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Durable Control of Autoimmune Diabetes in Mice Achieved by Intraperitoneal Transplantation of "Neo-Islets," Three-Dimensional Aggregates of Allogeneic Islet and "Mesenchymal Stem Cells"

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ABSTRACT

Novel interventions that reestablish endogenous insulin secretion and thereby halt progressive end-organ damage and prolong survival of patients with autoimmune Type 1 diabetes mellitus (T1DM) are urgently needed. While this is currently accomplished with allogeneic pancreas or islet transplants, their utility is significantly limited by both the scarcity of organ donors and life-long need for often-toxic antirejection drugs. Coadministering islets with bone marrow-derived mesenchymal stem cells (MSCs) that exert robust immune-modulating, anti-inflammatory, anti-apoptotic, and angiogenic actions, improves intrahepatic islet survival and function. Encapsulation of insulin-producing cells to prevent immune destruction has shown both promise and failures. Recently, stem cell-derived insulin secreting β-like cells induced euglycemia in diabetic animals, although their clinical use would still require encapsulation or anti-rejection drugs. Instead of focusing on further improvements in islet transplantation, we demonstrate here that the intraperitoneal administration of islet-sized "Neo-Islets" (NIs), generated by in vitro coaggregation of allogeneic, culture-expanded islet cells with high numbers of immuno-protective and cyto-protective MSCs, resulted in their omental engraftment in immune-competent, spontaneously diabetic nonobese diabetic (NOD) mice. This achieved long-term glycemic control without immunosuppression and without hypoglycemia. In preparation for an Food and Drug Administration-approved clinical trial in dogs with T1DM, we show that treatment of streptozotocin-diabetic NOD/severe combined immunodeficiency mice with identically formed canine NIs produced durable euglycemia, exclusively mediated by dog-specific insulin. We conclude that this novel technology has significant translational relevance for canine and potentially clinical T1DM as it effectively addresses both the organ donor scarcity (>80 therapeutic NI doses/donor pancreas can be generated) and completely eliminates the need for immunosuppression. STEM CELLS TRANSLATIONAL MEDICINE *2017;6:1631–1643*

SIGNIFICANCE STATEMENT

Mesenchymal stem cells (MSCs) possess potent immune-modulating, anti-inflammatory, pro-survival, and repair-stimulating activities. In patients with juvenile Type 1 diabetes mellitus (T1DM), the insulin-producing cells of the pancreas are destroyed by auto-immune attacks. Treatment with insulin, a pancreas or pancreatic islet transplant will enhance patient survival and reduce serious complications. However, transplants depend on potentially toxic anti-rejection drugs, and there is a shortage of pancreas donors. We tested whether the therapeutic activities of MSCs could be harnessed by combining them with healthy islet cells in cell clusters ("Neo-Islets") that are administered to a mouse model of T1DM. We show that Neo-Islets eliminate the need for exogenous insulin in experimental T1DM and conclude that this novel therapy has significant promise for the treatment of veterinary and human T1DM.

INTRODUCTION

The clinical need for novel technologies that effectively treat patients with Type 1 diabetes mellitus (T1DM) and render them insulin-independent is great and well documented. Endogenous insulin replacement by pancreas or islet of Langerhans transplants is currently the only treatment that can achieve insulin-independence and provide significant end organ protection in patients with

autoimmune-mediated T1DM. However, the great shortage of suitable pancreas donors combined with the need for repeated islet transplants, requiring up to five donors each, continue to limit the general availability of these expensive therapies [1, 2]. In addition, both transplant modalities depend on the permanent use of potentially toxic antirejection drugs [3–6].

Novel approaches to address these major limitations of islet transplantation therapies have shown significant progress. Auto- and allo-immune isolation of transplanted islet cells (ICs) is currently tested with various encapsulation technologies. Several of these are showing promise while others have failed due to foreign body reactions [7–11]. When insulin-producing β-cells are culture expanded through outgrowth from freshly isolated islets, they progressively de-differentiate and lose their ability to secrete insulin [12–14]. Although partial in vitro redifferentiation is feasible, this process is relatively inefficient [15]. For this reason, pancreatic progenitor, embryonic stem and induced pluripotent stem cell lines have recently been successfully used to generate cells that closely resemble β-cells and that induce euglycemia in diabetic animal models [16, 17], while their therapeutic use would still require either encapsulation or anti-rejection drugs. Other significant preclinical studies used the pleiotropic actions of bone marrow or adipose-derived mesenchymal stem cells (MSCs), that is, their well-documented immune-modulating, anti-inflammatory and complex trophic activities, and showed that the survival and function of transplanted islets was improved when islets were cocultured with MSCs and coadministered with islets or when administered islets were precoated with MSCs [18–22]. A clinical trial in which MSCs alone were administered to patients with T1DM demonstrated a modest improvement in β-cell function, a response that was previously observed in preclinical studies [23]. This approach by several groups clearly demonstrated that inclusion of MSCs in islet transplantation technologies does modestly reduce the number of needed islet donors.

Mindful of these important observations, we chose in the current study a new strategy that did not focus on the further improvement of islet transplantation technologies but instead tested whether the inclusion of higher numbers of healthy MSCs (adipose or bone marrow derived) in freshly formed "Neo-Islets" (NIs), Three-dimensional (3D) aggregates of culture-expanded allogeneic ICs and MSCs, could be used to potentiate the pleiotropic effects that the small numbers of MSCs, as pericytes, physiologically exert in islets [24]. In this fashion, we reasoned a substantially expanded MSC component (from ∼2% in normal isles to ∼50%) in these NIs should immune-protect, through close range signaling, culture-expanded and coaggregated islet and stem cells in vivo. In addition, we postulated that this approach would also make available the robust anti-apoptotic paracrine actions of MSCs and their released nanovesicles to neighboring ICs, combined with their pro-angiogenic and potent anti-inflammatory activities [25]. Together, allogeneic NIs that are engineered in this fashion should possess, we hypothesized, the ability to provide adequate auto- and allo-immune isolation of their cell components in vivo by creating a protective microenvironment where culture expanded ICs can resume their physiological endocrine and other functions, that is, reestablish euglycemia in the clinically relevant NOD mouse model of auto-immune T1DM.

Accordingly, NIs of approximate islet size were generated in vitro from culture expanded, dedifferentiated ICs [12–14] and bone marrow-derived MSCs of C57Bl/6 mice. NIs were administered to spontaneously diabetic, immune-competent NOD mice that develop auto-immune T1DM that largely resembles human T1DM [26]. This allogeneic treatment protocol was chosen as it models the most common clinical situation in recipients of pancreas or islet transplants. By not using anti-rejection drugs or encapsulation devices, we directly tested our hypothesis that high numbers of MSCs in NIs do enable ICs to survive and redifferentiate into normally functioning endocrine cells. This treatment established long-term glycemic control in NOD mice, which demonstrates that NIs survive, engraft and redifferentiate into functional endocrine cells in vivo, and that both allo- and autoimmune protection is achieved. Importantly, following i.p. administration the NIs were taken up by the well-vascularized omentum [27, 28] where they engrafted long term and redifferentiated into physiologically insulin-secreting cells, delivering insulin into the portal system of the liver [29]. Simultaneously, re-expression of other islet-specific hormones occurred. Identical injection of NIs into nondiabetic animals resulted in omental engraftment without causing hypoglycemia, further demonstrating regulated islet hormone secretion. In preparation for a pilot study in pet dogs with T1DM (INAD # 021776), streptozotocin-diabetic nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were treated by identical protocol with canine NIs (cNIs). In these, euglycemia was readily and durably induced and intraperitoneal Glucose Tolerance Tests (i.p. GTTs) were normalized by the exclusive release of canine-specific insulin. Taken together, the present data demonstrate that the complex pleiotropic actions of MSCs, as hypothesized, can be readily harnessed to protect cultured ICs, and when coaggregated with them in NIs and administered i.p., facilitate long-term glycemic control in mice with autoimmune T1DM. We conclude that these novel observations have significant translational relevance for the treatment of both canine and human T1DM.

