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Microencapsulated islet allografts in diabetic NOD mice and nonhuman primates

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Abstract. – OBJECTIVE: Our goal was to assess the efficacy of encapsulated allogeneic islets transplanted in diabetic NOD mice and streptozotocin (STZ)-diabetic nonhuman primates (NHPs).

MATERIALS AND METHODS: Murine or NHP islets were microencapsulated and transplanted in non-immunosuppressed mice or NHPs given clinically-acceptable immunosuppressive regimens, respectively. Two NHPs were treated with autologous mesenchymal stem cells (MSCs) and peri-transplant oxygen therapy. Different transplant sites (intraperitoneal [i.p.], omental pouch, omental surface, and bursa omentalis) were tested in separate NHPs. Graft function was monitored by exogenous insulin requirements, fasting blood glucose levels, glucose tolerance tests, percent hemoglobin A1c (% HbA1c), and C-peptide levels. *In vitro* assessment of grafts included histology, immunohistochemistry, and viability staining; host immune responses were characterized by flow cytometry and cytokine/chemokine multiplex ELISAS.

RESULTS: Microencapsulated islet allografts functioned long-term i.p. in diabetic NOD mice without immunosuppression, but for a relatively short time in immunosuppressed NHPs. In the NHPs, encapsulated allo-islets initially reduced hyperglycemia, decreased exogenous insulin requirements, elevated C-peptide levels, and lowered % HbA1c in plasma, but graft function diminished with time, regardless of transplant site. At necropsy, microcapsules were intact and non-fibrotic, but many islets exhibited volume loss, central necrosis and endogenous markers of hypoxia. Animals receiving supplemental oxygen and autologous MSCs showed improved graft function for a longer post-transplant period. In diabetic NHPs and mice, cell-free microcapsules did not elicit a fibrotic response.

CONCLUSIONS: The evidence suggested that hypoxia was a major factor for damage to encapsulated islets *in vivo*. To achieve long-term function, new approaches must be developed to increase the oxygen supply to microencapsulated islets and/or identify donor insulin-secreting cells which can tolerate hypoxia.

Key Words:
Islet transplantation, Microencapsulation, Diabetes, Hypoxia, Nonhuman primates.

Introduction

Our goal was to assess the efficacy of encapsulated allogeneic islets transplanted in diabetic mice and nonhuman primates (NHP). Human islet allografts reverse diabetes in patients with type 1 diabetes (T1DM)[1], and in the last decade there have been significant improvements in outcomes after islet transplantation[2,3]. Donor islet encapsulation may be valuable clinically because immunoisolation may reduce or eliminate the need for immunosuppression. However, encapsulation may put islets at risk of hypoxic damage due to the diffusion gradient of oxygen within non-vascularized capsules[4,5].

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Encapsulation promoted the survival of a murine beta-cell line transplanted intraperitoneally (i.p.) in diabetic NOD mice without immunosuppression[6], suggesting that preventing contact between host immune cells and donor islets might prevent allo-islet rejection. Therefore, we first tested the premise that encapsulated islet allografts would correct hyperglycemia long-term in diabetic mice and then in streptozotocin (STZ)-diabetic NHPs. For comparison with islet transplantation in diabetic patients, and because results in outbred NHPs may not mirror those in inbred mice, we used targeted immunosuppression with CTLA4-Ig and anti-CD154 mAb (dual costimulatory blockade), or other clinically acceptable regimens, for all NHP transplants. The oxygen consumption rate of islets is relatively high compared to other cell types[7], and even moderate hypoxia damages islet viability and function[8,9]. Since the i.p. site in NHPs is marginally hypoxic[10], we tested additional transplant sites using the omentum (within an omental pouch[11,12], within the bursa omentalis[13,14], and in a biologic scaffold on the surface of the omentum). Because oxygen inhalation therapy improved the outcome of islet transplantation in a rat model[15], we performed pilot transplants exposing recipient NHPs to relatively high ambient oxygen (45-85%) for the first week post-transplant and administered bone-marrow derived autologous mesenchymal stem cells (MSCs), which have beneficial effects on islet survival[16], during the first month post-transplant.

