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

CHRISTOF WESTENFELDER,†,‡, ANNA GOOCH,§ ZHUMA HU,∥ JON AHLSTROM,∥ PING ZHANG

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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...
MSC-Rich Neo-Islets Control Autoimmune T1DM

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 allo- genic 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 islets 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, allogenic 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 C57B/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 auto- immunity 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 nongenetic 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 nongenetic subjects (Proto 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 rPCR 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.mdysystems.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 IC 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 1x10^6 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 ABI 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, p H 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 × 10^5 NIs/kg b.w.t., 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 N1-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 (osseous, adipose- or chondrogenic).

**Allo-IgG Response**

Aliquots of ~5×10^4 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 (600g for 5 minutes), incubated with cy3-conjugated goat-anti-mouse IgG antibody (Jackson ImmunoResearch, www.jacksonimmuno.com), and analyzed by FACS (BD FACScan Analyzer, BD Biosciences, www.bdbiosciences.com) for percent green (MSCs) versus unstained (ICs) cells.

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Table 1. Treatment protocols

| Study question                                                                 | Recip. mice diabetes type | Recip. strain | Recip. age (wks) | Donor strain/species  | Islet cell passage | 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 rPCR for islet associated gene expression |
|                                                                                |                            |               |                  | NA                    | NA                | NA            | Vehicle control  | 6          | 12                               |                                                                           |
|                                                                                |                            |               |                  | wt C57Bl/6            | NA                | NA            | MSC only cluster | 5          | 12                               |                                                                           |
|                                                                                |                            |               |                  | wt C57Bl/6            | P1 wt             | C57Bl/6       | IC only cluster  | 5          | 12                               |                                                                           |
| Do NIs release insulin physiologically or cause hypoglycemia?                  | Non-diabetic               | C57Bl/6       | 12               | C57Bl/6 wt and egfp+ | P1 wt             | P5 egfp+ MSCs  | NI               | 14         | 0.5-12                           | Blood glucose levels, cell tracking                                     |
|                                                                                |                            |               |                  | NA                    | NA                | NA            | Vehicle          | 3          | 3                                | Blood glucose levels                                                    |
|                                                                                |                            |               |                  | wt C57Bl/6            | NA                | NA            | Vehicle          | 3          | 10                               | Blood glucose levels                                                    |
|                                                                                |                            |               |                  | NOD/SCID              | 9 Dog             | P1 P2 MSCs     | cNI              | 6          | 10                               |                                                                           |
| 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                             |                                                                           |

Abbreviations: 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.
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). 0.5×10^6 cells were stained per antibody, and 1×10^4 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 out 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 T1DM 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
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 /C24 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.w.t., 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 malign differentiation (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 Tgfβ1 expression (Supporting Information Figs. S2, S4) and IDO-1 upregulation (Supporting Information Fig. S3C) [54, 58, 62–65]. To explore the putative immunomodulating 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.

**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
summarized in Figure 5 (see also Table 1). First, STZ-diabetic C57Bl/6 mice were treated i.p. with $2 \times 10^5$ kg b.wt. syngeneic NIs ($N = 6$) or with vehicle ($N = 6$). Second, STZ-diabetic C57Bl/6 mice were treated i.p. with $2 \times 10^5$ 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 ($\sim 1,000$ 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 12 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]. We were 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 $2 \times 10^5$ 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 $8 \times 10^4$ or $2 \times 10^5$ cNIs/kg b.wt. (cNIs) were administered i.p. to STZ diabetic NOD/
SCID mice as indicated in Table 1. cNIs lower blood glucose dose dependently (Supporting Information Fig. S7A). Intraperitoneal administration of $2 \times 10^5$ 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 $2 \times 10^5$ cNI/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, 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...
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, intravenously 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, immunoprotective and anti-inflammatory actions [32, 75, 76]. Chronic hyperglycemic states result through various pathomechanisms 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 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 NIs, 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

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 2x10⁵ 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.
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 STZ-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 functional 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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