Materials and Methods

Reagents

All reagents used and their sources are listed in Supporting Information Table S1.

Cell Isolation and Culture

Islets and adipose derived MSCs were Isolated and cultured from mice and dogs as previously reported [30–34]. Purified human islets from nondiabetic subjects (Prodo Laboratories, Irvine, CA), and human adipose derived MSCs (P1, Lonza, Walkersville, MD) were identically cultured. See Supporting Information data. Prior to NI formation, cultured MSCs were characterized as in our previous publication [35]. Cell viability was assessed using fluorescein diacetate (FDA) and propidium iodide as per the manufacturers' instructions.

Induction of Indoleamine 2,3 Dioxygenase

Canine MSCs were tested by rtPCR at passage 2 (P2) for induction of indoleamine 2,3 dioxygenase (IDO-1) in response to overnight culture in DMEM-F12 (Sigma, www.sigmaaldrich.com) +10% canine serum (Golden West Biologicals, www.goldenwestbio.com) + 10 ng/ml canine interferon gamma (IFNγ, R&D Systems, www.rndsystems.com). Results from IFNγ treated cultures were normalized to those of identically passaged and cultured (but without IFNγ) cells, and expressed as Log10RQ ($n = 4$ independent experiments).

Neo-Islet Formation

MSCs (P1 to P5) and ICs (P1 to P2) were cocultured in DMEM-F12 + 10% Fetal Bovine Serum (FBS; Hyclone, www.fishersci.com, for murine or human cells) or dog serum (for canine cells) at a 1:1 ratio in ultra-low adhesion surface culture dishes (Corning, www.corning.com), and NIs formed overnight. Control MSC and Islet cell clusters were formed by the same method. Prior to in vivo administration, NIs were tested by reverse transcription polymerase chain reaction (rtPCR) for expression of islet and MSC associated genes (see below), and Fluorescence-activated cell sorting (FACS) for determining the ratio of MSCs to ICs post-formation.

Staining of Cells and Neo Islets

Where indicated, MSCs were stained with Cell Tracker Green (green, Life Technologies, www.thermofisher.com), and passaged ICs were stained with Lipophilic Tracer DiI (red, Life Technologies) according to the manufacturers' instructions.

Neo-Islet Cellular Ratio Assessment

NIs were formed overnight from cell tracker green stained MSCs and unstained ICs, collected and dissociated to single cell preparations (30 minutes Accumax Innovative Cell Technologies, www.accutase.com), and analyzed by FACS (BD FACScan Analyzer, BD Biosciences, www.bdbiosciences.com) for percent green (MSCs) versus unstained (ICs) cells.

Immunohistochemistry

Harvested organs were fixed, paraffin embedded, sectioned and deparaffinized as previously described [35], then stained for indicated antigens by standard methods (see Supporting Information data).

rtPCR

RNA was extracted from 1x10e6 cells (Qiagen RNeasy Mini Kit, Qiagen, www.qiagen.com). Reverse transcription was performed using SuperScript II Reverse Transcriptase (Applied Biosystems, www.thermofisher.com) for 60 minutes at 42°C. rtPCR was carried out in duplicate using species-specific TaqMan primers (Applied Biosystems; see Supporting Information Table S2) and the ABS 7500 Real Time PCR System. RQ was calculated through normalization to internal controls (beta actin and beta 2 microglobulin, and the machine's software. Results are presented as log10(RQ) ± log10(RQmin and RQmax). Differences greater or less than log10(RQ) 2 or −2 were considered significant. (see Supporting Information data).

Animal Care and Models

Animal studies were conducted in adherence to the NIH Guide for the Care and Use of Laboratory Animals, and were supervised and approved by an institutional veterinarian and member of the IACUC. Mouse experiments used female (a) C57Bl/6 (Harlan, www.envigo.com), (b) NOD (Jackson Laboratory, www.jax.org), (c) Nonobese diabetic/severe combined immunodeficiency mice (NOD/SCID, Harlan), weighing between 15 and 35 g. All treatments were conducted under isoflurane anesthesia. Care and anesthesia details are in Supporting Information .

Insulin Treatment

Where indicated, insulin was administered via slow-release, subcutaneous insulin pellets (Linbits, LinShin, www.linshincanada.com; see Supporting Information data) following the manufacturer's instructions.

Blood Glucose Monitoring

In all in vivo studies, blood glucose concentrations were assessed twice per week via tail vein sampling, using a OneTouch Ultra 2 glucometer (Johnson and Johnson, www.jnj.com, level of detection, 20–600 mg glucose/dl).

Spontaneous Diabetes

Female NOD mice develop T1DM spontaneously between 12 and 20 weeks of age. Diabetes was confirmed by nonfasting blood glucose levels of >300 mg/dl on 3 separate days. Mice entered experimental or control groups at ages 13–21 weeks of age.

Streptozotocin-Induced T1DM

C57Bl/6 and NOD/SCID mice were rendered diabetic with 3–5 i.p. doses (1 per day) of 50–75 mg/kg body weight (b.wt.) STZ (Sigma), freshly dissolved in 20 mM citrate buffer, pH 4.5, and randomized after diabetes was confirmed as above.

Treatment Protocols

See Table 1 for details. For all diabetes models, blood glucose levels were controlled with Linbits administration prior to treatment. Therapies were administered under light isoflurane anesthesia once blood glucose levels were controlled, and prior to Linbits expiring (1–3 weeks post-implantation). After Linbits expired, no further insulin was given. Unless otherwise indicated, NIs were dosed at 2 × 10e5 NIs/kg b.wt., and administered i.p. suspended in vehicle (0.5 ml serum free DMEM). Where stained or fluorescent cells were administered, omenta, livers, spleens, lungs, kidneys, and pancreata were harvested upon euthanasia and examined by fluorescence microscopy for the presence of fluorescently labeled NIs. Other endpoints are given in Table 1 and Results.

In Vivo Imaging

In vivo imaging of DiR (Life Technologies) stained NIs was performed in anesthetized mice using the Li-Cor, Pearl Impulse imager (LiCor, www.licor.com).

IP Glucose Tolerance Tests

i.p. GTTs were conducted in 3 vehicle-treated and 5 cNI-treated STZ-diabetic NOD/SCID mice by standard procedures (see Supporting Information data), and canine- and mouse-specific insulin levels were assayed by enzyme-linked immunosorbent assay (ELISA).

Tumor Formation and Ectopic Maldifferentiation of MSCs

Upon euthanasia, all harvested organs were examined histologically for tumors or evidence for ectopic maldifferentiation (osteo-, adipo- or chondrogenic).

Allo-IgG Response

Aliquots of ~5×10e4 C57Bl/6 MSCs, ICs, dissociated NIs or dissociated ICs (Accumax) were incubated with 500 μl of serum obtained upon euthanasia from NI-, vehicle-, or islet-treated NOD mice (sera obtained at day 77 for NI and vehicle-treated, and day 14 for islet-treated mice), for 30 minutes at room temperature, centrifuged (600*g* for 5 minutes), incubated with cy3-conjugated goat-anti-mouse IgG antibody (Jackson ImmunoResearch, www.

