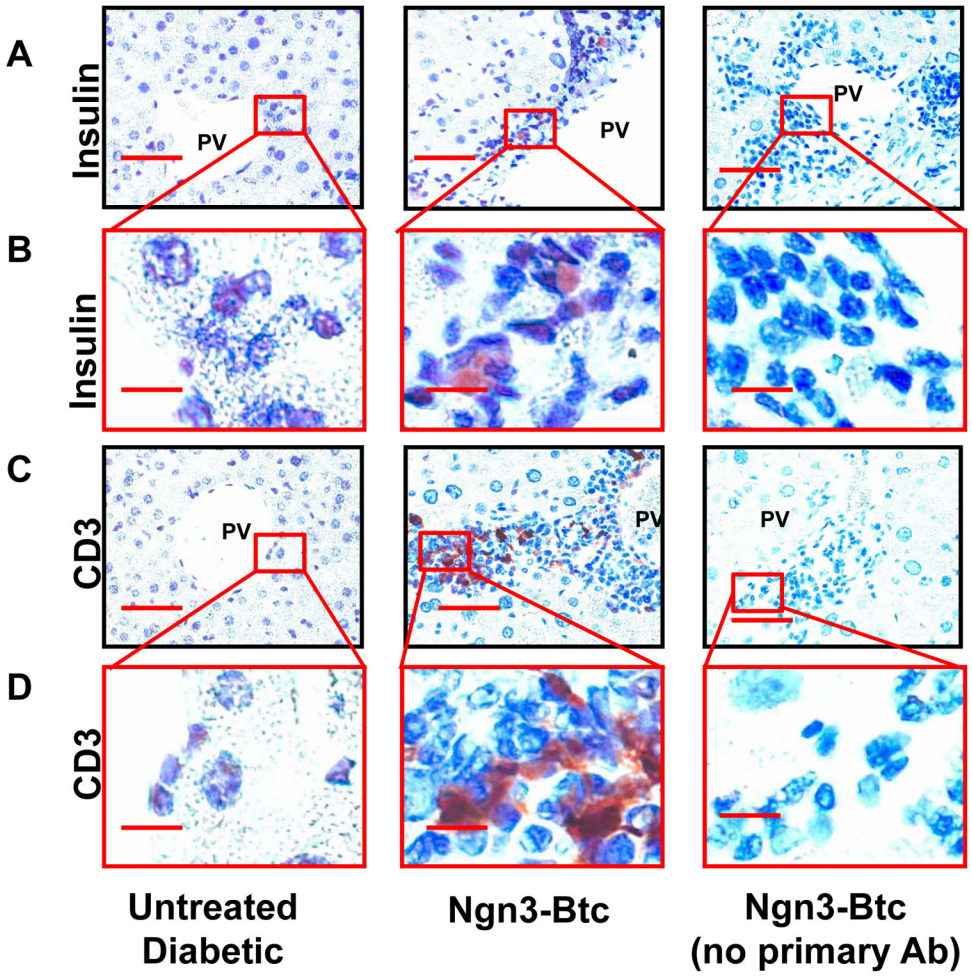

Fig. 2. Ngn3-Btc gene therapy induces insulin-expressing cell clusters in the periportal regions of the liver

(A). Immunohistochemistry for insulin reveals periportal insulin-positive cell clusters only in the diabetic mice treated with Ngn3-Btc. (C) CD3 immunostaining reveals these periportal insulin-positive clusters infiltrated by lymphocytes in the Ngn3-Btc treated mice. A control no primary antibody staining is also shown to demonstrate specificity of the antibody. Higher magnification of the red boxed area in (A) and (C) are shown in panels (B) and (D) respectively. PV – portal vein. Scale bars represent 50 μm (A&C) and 10μm (B&D).