Materials and Methods

Animals

All mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and SPF Mauritian cynomolgus NHPs were purchased from the Mannheimer Foundation (Homestead, FL, USA) or Charles River Laboratories (Houston, TX, USA). NHPs were tissue-typed and recipient-donor combinations were matched as closely as possible for MHC alleles (see Supporting Information, ***Supplementary Table I***).

Murine and NHP Donor Islet Isolation, Recipient Management and Transplantation

All murine and NHP protocols were approved by the Institutional Animal Care and Use Committees of Emory University and The University of Miami, respectively. Murine and NHP pancreatic islets were isolated, microencapsulated and transplanted in various sites as described in Supporting Information. The function of encapsulated islets was documented by i.p. transplantation in SZN-diabetic nude mice (***Supplementary Figure 1***).

Immunosuppression, MSCs, and Inspired Oxygen Therapy

NHPs received various immunosuppressive regimens (Table I). CTLA4-Ig and anti-CD154 mAb were administered as previously described[17]. NHPs were treated with liraglutide, thymoglobulin, Enbrel, FK506, rapamycin, basiliximab, autologous MSCs, and supplemental inspired oxygen as described in Supporting Information.

In vitro Assessments

Assessment of capsule immunogenicity was performed using a bioassay as previously described[18]. Whole blood cell phenotyping, cytokine/chemokine level measurement, islet viability determination, and histologic and immunohistochemical evaluation of encapsulated islets were performed as described in Supporting Information.

Statistical Analysis

All data were analyzed using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA, USA). Graft survival data as well as cytokine/chemokine levels were reported as the mean ± the standard error of the mean (SEM). The statistical significance of differences in quantitative variables between groups was analyzed by unpaired (two-tailed) Mann Whitney *t*-tests with Welch correction. The statistical significance of differences among more than two groups was analyzed by the Tukey-Kramer Multiple Comparisons Test (parametric ANOVA). In all cases, p-values ≤ 0.05 were considered significant.

Results

Biocompatibility of Cell-Free Microcapsules

Cell-free double alginate capsules composed of an inner calcium-gelled compartment coated with poly-L-lysine and an outer strontium-gelled layer (see ***Supplementary Information***) did not elicit a response by a reporter cell line (THP1-XBlue™-MD2-CD14, InvivoGen, Corning, NY, USA) in a bioassay[18] (Figure 1A) (see ***Supplementary Information***), indicating very low immunogenicity. There was no fibrotic response in

Table I. Double capsule implant with or without allogeneic islets.

NHP ID	Txpl Sites	IEQ/kg	Treatment	Days Plasma CP	i.p. CP (ng/ml)	Reduced EIR (POD)	Reduced FBG (POD)
H14C103	4 sites[ł]	None	None	NA	NA	NA	NA
14C88	4 sites[ł]	None	None	NA	NA	NA	NA
H10C19	i.p.	9,000	CTLA4-Ig α-CD154 mAb	*63	10.86 (necropsy)	None	None
11C127[¥]	i.p.	13,452	Liraglutide CTLA4-Ig α-CD154	59	26.9 (Day 34 biopsy)	None	None
H10C71[¥]	i.p.	14,243	CTLA4-Ig α-CD154 mAb	*101	207.1 (necropsy)	[ǂ]63% (9-29)	[ǂ]34% (9-29)
10C156[¥]	i.p.	19,653	Liraglutide CTLA4-Ig α-CD154	*107	14.2 (necropsy)	[£]55% (9-69)	[£]55% (9-69)
H10C60[¥]	Omental Pouch	2,312	Liraglutide CTLA4-Ig α-CD154	0	Not done	None	None
H12C109[¥]	Omental Pouch	10,000	Liraglutide CTLA4-Ig α-CD154	83	Not done	[Ω]32% (20-57); 31% (98-124)	[Ω]23% (20-57); 45% (98-124)
H14C59[¥]	Omental Pouch	11,215	Basiliximab Enbrel, FK Rapa	28	Not done	None	None
14C12[¥]	Omental Pouch	12,322	Basiliximab Enbrel, FK Rapa	3	Not done	None	None
H14C19	In Bursa	12,000	Thymo, Enbrel, FK, Rapa	*21	Not done	None	None
H14C48	In Bursa	12,000	Thymo, Enbrel, FK, Rapa	68	Not done	38% (28-38)	53% (28-38)
14C72[¥]	On Omental Surface	11,688	CTLA4-Ig α-CD154 MSCs + O2	167	Not done	[€]42% (90-100)	[€]48% (90-100)
H14C126[¥]	On Omental Surface	17,303	CTLA4-Ig α-CD154 MSCs + O2	140*	Not done	91% (23-34)	56% (23-34)