Table 1. Treatment protocols

Study question	Recip.mice diabetes type	Recip. strain	Recip. age (wks)	Donor strain/ species	NIs/Clusters composed of: Islet cell passage	NIs/Clusters composed of: MSCs passage	Treatment groups	Mice/ group	End of study (wks post NI admin.)	Endpoints
Do NIs reverse hyperglycemia in spont. diabetic mice?	Spont. T1DM	NOD	13-21	C57Bl/6, wt and *egfp*+	P1 wt	P5 *egfp*+ MSCs	NI	6	10	Sera collected to test for allo Ig-G response to cells that make up NIs. Omenta examined for T cells.
				NA	NA	NA	Vehicle	6	10	
				wt C57Bl/6	NA	NA	2x10e5 islets	3	2	Sera harvested and assessed as above
Are both MSCs and Islet Cells required for clusters to reverse hyperglycemia?	STZ	wt C57Bl/6	10	C57Bl/6, wt and *egfp*+	P1 wt	P5 *egfp*+ MSCs	NI	6	12 ($n = 3$); 21 ($n = 3$)	Omenta, pancreata examined by rtPCR for islet associated gene expression
				NA	NA	NA	Vehicle control	6	12	
				wt C57Bl/6	NA	P1 MSCs	MSC only cluster	5	12	
				wt C57Bl/6	P1 wt C57Bl/6	NA	IC only cluster	5	12	
Do NIs release insulin physiologically or cause hypoglycemia?	Non-diabetic	wt C57Bl/6	12	C57Bl/6, wt and *egfp*+	P1 wt	P5 *egfp*+ MSCs	NI	14	0.5-12	Blood glucose levels, cell tracking
		wt C57Bl/6	12	NA	NA	NA	Vehicle	3	3	Blood glucose levels
		NOD/SCID	9	Dog	P1	P2 MSCs	cNI	6	10	Blood glucose levels
		NOD/SCID	9	NA	NA	NA	Vehicle	3	10	Blood glucose levels
Can NIs derived from canine cells reverse hyperglycemia?	STZ	NOD/SCID	20	Dog	P1	P2 MSCs	cNI;	5	12.5	Dose finding. Remote onset efficacy. IP GTT at 8 wks, NIs removed at 10 wks. Sera examined for canine specific insulin during IP GTT.
				NA	NA	NA	Vehicle	5	12.5	

Abbreviaitons: cNI, canine neo-islets; *egfp*+, bears the green fluorescent protein gene, expressed in all cells; IP GTT, intra peritoneal glucose tolerance test; MSCs, mesenchymal stromal cells; NA, not applicable; NI, neo-islets; admin. administration; NOD, nonobese diabetic; NOD/SCID, nonobese diabetic/severe combined immuno-deficient; P, Passage number; Recip., recipient; spont., spontaneous; STZ, streptozotocin; wks, weeks; wt, wild type.

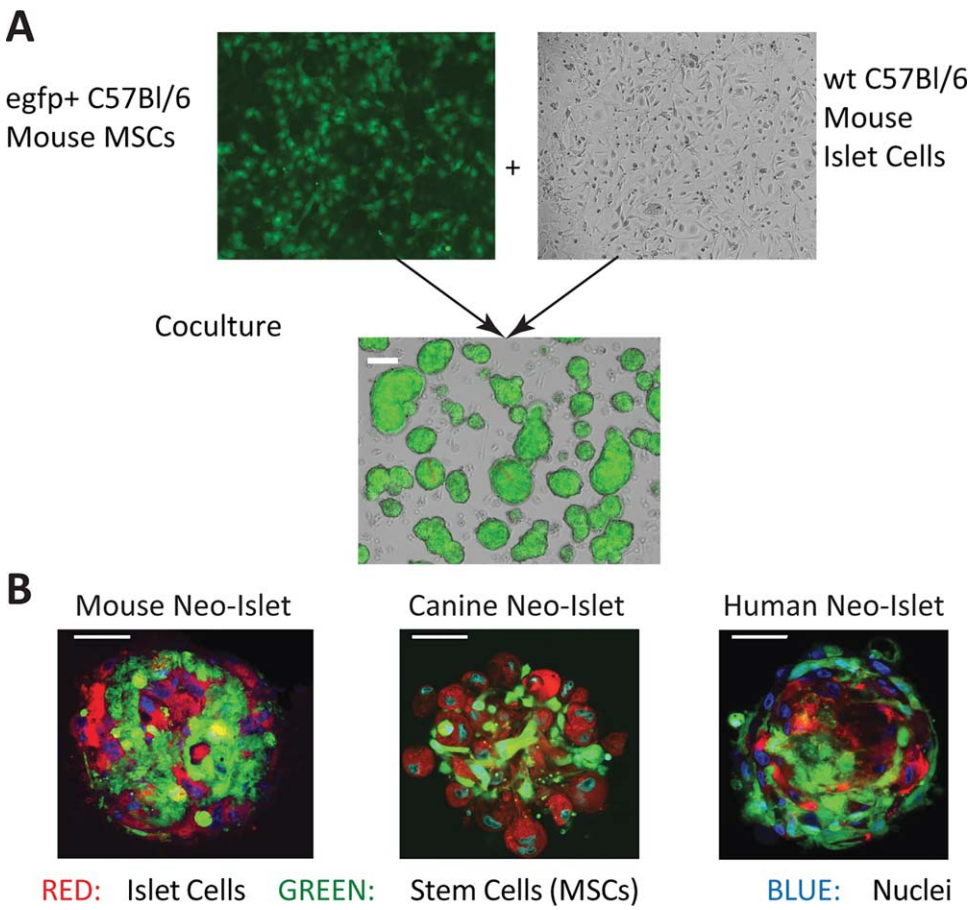

Figure 1. Mouse Neo-Islets (NI) formation and confocal microscopy. **(A)**: Images (×10) and a schematic representation of mouse cells undergoing NI formation. Approximately 500 green fluorescent protein positive (*egfp*+) C57Bl/6 MSCs (left, top) and approximately 500 C57Bl/6 islet cells (right, top) were culture expanded, then cocultured in ultra-low-adhesion plates where they readily formed NIs overnight (bottom; white scale bar = 150 μm). Average NI-formation (cells in low adhesion plates incorporated into NIs) efficiency ≥95%; viability ≥ 90%; replicates, $N > 25$. **(B)**: Confocal microscopic images (×63) of murine (left), canine (middle), and human (right) NIs. Within the NIs, MSCs were Cell Tracker Green stained (green), islet cells were DiI stained (red) and nuclei were stained with DAPI (blue). Morphology and cell composition (approximately 1,000 cells per NI) did not differ significantly among murine, canine, and human NIs. Each depicted NI was approximately 150 μm in diameter. White scale bar = 50 μm. Abbreviation: MSC, mesenchymal stem cell.

jacksonimmuno.com) or isotype control (1:100 dilution) for 30 minutes fixed, and analyzed by FACS.

Spleen Cell Preparation and T Cell FACS Analysis

Spleens and omenta were sectioned into small pieces, triturated in 1× Phosphate Buffered Saline (PBS, Roche, www.roche.com), passed through a sterile 40 μm strainer (BD) and washed with PBS. Red blood cells were lysed with 1× ACK (Life Technologies) for 10 minutes. Cells were washed with 1× PBS and used directly for FACS staining assays. T and Treg cells were identified using a Mouse T Lymphocyte kit (BD) and a Treg Detection kit (Miltenyi Biotech, www.miltenyibiotec.com). 0.5×10e6 cells were stained per antibody, and 1×10e4 events were counted by FACS (see Supporting Information data).