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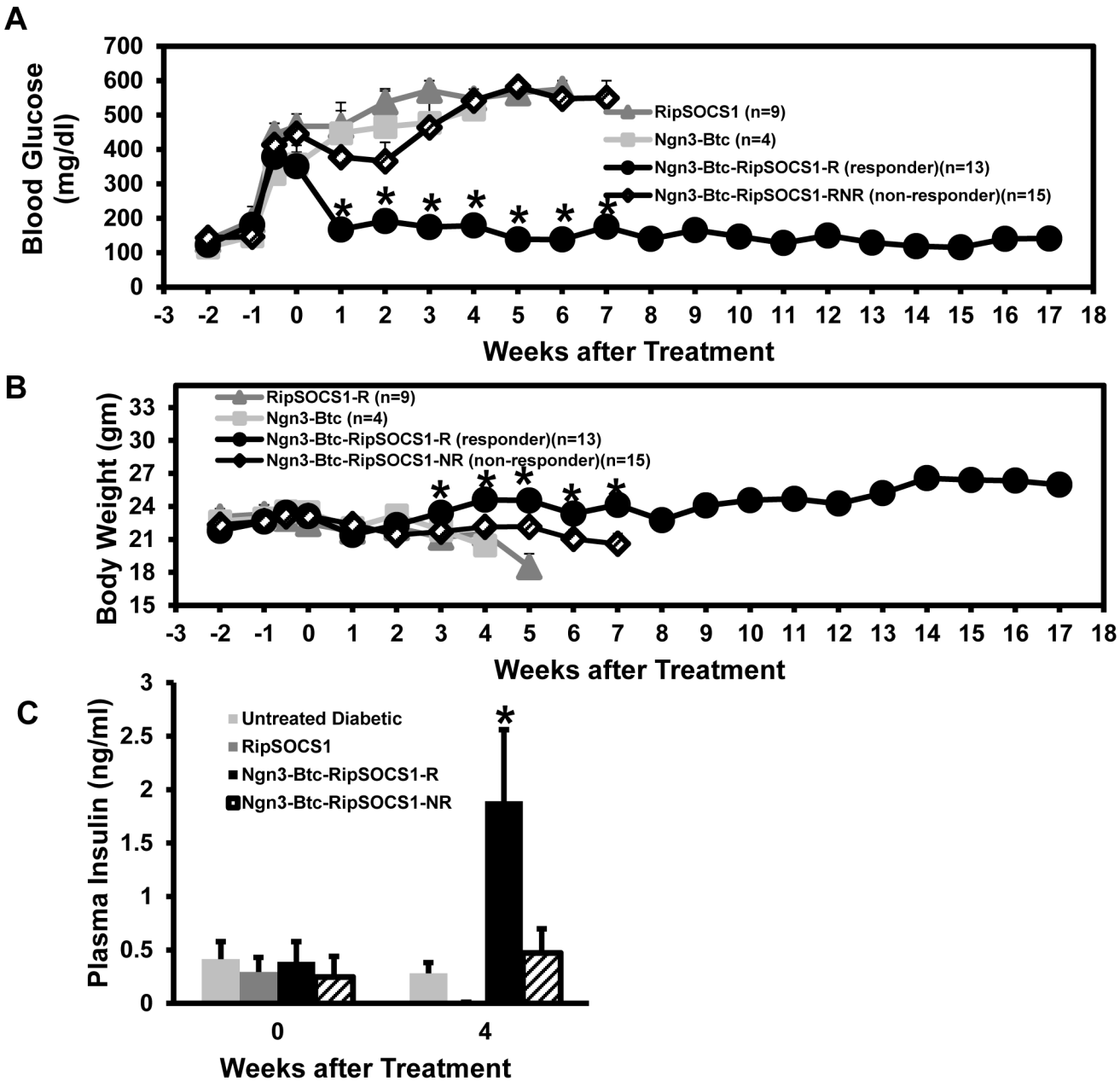

Fig. 3. Ngn3-Btc-RipSOCS1 gene therapy leads to reversal of diabetes in about half of diabetic NOD mice

(A–B) Blood Glucose and body weight display a sustained normalization in 13 of 28 diabetic NOD mice treated with Ngn3-Btc-RipSOCS1 (responders - Ngn3-Btc-RipSOCS1-R). (C) Plasma insulin levels increase significantly only in the responder mice treated with Ngn3-Btc-RipSOCS1; n=3–5. Values represent mean±SEM. * p≤0.05 as compared to controls (SOCS-1 only or Ngn3-Btc).