Shown are the NHP ID number, transplant site, islet equivalents (IEQ) per kg, immunotherapy, type of capsule, number of days CP was detected in plasma, concentration of CP in PF at necropsy. *CP positive at necropsy, [¥]Capsules contained CXCL12 (1 μg/ml) in both the inner and the outer compartments, [ł]Cell-free capsules were implanted i.p., on the omental surface, in the bursa omentalis, and subcutaneously, [ǂ]H10C171: EIR mean ± SEM: 1.57 ± 0.06 *vs.* 4.19 ± 0.03 IU/kg/day; FBG mean 87 ± 9 *vs.* 131 ± 21 mg/dl, [£]10C156: EIR mean ± SEM: 1.82 ± 0.08 *vs.* 4.04 ± 0.10 IU/kg/day; FBG mean77 ± 5 *vs.* 173 ± 19 mg/dl, [Ω]H12C109: EIR mean ± SEM: 1.98 ± 0.03 *vs.* 2.92 ± 0.16 IU/kg/day (POD 20-57) and 2.01 ± 0.04 *vs.* 2.92 ± 0.16 IU/kg/day (POD 98-124) ; FBG mean 98 ± 51 *vs.* 128 ± 17 mg/dl (POD 20-57) and 70 ± 9 mg/dl *vs.* 128 ± 17 mg/dl (POD 98-124), anti-CD154 mAb withdrawn POD 98; CTLA4-Ig discontinued on POD 167. €14C72: EIR mean ± SEM: 2.34 ± 0.04 *vs.* 4.05 ± 0.4 IU/kg/day (POD 90-100) and 2.75 ± 0.02 *vs.* 4.05 ± 0.4 IU/kg/day (POD 170-190); FBG 78 ± 10 *vs.* 150 ± 12 mg/dl (POD 90-100).

BALB/c mice for up to 24 weeks or in diabetic NOD mice for up to 28 days after i.p. implantation of cell-free capsules (Figure 1B and inset). In two STZ-diabetic NHPs (H14C103 and 14C88, Table I), cell-free capsules were non-fibrotic after a month i.p. (Figure 1C and inset), demonstrating their biocompatibility.

Murine Islets In Single Capsules Transplanted i.p. in Diabetic NOD Mice Without Immunosuppression

We showed previously that an allogeneic beta-cell line in single calcium-gelled alginate-PLL-alginate (APA) capsules corrected diabetes in non-immunosuppressed diabetic NOD mice[6]. In our new