Statistical Analysis

Data are expressed as Mean ± SEM or Mean ± 95% confidence interval, as indicated. Primary data were collected using Excel (Microsoft, Redmond, WA), and statistical analyses were carried our using Prism (GraphPad, San Diego, California). Two-tailed *t* tests and one way ANOVA with Bonferroni Post Test analysis and confidence interval of 95% were used to assess differences between data means. A p value of $< .05$ was considered significant.

Results

To test our central hypothesis in a clinically informative autoimmune TIDM model, we first examined whether the i.p. administration of in vitro generated allogeneic NIs could reestablish euglycemia in spontaneously diabetic NOD mice as a reflection of (a) their survival, (b) the redifferentiation of ICs contained in the NIs into functional insulin-producing cells in vivo and re-expression of other islet-specific genes, and (c) the MSC-mediated cyto-, allo- and auto-immune protection of the transplanted NIs [36–43]. Like humans, NOD mice develop a T-cell mediated, autoimmune form of T1DM [26, 44, 45].

Formation of NIs

NIs of approximate islet size (150 μm) were prepared as illustrated in Figure 1A. We furthermore confirmed that comparable NIs could be generated from both canine and human ICs and MSCs (Fig. 1B). At 24 hours. post-formation, NIs remained comprised of

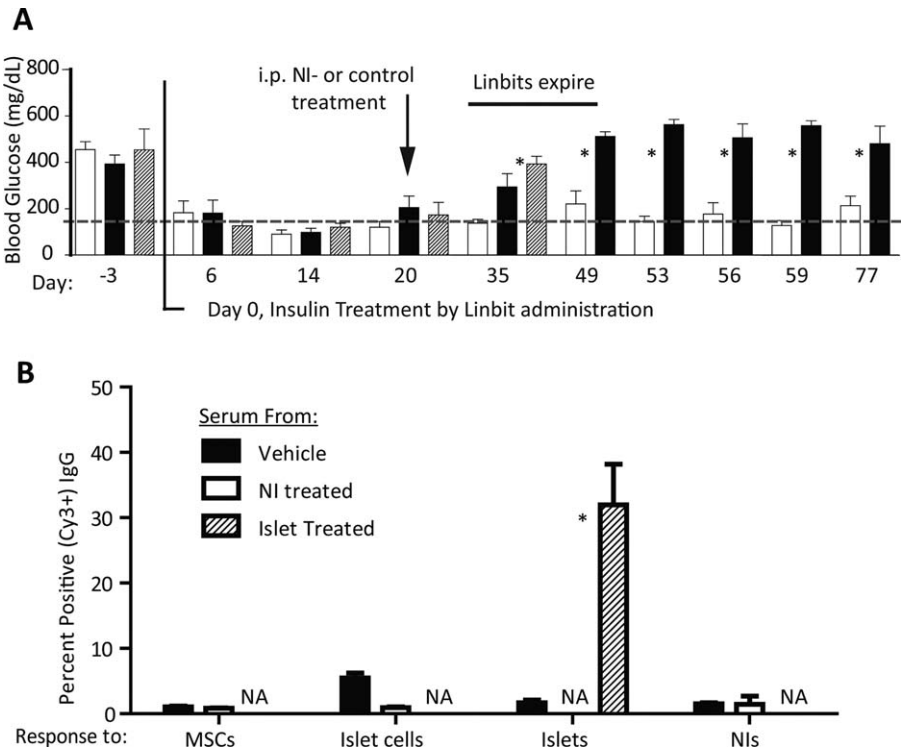

Figure 2. Allogeneic NI-treatment established euglycemia in spontaneously diabetic nonobese diabetic (NOD) mice without inducing an IgG antibody response. **(A)**: Blood glucose levels (mean ± SEM) of NOD mice normalized with Linbits (day 0), then infused i.p. on day 20 post Linbit therapy with 2×10e5 C57Bl/6 NI/kg b.wt. ($N = 6$; open bars), vehicle ($N = 6$; black bars), or 2×10e5 islets ($N = 3$; hashed bars). While vehicle- and islet-treated mouse blood glucose levels increased when Linbits expired (approximately day 35), euglycemia was maintained long term in NI-treated mice, implying IC redifferentiation into insulin producing cells and protection from allo- and autoimmune attacks. Normal blood glucose level, hashed line. *, $p < .05$ versus vehicle treated group. **(B)**: IgG responses (FACS; Mean ± SEM) in treated-NOD mouse sera to C57Bl/6 cells or islets ($N = 3$ per group). Shown are percentages of cy3+ cells when P5 C57Bl/6 MSCs and P1 C57Bl/6 cultured ICs were incubated with serum and cy3-labeled anti-mouse IgG antibody. Sera were from vehicle-treated and NI-treated NOD mice from the experiment depicted in (A) collected upon euthanasia (day 77), and from intact C57Bl/6 (allogeneic) islet-treated NOD mice (positive control) 14 days post i.p. islet administration. Treated-NOD mice show robust IgG responses to islets, but not to NIs nor to the cells from which NIs are composed. Antibody mediated rejection of NIs appears unlikely since (a) NOD mice remained euglycemic, and (b) FACS data show no IgG response to these cells in otherwise immune competent NOD mice. *, $p < .05$ versus the other treatments. Abbreviations: NI, neo-islets; MSC, mesenchymal stem cell; NA, not applicable.

approximately 50% MSCs and 50% ICs (Supporting Information Fig. S1).

Starting Materials for NIs: Growth and Characterization of ICs and MSCs

Upon culture of islets, ICs proliferate and dedifferentiate [12–14]. Upon passaging, IC-associated gene expression levels decreased. Their gene expression pattern was distinct from that of cultured MSCs (Supporting Information Fig. S2). All ICs were used at P1-P2 for NI formation. By expanding ICs to P2 and using them at this passage, one canine pancreas, assuming ~45,000 islets per pancreas, will yield at least 80 therapeutic doses.

All MSCs met the minimal criteria [46–48] and were used at P1–P5. See Supporting Information data and Supporting Information Figures S3 and S4 for details of epitope expression, trilineage differentiation, INFγ-induced IDO-1 expression, role of passage number on gene expression and NI formation, and glucose stimulated insulin secretion by freshly formed NIs.

Treatment of Spontaneously Diabetic NOD Mice with Allogeneic, C57Bl/6 NIs

Allogeneic, C57Bl/6 mouse NIs (2x10e5/kg b.wt., $N = 6$, see Table 1 and Fig. 1A), 2x10e5 C57Bl/6 mouse islets ($N = 3$), or vehicle ($N = 6$) were administered i.p. to spontaneously diabetic NOD mice after blood glucose levels were normalized with slow-release insulin pellets (Linbits) in order to reduce glucotoxic effects on the transplanted cells [14, 49, 50] and to enhance their in vivo redifferentiation [14, 16, 17]. By day 35–40 post-Linbit treatment, Linbits are depleted, and hyperglycemia redeveloped in both islet- and vehicle-treated NOD mice, as expected [51]. Strikingly, blood glucose levels in NI-treated animals remained near normal (Fig. 2A). These data suggest that (a) the NIs engraft and survive, (b) the ICs within the NIs redifferentiate in vivo, providing the mouse with a new, endogenous source of insulin and potentially other islet hormones, and (c) the MSCs contained in the NIs effectively provide cyto-protection and allo- and auto-immune-isolation of the NIs in NOD mice, and together establishing glycemic control in this clinically relevant T1DM model. Next, we explored mechanisms by which this was achieved.