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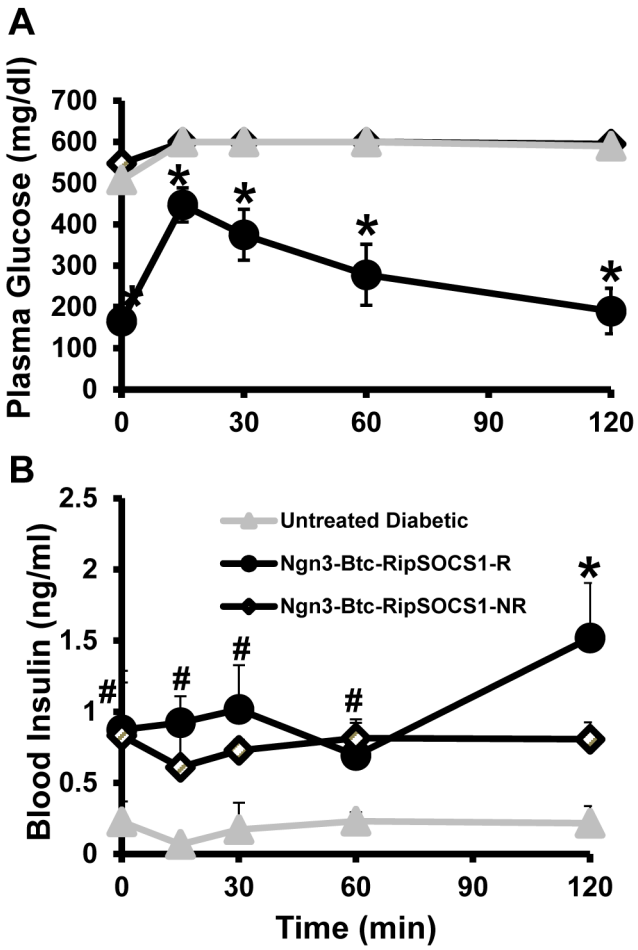

Fig. 4. Ngn3-Btc-RipSOCS1 gene therapy leads to restoration of glucose tolerance in diabetic NOD mice

(A) Plasma glucose during an IP-GTT, 4 weeks after treatment. The Ngn3-Btc-RipSOCS1 treated responder mice (Ngn3-Btc-RipSOCS1-R) display normal glucose tolerance as compared to the untreated diabetic mice. (B) Plasma insulin during this GTT revealed a significant improvement over untreated diabetic mice indicating *in vivo* glucose-stimulated insulin secretion. n=3–5. # $p \leq 0.06$ as compared to untreated diabetic controls.

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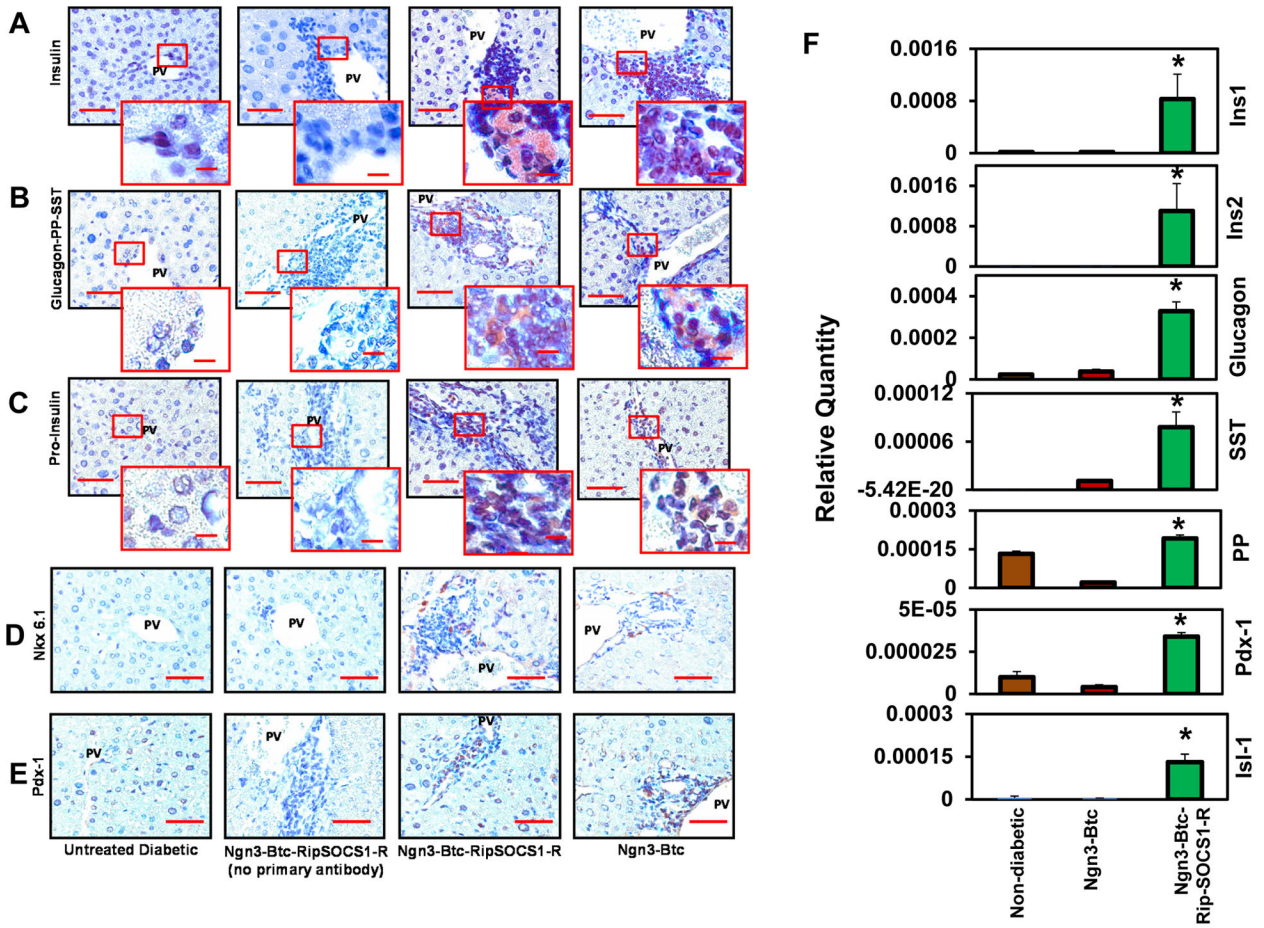