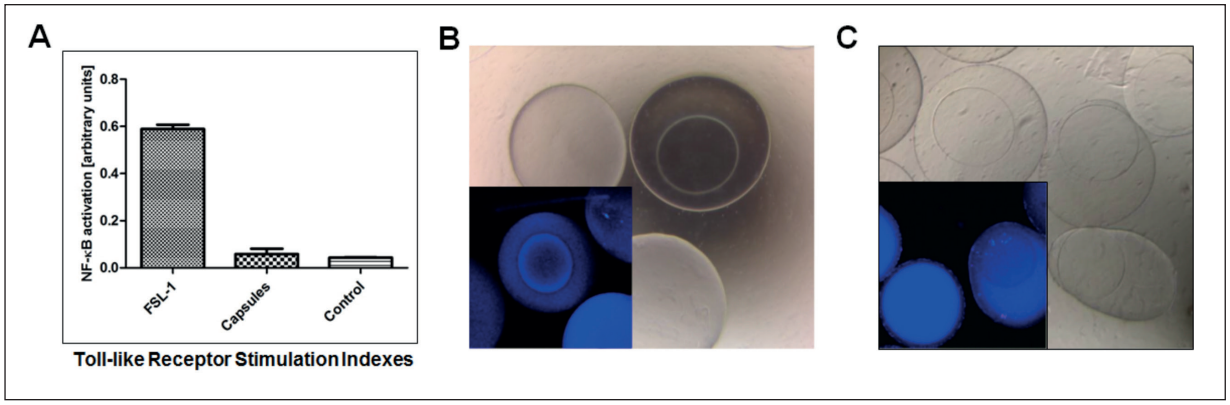

Figure 1. Biocompatibility of cell-free double capsules in vitro and in vivo. **A**, A reporter cell line was not activated by double alginate capsules in vitro, nor by the negative control (RPMI culture medium alone) but was activated by the positive control (FSL-1, synthetic diacylated lipoprotein recognized by toll-like receptors on the reporter cells). **B**, Cell-free double capsules from peritoneal cavity of diabetic NOD mouse, POD 28, phase contrast microscope, 4X mag, Inset: no live cells present on the surface of the capsules as indicated by Live/Dead stain, confocal microscope 5X mag. **C**, Cell-free double capsules from the peritoneal cavity of a STZ-diabetic NHP on POD 28, phase contrast 4X mag, Inset: no live cells present on the surface of the capsules as indicated by Live/Dead stain, confocal, 5X mag.

experiments, single barium-gelled alginate-encapsulated C57BL/6 (H-2^b) islets functioned i.p. in diabetic NOD mice (H-2^{g7}) for up to 553 days (329 ± 38 days, n=23), BALB/c (H-2^d) islets for up to 515 days, (278 ± 46 days, n=12, Figure 2A), and C3HeB/FeJ (H-2^k) islets for up to 534 days (313 ± 40 days, n=13) without immunosuppression. Microcapsules from mice with long-term functioning grafts were intact with little, if any, host-cell overgrowth (Figure 2A inset). Non-encapsulated C57BL/6 islets functioned for 6 ± 1 days (n=11), BALB/c islets for 3 ± 1 days (n=5), and C3HeB/FeJ islets for 1 ± 0.3 days (n=4). Encapsulation prevented destruction of syngeneic islets in two diabetic NOD recipients for 330 and 480 days (Figure 2B), unlike non-encapsulated syngeneic grafts (7 ± 1 days, n=3), suggesting that encapsulation inhibits host autoimmune-mediated damage to donor islets.