NOD Mice Do Not Mount an Allo-Immune IgG Response to the MSCs and ICs of NIs

To examine whether ICs and MSCs contained in the NIs are protected from a humoral immune attack [52], we assessed whether sera from normoglycemic, NI-treated NOD mice contained IgG antibodies directed against either the MSCs or cultured ICs in the

NIs. Sera from NI-treated, normoglycemic NOD mice contained neither IgG antibodies directed at MSCs nor at cultured ICs, while the i.p. administration of identical numbers of allogeneic (C57Bl/6), freshly isolated islets used as a positive control, elicited a robust antibody response (Fig. 2B). The lack of an IgG antibody response to the cells that are used to form the allogeneic NIs, along with the achievement of long term euglycemia, indicates that the NIs also provide humoral, allo-immune protection to the transplanted cells.

NIs Spontaneously Engraft in the Murine Omentum and Produce Insulin

As shown in Figure 3A, fluorescence in vivo imaging of a euglycemic NOD mouse treated 10 weeks previously with DiR-labeled, *egfp*+ NIs demonstrates their persistent location in the upper abdomen. Histological examination upon euthanasia of omenta, pancreata, spleens, livers, lungs, and kidneys from NI-treated NOD mice from Figure 2A revealed the presence of the *egfp*+ NI only in the animals' omenta (Fig. 3B). Furthermore, sections of the omentum stained positive for insulin (Fig. 3C left panel), while negative controls (Fig. 3C inset) and omenta from vehicle-treated, diabetic NOD mice did not (Fig. 3C right panel). Their pancreata showed high-grade insulitis, as expected (Supporting Information Fig. S5), indicating that euglycemia was achieved through physiologic insulin secretion by the NIs, not islet recovery. Importantly, there was no histologic evidence for tumor formation or ectopic maldifferentiation (adipo-, osteo-, chondrogenic) in any examined organs. Additionally, Ki67 staining showed no evidence of proliferation of administered NIs in the omenta.

Inhibition of Autoimmune Response

Critical to effectively treating autoimmune T1DM with insulin producing cells is the autoimmune isolation of those cells. The results presented in Figure 2A demonstrate that the ICs within the NIs are protected from NOD mouse autoimmune attack. As in human T1DM, autoimmune beta cell destruction in NOD mice is mediated by autoreactive CD4+ Th1 cells, and is characterized by insulitis involving macrophage, CD4+ and CD8+ T-cell infiltration [26, 44, 45, 53]. It has been shown that allo-MSC administration either alone [54–59] or with islets [18, 21–23, 60, 61] alleviates hyperglycemia in diabetic animals and humans partly by promoting expansion of regulatory T cells and suppressing expansion of immune cells through here confirmed Tgfb1 expression (Supporting Information Figs. S2, S4) and IDO-1 upregulation (Supporting Information Fig. S3C) [54, 58, 62–65]. To explore the putative immune-modulating role of NI-contained MSCs in shielding the NIs from autoimmune attack, we examined a select set of known MSC immunomodulatory mechanisms as follows. Diabetic NOD mice were treated i.p. with allogeneic C57Bl/6 islets ($N = 3$) or with allogeneic NIs ($N = 3$). After 14 days, mice were euthanized. Spleens and omenta were harvested and tested by FACS for the percentages of CD3, CD4, CD8, FOXP3, CD25 positive cells. The percent of CD3/CD4 and CD3/CD8 double positive cells (helper and cytotoxic T lymphocytes) were significantly lower in spleen cells of NI-treated versus Islet treated NOD mice, while the percent of CD4/CD25/Foxp3 triple positive Tregs were significantly increased in the spleens and omenta of NI-treated versus Islet treated NOD mice (Fig. 4 and Supporting Information Fig. S6A, S6B). While the number of animals tested is small, these results are in agreement with others' findings [54–56, 58, 60, 61] and with our hypothesis that NIs, and specifically their MSC component, promotes euglycemia in T1 diabetic mice through modulation of the diabetogenic auto-immune response.

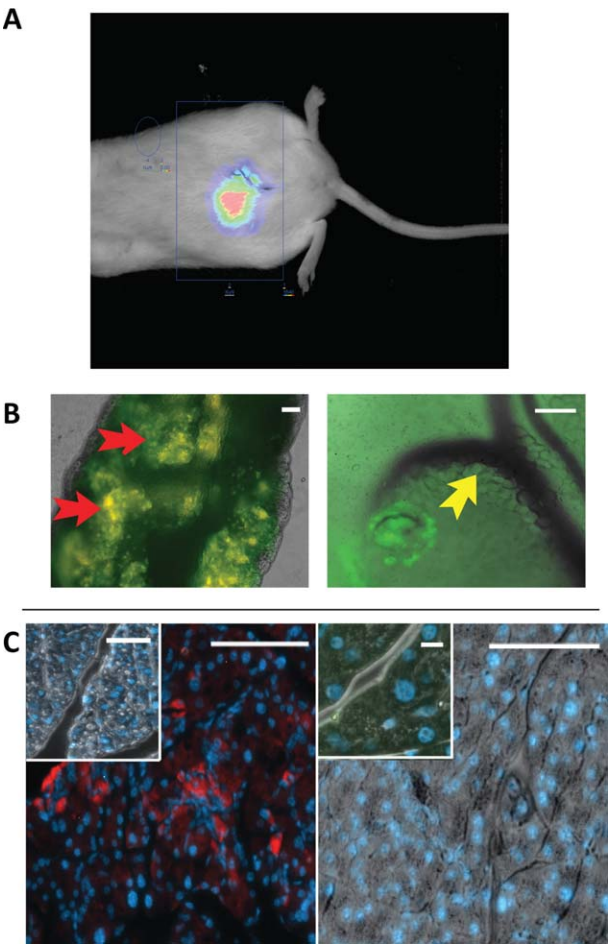

Figure 3. Neo-Islets (NI) engraftment, survival, and insulin expression in nonobese diabetic (NOD) mice omenta. **(A)**: Fluorescence in vivo imaging of a NOD mouse treated 10 weeks previously with DiR labeled, green fluorescent protein positive (*egfp*+) NIs demonstrates their location in the upper abdomen. **(B)**: *egfp*+ C57Bl/6 mouse NIs given i.p. remained engrafted in the omentum and maintained euglycemia in spontaneously diabetic NOD mice at 11 weeks post treatment (see Fig. 2). **Left image** (×10): representative omentum of a NOD mouse treated with C57Bl/6 *egfp*+ NIs (green; see red arrows). This image demonstrates that the NIs homed to and engrafted in the omentum, and indicates there is no rejection of the NIs. **Right image** (×10): enlarged image of a single, engrafted NI. Its location, close to capillaries (yellow arrow) is shown. **(C): Left panel, Main image**: Sections of the omentum (×10 image) depicted in (B) stained by immunohistochemistry for DNA (Dapi, blue), and insulin protein (red). Insulin protein was clearly detected. **Inset**, negative control in which the primary, anti-insulin antibody was omitted. **Right panel, Main image**: Sections of the omentum (×10) of a vehicle treated, diabetic NOD mouse stained for DNA (blue), and insulin protein (red). **Inset**: ×40 magnification of the same section (scale bar = 10 μm). No insulin was detected at either magnification. These images demonstrate the omental location and insulin synthesis by engrafted NIs. Except where indicated above, scale bars = 100 μm.