Fig. 5. Ngn3-Btc-RipSOCS1 gene therapy leads to islet hormone-positive periportal 'neo-islets'

(A–E) Immunohistochemistry for insulin (A), proinsulin (C) and combined staining of other islet hormones (B) reveal the presence of hormone-positive cell clusters in the periportal regions of the liver in Ngn3-Btc-RipSOCS1-R mice. The higher magnification of the red boxed area is shown in the inset below each panel in A–C. β-cell transcription factors, Nkx6.1 and Pdx-1 are also seen in the Ngn3-Btc-Rip-SOCS1-R mice (D&E). (F) RT-qPCR for islet hormones and transcription factors from the livers of treated mice 6 weeks after treatment (n=3–5). Values (mean±SEM) are expressed after normalization relative to GAPDH and eEF1γ. PV – portal vein. Scale bars represent 50 μm in all except for the higer magnification insets in A–C wherein the scale bars represent 10μm. * p≤0.05 as compared to controls (non-diabetic and Ngn3-Btc).

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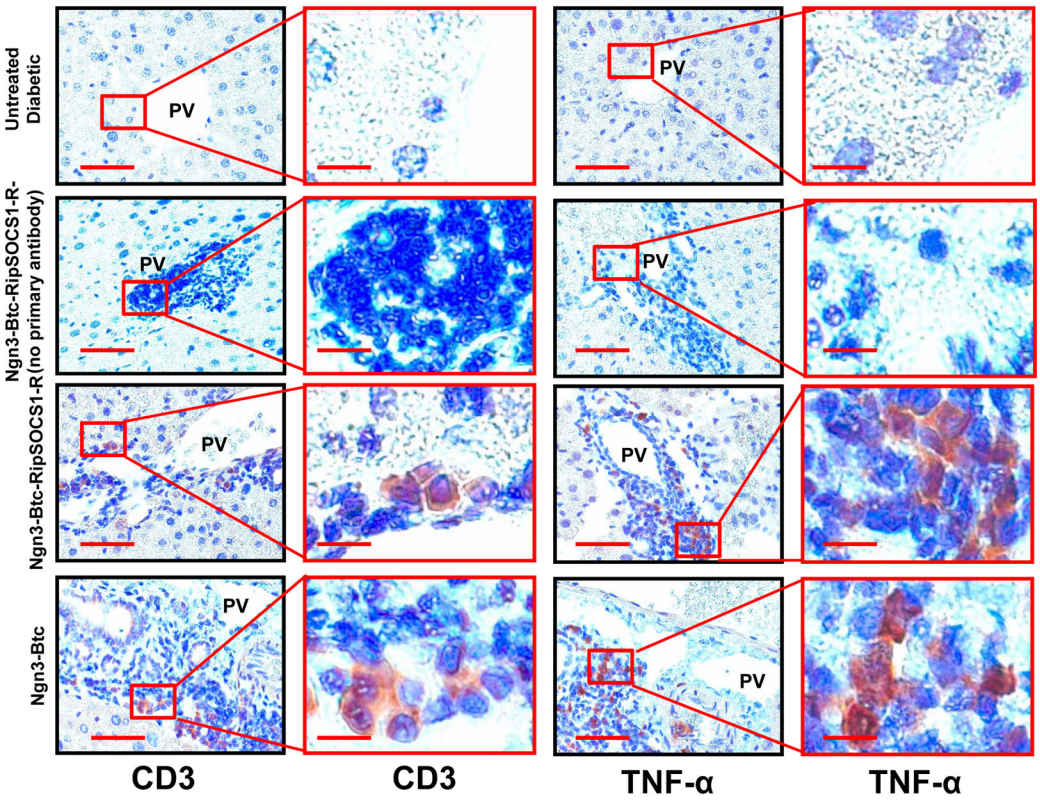

Fig. 6. Ngn3-Btc-RipSOCS1 gene therapy leads to islet hormone-positive periportal 'neo-islets'

Immunohistochemistry for CD3 and TNF-α reveal the presence of activated lymphocytes in the periportal regions of the liver in treated mice (Ngn3-Btc-RipSOCS1 and Ngn3-Btc) but not in control untreated diabetic mice. The higher magnification of the red boxed area in columns 1&3 are shown in columns 2&4 respectively. PV – portal vein. Scale bars represent 50 μm for images in columns 1&3 and 10μm for images in columns 2&4.

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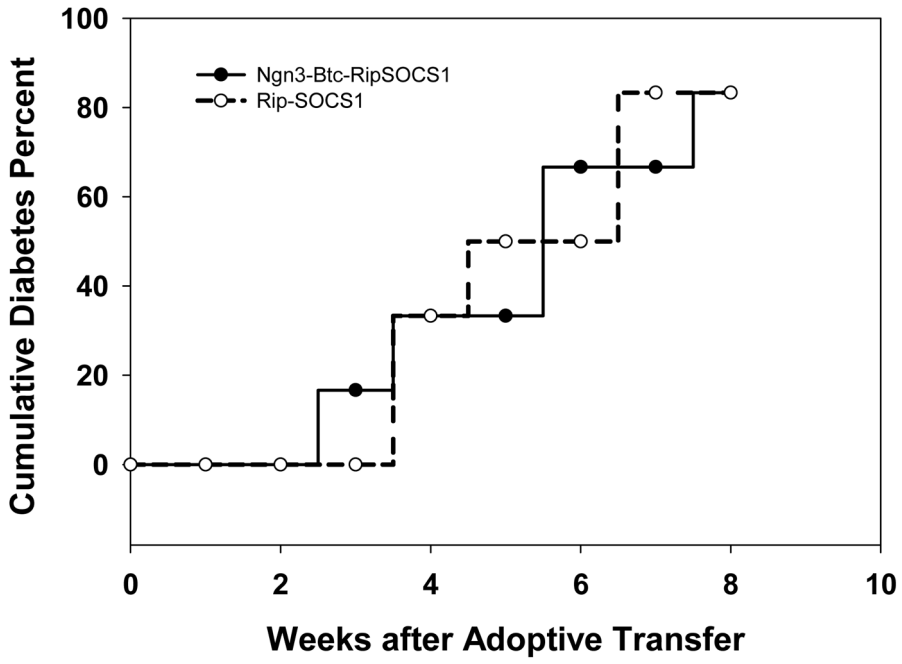

Fig. 7. Ngn3-Btc-RipSOCS1 therapy does not alter the diabetogenicity of splenocytes

No difference is observed in the induction of diabetes on adoptive transfer of splenocytes from Ngn3-Btc-RipSOCS1 and RipSOCS1 treated mice into 6 week old female NOD-Scid mice.