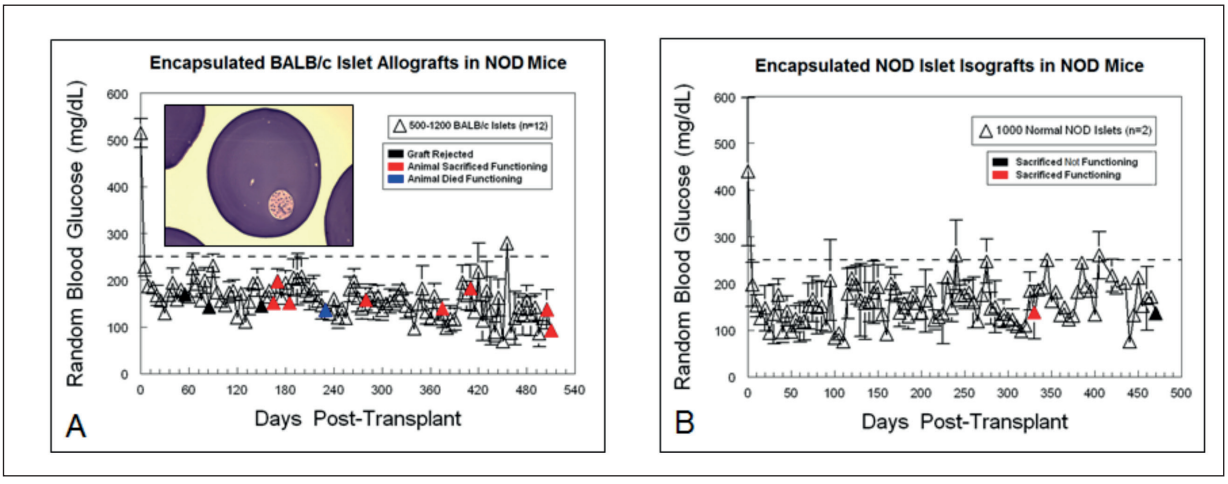

Figure 2. A, Function of BALB/c islet allografts in single Ba+-gelled microcapsules in diabetic NOD mice without immunosuppression. Average BG levels of NOD mice transplanted i.p. with 1000 IEQ encapsulated BALB/c islets (n=12) are shown. Inset, H&E staining of single capsule from NOD with a functioning islet allograft on Day 280 post-transplantation, showing viable islet with no host-cell overgrowth on the capsule. **B**, Function of encapsulated normal NOD islet isografts in diabetic NODs. Average BG levels in mice transplanted i.p. (n=2) with 1000 IEQ encapsulated NOD islets are shown. One mouse was sacrificed with a functioning graft on day 330 post-transplant, and the other was sacrificed when the graft failed after >450 days of function.

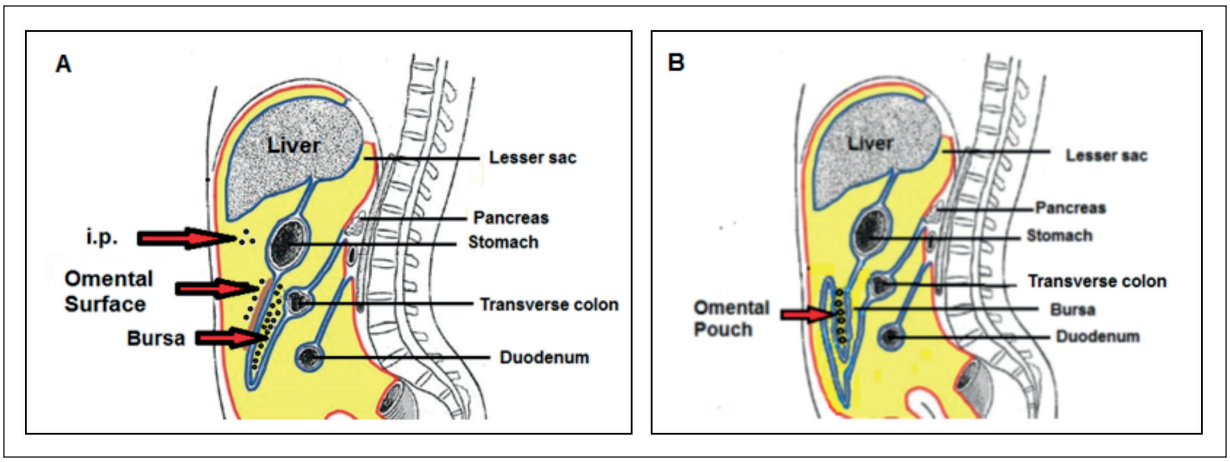

Figure 3. Diagram of primate peritoneal cavity showing 4 sites for transplantation of encapsulated islets (saggital plane). **A**, Locations of capsules transplanted i.p., on the omental surface, or within the bursa. **B**, Location of capsules transplanted in an omental pouch. The figure was adapted by one of us (SAS) from an illustration in Teach Me Anatomy, part of the Teach Me Series Educational Healthcare Resources (teachmeseries.com).