Collaboration of ICs and MSCs within NIs Is Essential to Establishing Normoglycemia in Diabetic Animals

To explore the collaboration between ICs and MSCs in NIs, two experiments using a readily controllable Streptozotocin (STZ) model of T1DM in C57Bl/6 mice were conducted and are

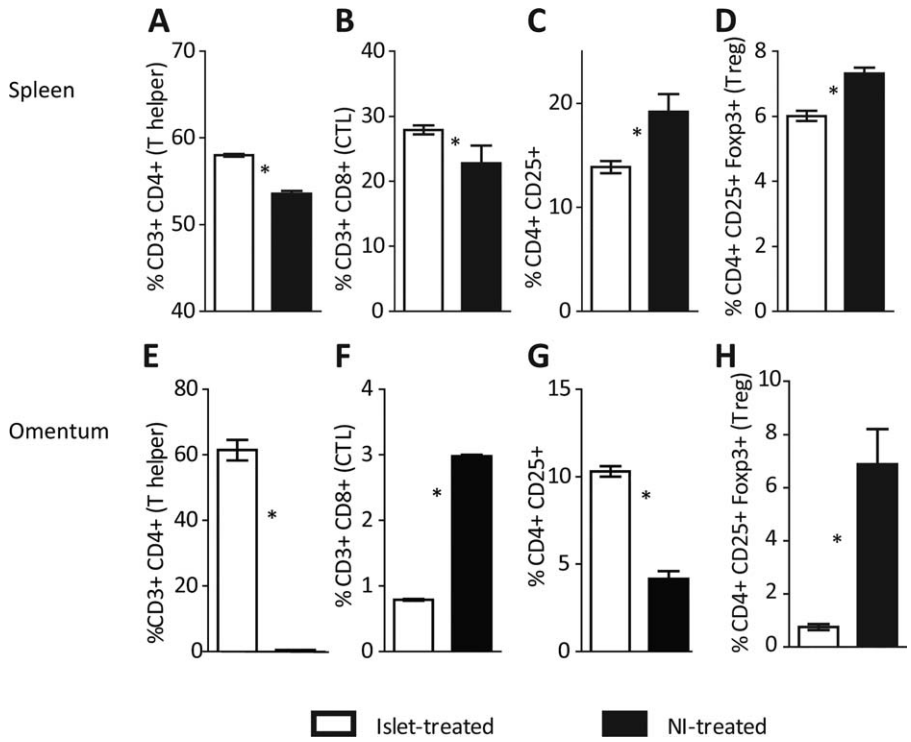

Figure 4. Percent of helper, cytotoxic, and regulatory T (CTL) cells from spleens **(A–D)** and omenta **(E–H)** of islet-treated ($N = 3$) versus Neo-Islets (NI)-treated nonobese diabetic (NOD) mice ($N = 3$) 14 days post i.p. administration. For (A), (E), (B), and (F), shown are the percent of CD3+ cells that are also (A) and (E) CD4+ or (B) and (F) CD8+. For (C), (G), (D), and (H), shown are the percent of CD4+ cells that were also (C) and (G) CD25+ or (D) and (H) CD25+Foxp3+. While the percentages of helper T cells were lower in NI-treated mice than in islet treated mice, the percentages of regulatory T cells (Treg) were significantly increased, suggesting that NIs helped restore normoglycemia in NOD mice (see Fig. 2) in part through immune-modulation. *, $p < .05$ versus islet-treated group. Representative FACS histograms of (A) through (H) are shown in Supporting Information Figure 6A and 6B. Abbreviations: CTL, cytotoxic lymphocytes; Treg, regulatory T cells.

summarized in Figure 5 (see also Table 1). First, STZ-diabetic C57Bl/6 mice were treated i.p. with 2×10e5/kg b.wt. syngeneic NIs ($N = 6$) or with vehicle ($N = 6$). Second, STZ-diabetic C57Bl/6 mice were treated i.p. with 2×10e5/kg b.wt. control clusters composed of either MSCs (P1; $N = 5$) or passaged ICs (P1; $N = 5$) alone. Importantly, the total number of cells in each generated cell cluster was identical to that in NIs (~1,000 cells per cluster). Three mice from the NI-treated group, and all mice from the control groups were euthanized at 12 weeks. The remaining 3 NI-treated mice were followed for 21 weeks. Long-term (21 weeks) euglycemia was obtained only in NI treated mice. Treatment with control clusters only minimally reduced blood glucose levels when IC clusters were given (Fig. 5A), demonstrating that both cell types must be present within NIs to facilitate optimal glycemic control.

In Vivo Redifferentiation

Data from the NOD mouse experiment (Fig. 2), as well as from their retrieved omenta (Fig. 3B) imply that the NIs redifferentiate in vivo to produce sufficient insulin to render mice euglycemic. Indeed, omenta retrieved from the euglycemic, C57Bl/6 NI-treated mice at 21 weeks showed both engraftment of NIs and significantly increased insulin, glucagon, somatostatin and Pdx1 gene expression compared to freshly formed NIs (Fig. 5B), demonstrating effective in vivo redifferentiation of islet hormone-expressing ICs. Furthermore, expression of Ins1 and Ins2 in whole pancreata of STZ-diabetic mice was, as expected, significantly reduced in all animals (Fig. 5C), indicating that euglycemia in NI-treated mice was achieved by physiological insulin secretion provided by omentally-engrafted NIs and not by residual pancreatic insulin.

Canine-Specific Insulin Secretion from cNIs in STZ-Diabetic NOD/SCID Mice, and Return of Hyperglycemia Upon Their Removal

Spontaneous diabetes mellitus in pet dogs is treated with insulin, but up to 40% are euthanized primarily due to the burden associated with their care [66]. Were we to demonstrate, in our Food and Drug Administration approved pilot study (INAD 012776), that cNI therapy was effective in dogs with T1DM, this would reduce euthanasia rates and the burden on dog owners. Furthermore, dogs with T1DM represent a clinically relevant large mammal model, providing potentially valuable information for envisioned clinical trials.

Treatment of NOD/SCID mice (routinely used for xenogeneic protocols) with 2x10e5 cNI/kg b.wt. maintained euglycemia, and significantly, surgical removal of the cNIs from treated, euglycemic mice caused the reappearance of hyperglycemia (Fig. 6A). When this group of mice was subjected to an i.p. GTT (Fig. 6B), only cNI-treated mice had a normal response, and only these released canine-specific insulin (Fig. 6C). Taken together, these data demonstrate further that the NIs redifferentiate in vivo to produce and secrete insulin physiologically in response to glucose.