Encapsulated NHP Islets Transplanted i.p. in Immunosuppressed Diabetic NHPs

To prevent potential antibody-mediated damage, NHP donor islets were encapsulated in double alginate capsules which exclude IgG[17]. Four NHPs were transplanted i.p. (Figure 3A) and given CTLA4-Ig and anti-CD154 mAb with or without liraglutide, a glucagon-like peptide-1 receptor agonist that stimulates insulin release from islets[19,20]. Three of these animals also received intracapsular CXCL12, a chemokine with pro-survival and immunomodulatory properties beneficial to islets[21] (Table I).

In contrast to long-term BG control in most mice with encapsulated allogeneic islets, i.p. transplanted encapsulated allo-islets functioned for less time (Table I). While fasting C-peptide (CP) increased in all 4 animals, glycemic control did not improve in 2 NHPs receiving 9,000 (H10C19) or 13,452 IEQ/kg (11C127) and having CP values of ≤1.0 ng/ml (Figure 4A, B and C, D). NHP 11C127 received a second, marginal islet mass transplant (6,510 IEQ/kg) on POD 95 in the external oblique muscle, which did not improve graft function. Biopsies (POD 34, POD 110 and at elective necropsy on POD 118) showed no adherent host cells, fibrosis, or broken capsules, and some of the islets remained viable until elective necropsy (***Supplementary Figure 2***).

Two NHPs transplanted i.p. with a larger islet mass (H10C71 and 10C156, 14,243 and 19,653 IEQ/kg respectively) and CP >1.0 ng/ml in the peri-transplant period had transient improved graft function compared to pre-transplant (Figures 4 E, F, G, H). NHP H10C71 had improved glycemic control for 20 days with a 63% reduced exogenous insulin requirement (EIR) and 34% reduced fasting blood glucose (FBG) (Table I), followed by steady increases in both parameters, and decreasing CP (Figure 4E, F). For NHP 10C156, with the largest islet mass, graft function lasted for 60 days, with a 55% decrease in both EIR and FBG (Table I), despite more extensive MHC mismatch as compared to the previous three recipients (***Supplementary Table I***). FBG increased and CP decreased thereafter.

To investigate potential mechanisms for graft failure, we compared cytokines and chemokines in peritoneal fluid (PF) at necropsy of all 4 transplanted NHPs and 2 healthy (non-transplanted) NHPs, and in pre- and post-transplant serum in NHP H10C19 (POD-1, 14 and 58) and NHP H10C71 (POD -1, 15, 88 and 101) and in 6 healthy NHPs. Minimal levels of cytokines IL-1β, IL-6, IL-8, IL-10, IL-15 and IFNγ, and TNFα were detected in the serum and PF of all transplanted animals evaluated (***Supplementary Figure 3***). Chemokine levels in PF and serum were statistically comparable between transplanted NHPs and controls, except in IP-10 which was lower in serum after transplant. For one animal, HC1071, we noted an increase in Eotaxin, IP-10, and RANTES in the post-transplant period (***Supplementary Table II***).

At necropsy, non-fibrotic capsules (Figures 5A and 5B) were free-floating or resting on the omentum but not attached by fibrosis (Figure 5C).