Dose Dependency of Glycemic Control, and Control of Remote Onset T1DM by cNIs

To prepare for the canine pilot study, either 8×10e4 or 2×10e5 cNIs/kg b.wt. (cNIs) were administered i.p. to STZ diabetic NOD/

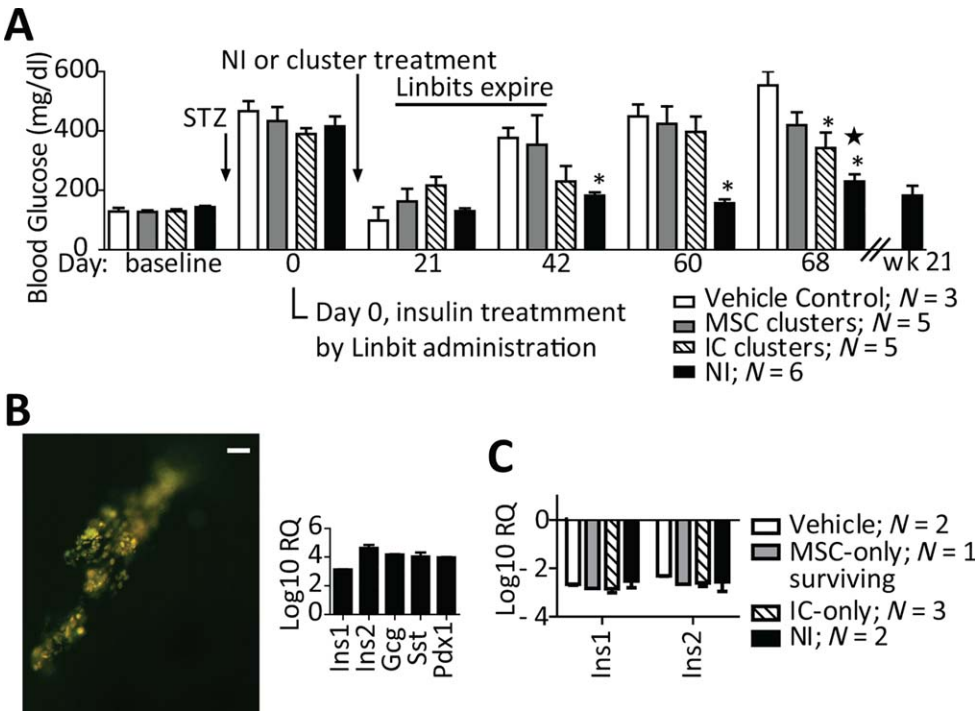

Figure 5. Blood glucose levels of NI versus MSC- or IC-cluster treated, STZ diabetic C57Bl/6 mice and in vivo redifferentiation of ICs into endocrine cells contained in the NIs. **(A)**: Blood glucose levels over time are shown in groups of STZ-diabetic mice all treated i.p. on day 7 with (i) vehicle, (ii) 2×10e5 MSC clusters/kg b.wt., (iii) 2×10e5 IC clusters/kg b.wt. or (iv) 2×10e5 NIs/kg b.wt. *, $p < .05$ versus vehicle-treated group. *, $p < .05$ versus MSC-cluster treated group. NIs maintain euglycemia, while MSC- and IC- clusters do not. **(B)**: Left: Fluorescence image (green, *egfp*+ cells) of a representative omentum from an NI-treated, euglycemic mouse 21 weeks post NI injection (scale bar = 200 μm). Right: omental gene expression profile (mean ± SEM) normalized to that of fresh NIs prior to administration, demonstrating NI engraftment, and significant endocrine redifferentiation. **(C)**: Ins1 and Ins2 expression profiles (mean ± SEM) from whole pancreata of MSC-cluster, IC-cluster, and NI-treated versus vehicle-treated diabetic mice normalized to those of nondiabetic mice. Since pancreatic insulin gene expression levels were similarly decreased in all treatment groups versus those of hyperglycemic, vehicle-treated mice, it follows that the blood glucose control seen in NI-treated mice was achieved by insulin secretion from omental NIs. NI + Neo-Islet. Abbreviations: IC, islet cell; MSC, mesenchymal stromal cell; NI, Neo-Islets; STZ, Streptozotocin.

SCID mice as indicated in Table 1. cNIs lower blood glucose dose dependently (Supporting Information Fig. S7A). Intraperitoneal administration of 2x10e5 cNI/kg b.wt. to NOD/SCID mice with remote onset STZ-induced DM, a potential model of therapy initiation later in the course of the disease, similarly restored euglycemia (Supporting Information Fig. S7B).

Intraperitoneal NIs Do Not Cause Hypoglycemia in Nondiabetic Mice

To further ascertain that NIs' insulin delivery is physiologic and does not cause hypoglycemia, nondiabetic C57Bl/6 mice were treated i.p. either with 2x10e5/kg b.wt. syngeneic NIs or vehicle. No animals developed hypoglycemia over time, and blood glucose levels were identical to those in vehicle treated controls (Supporting Information Fig. S8A). Analogous experiments wherein nondiabetic NOD/SCID mice were treated i.p. with either cNIs or vehicle also did not result in hypoglycemia at any time point (Supporting Information Fig. S8B).

Discussion

The present study was designed as a de novo attempt to overcome the principal hurdles that continue to limit the successful treatment of autoimmune-mediated T1DM with a readily available, progenitor or stem cell based therapy [1, 44]. Specifically, our investigations addressed (a) the shortage of suitable pancreas donors for the preparation of adequate islet cell numbers, (b) the difficulty of expanding β-cells in culture, (c) the permanent need for potentially toxic anti-rejection drugs in islet and pancreas transplants, (d) the alternative use of encapsulation devices for the immune isolation of islet or progenitor cell transplants, (e) the physiological delivery of insulin into the portal system of the liver, all to be achieved with a minimally invasive mode of NI administration, and by directly harnessing the complex pleiotropic actions of MSCs, cell types that are free of ethical concerns [26, 36–40, 42, 67–69]. We reasoned that newly formed NIs in which high numbers of healthy MSCs are combined with culture expanded ICs that have undergone expansion and dedifferentiation, would enable these cells, potentially aided by preservation of their epigenetic memory, to re-differentiate in vivo to functioning β-cells [12, 14, 70], to survive, and to be shielded from inflammatory and auto- and allo-immune attacks, thereby avoiding the need for anti-rejection drugs or encapsulation devices. We previously tested the fusion of ICs with MSCs, the creation of heterokaryons, in order to achieve immune protection of the endocrine component of such hybrid cells. We found this approach effective both in vitro and in vivo. However, both the low fusion efficiency and recent reports on the development of malignancies by fused cells made us abandon this technology [71, 72].

Since both standard subcutaneous insulin injections and subcutaneously placed encapsulated endocrine cells deliver insulin

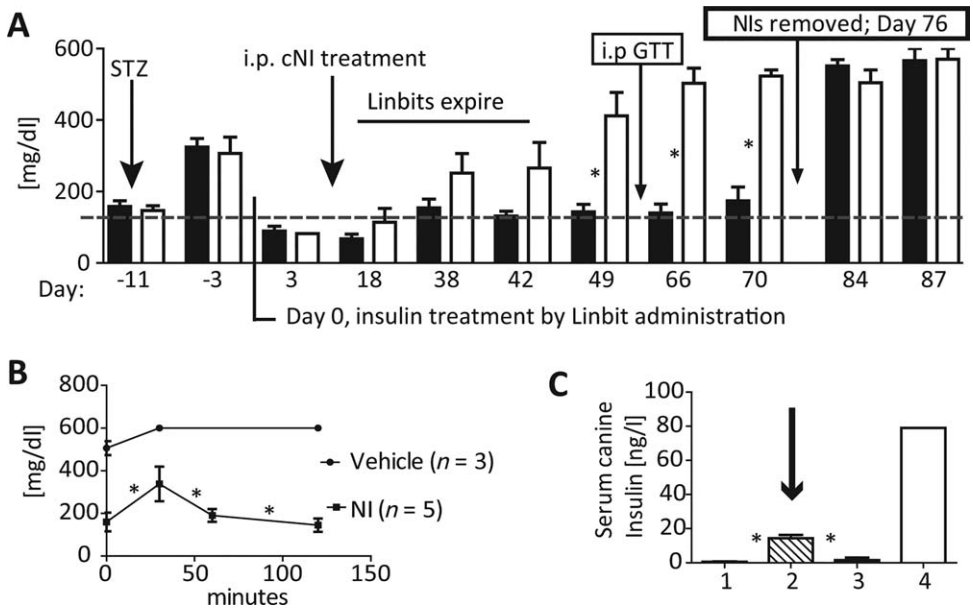

Figure 6. cNIs administered to diabetic nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice redifferentiate in vivo to control blood glucose levels. **(A)**: Blood glucose levels of Streptozotocin diabetic NOD/SCID mice treated with either 2x10e5 cNI/kg b.wt. (black bars, $N = 5$) or vehicle (open bars, $N = 5$). cNIs control blood glucose levels long term versus vehicle. Removing cNIs on day 76 resulted in the return of hyperglycemia. **(B)**: IP GTTs on day 63 (arrow) of cNI-treated NOD/SCID mice were normal versus those of vehicle treated animals. **(C)**: Canine-specific serum insulin (ELISA) levels rose during the IP GTT only in cNI-treated (column 2, cross-hatched bar, arrow, $N = 5$) NOD/SCID mice. Also shown are canine insulin levels in sera from vehicle-treated NOD/SCID mice (1st bar, $N = 3$); nondiabetic C57Bl/6 mice (3rd bar, $N = 2$, negative control for ELISA specificity) and a nondiabetic dog (4th bar, positive control). Together these data indicate that euglycemia was maintained as a consequence of canine insulin expression and secretion by cNIs. Data: mean ± SEM. *, $p < .05$ compared to control groups. Abbreviations: cNIs, canine NIs; i.p GTT, intra peritoneal glucose tolerance test; NI, neo-islets.

not into the portal vein of the liver, where up to 50% of it is inactivated, but exposes peripheral tissues to potentially harmful, supraphysiologic concentrations of this hormone [73, 74], we tested whether the unique biological functions of the well vascularized omentum, that is, the uptake of cells and foreign bodies, could be exploited to incorporate NIs that are intraperitoneally administered. Furthermore, as intrahepatic islet transplants are inefficient, requiring up to 5 donors per often repeated treatment, and being associated in high early losses of islets [1], a successful omental engraftment of NIs would be highly advantageous. If accomplished, it would facilitate their engraftment, redifferentiation and physiological function within the omentum. In this fashion, intraomentally secreted insulin would be, as is physiological, directly delivered into the portal system of the liver. An additional benefit the intraperitoneal location of NIs provides is the fact that glucose sensing from this location is superior to that from the subcutaneous space [29].

There is increasing evidence that MSCs that are located like pericytes in perivascular niches of all blood vessels exert in these microenvironments complex endothelial cell survival, immune-protective and anti-inflammatory actions [32, 75, 76]. Chronic hyperglycemic states result through various pathomechanisms in microvascular and macrovascular damage, which may also impair various functions of local MSCs and results in what has been termed a "pericytopathy," best described in the retinal microcirculation [24, 77, 78].

Finally, the novel microenvironment that is created in NIs that are composites of cultured ICs and equal numbers of MSCs, facilitates close range signaling by MSCs through the release of survival factors, cytokines, growth and angiogenic factors, anti-inflammatory miRNAs, the transfer of mitochondria and beneficial exosomes into adjacent ICs [26, 37, 42, 68, 79–82]. In addition, it would also enable cross talk between insulin producing and other islet endocrine ICs. In contrast to technologies that use monohormonal, that is, insulin-only producing cells, NIs are expected to contain all or most islet cell types, which may be advantageous. When MSCs are grown in 3D culture, their anti-inflammatory actions are potentiated, which may further enhance the therapeutic efficacy of the NI technology in subjects with T1DM [83, 84]. It is well established that MSCs respond to cues that arise from stressed or damaged cells, resulting in improved survival of such cells and repair through complex paracrine mechanisms, as has been shown in the acutely injured kidney, the bone marrow, and numerous other organs [68, 85]. Of further note is the fact that after an NI is formed none of its cells proliferate in vitro or in vivo, and MSCs do not undergo ectopic maldifferentiation or oncogenic transformation. Finally, if indicated by an unanticipated complication, the omentum that harbors NIs can be removed, as we show here, and standard insulin therapy can be resumed.

Although our NI technology appears to be an effective therapy in the tested rodent models, we expect that further refinements or modifications of this therapy will be needed. The i.v. administration of MSCs has been shown to have beneficial effects early in the course of T1DM [23, 26, 55, 56], and may thus be useful as an adjuvant therapy to NIs in subjects with a recent diagnosis of T1DM. The i.p. administration of NIs may be more efficient when delivered in hydrogel, Gelfoam or a thrombin clot, all of which can improve their initial adhesion to the omentum. Should there be evidence for premature rejection and loss of function of cNIs, a short initial course with rapamycin has been reported by others to improve islet survival and function [86]. If a potential recipient of this therapy lacks or has a damaged omentum due to a prior

intra-abdominal catastrophe, an intrahepatic transplant or a suitable encapsulation device for i.p. delivery would be required.

Ongoing studies regarding our NI technology are focused on analogous studies using human NIs in diabetic NOD/SCID mice, as well as on the characterization of the NI-intrinsic microcirculation post engraftment in the omentum, the long-term distribution of MSCs within the NIs in vivo, their potential differentiation into insulin-producing and vascular endothelial cells, the redifferentiation of alpha and other endocrine cells in vivo, in situ IDO (canine) and iNOS (murine) expression by MSCs, and a detailed analysis of the long-term histology and cell composition of functioning NIs.

Conclusion

In conclusion, the data from the present study demonstrate that efficient generation of NIs from mouse, canine and human cells is feasible and reproducible. The i.p. administration of NIs results in their engraftment, redifferentiation and survival in the omentum of spontaneously diabetic NOD mice, in SZT-diabetic mice, and equally well in allogeneic, syngeneic and xenogeneic treatment protocols. The fact that the therapy of diabetic NOD mice with allogeneic NIs results in durable euglycemia, and absent anti-islet cell and anti-MSC antibody production, up regulation of Tregs within the NI-carrying omentum, demonstrates that the utilized NIs provide both auto- and allo-immune isolation and importantly facilitate redifferentiation of ICs into insulin producing cells. Furthermore, since adequate capillary perfusion is essential for the function of islets in vivo, it follows that the potent angiogenic actions of MSCs induce the formation of a functioning capillary system within the NIs that connects to the omental microvasculature. Similarly, the successful glycemic control in STZ-diabetic NOD/SCID mice with cNIs provides a strong scientific basis for our Food and Drug Administration approved pilot studies in dogs with T1DM. We expect to generate from these pilot studies additional valuable information for potential future clinical trials.

Finally, the potential benefits NI technology could provide to patients with T2DM lies in the fact that the route of insulin delivery would once again be physiological, and thus would be expected to reduce insulin resistance, insulin-mediated lipogenesis and potentially harmful exposure of peripheral tissues to high concentrations of subcutaneously administered insulin [44, 45]. Respective preclinical studies that investigate these possibilities are currently underway.

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Author Contributions

C.W.: conception and design, financial, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript; A.G.: collection and assembly of data, data analysis and interpretation, manuscript writing; J.A. and Z.H.: collection of data; P.Z.: collection and assembly of data.

Disclosure of Potential Conflicts of Interest

A.G., J.A., P.Z., Z.H., and R.H., are employees of SCT, LLC; and C.W. is a consultant to SCT, LLC. C.W., A.G., P.Z., Z.H. are shareholders in SCT, LLC, and declare competing financial interests. Patent pending on the herein described technology.

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