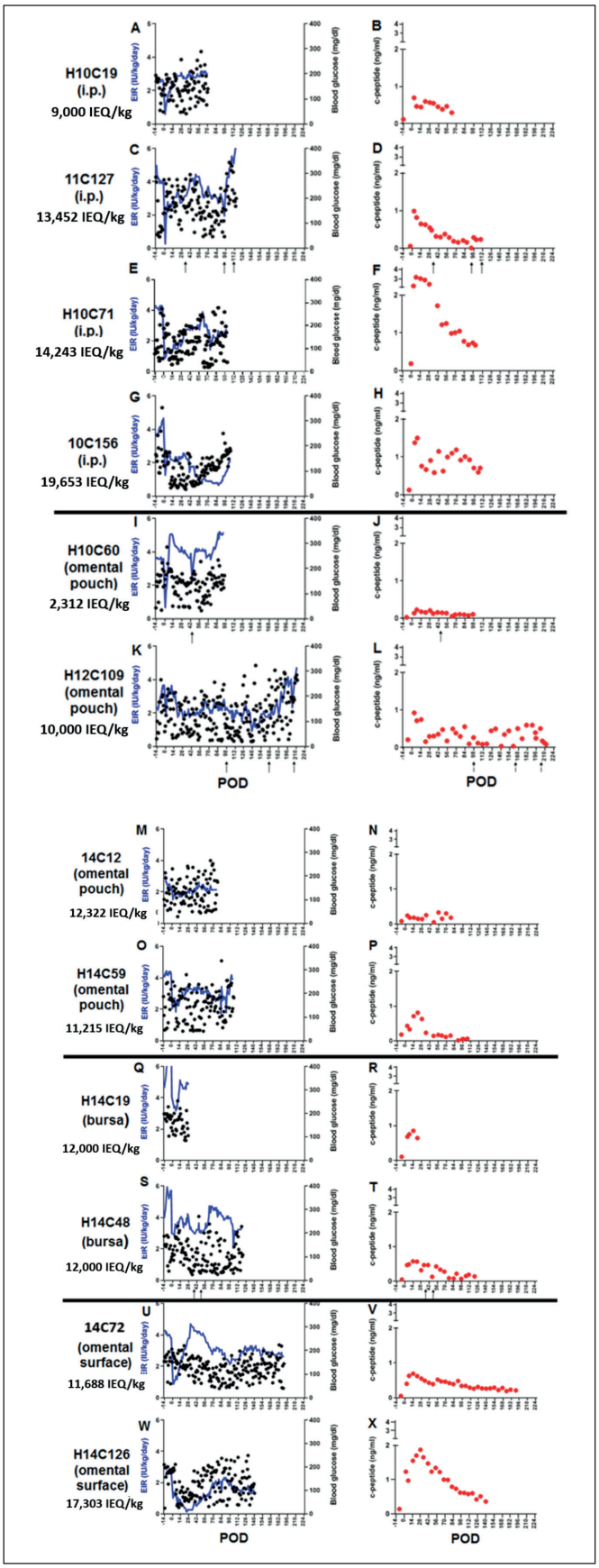

Figure 4. Function of encapsulated NHP islet allografts transplanted i.p or in an omental pouch in immunosuppressed STZ-diabetic NHPs. Left Column: FBG (black circles) and exogenous insulin requirements (EIR, blue line) over time. Right Column: Corresponding plasma CCP levels (red circles) over time. **A**, and **B**, NHP H10C19* transplanted i.p (9,000 IEQ) and treated with CTLA4-Ig and anti-CD154 mAb; **C**, and **D**, 11C127 transplanted i.p (13,452 IEQ) and treated with CTLA4-Ig and anti-CD154 mAb plus liraglutide. The arrows indicate an intraperitoneal biopsy on POD 34, an intramuscular islet transplant of 6,510 IEQ/kg on POD 95 and a biopsy of the intramuscular site on POD 110 (elective necropsy on POD 118). **E**, and **F**, H10C71 transplanted i.p (14,243 IEQ) and treated with CTLA4-Ig and anti-CD154 mAb. **G**, and **H**, 10C156 transplanted i.p (19,653 IEQ) and treated with CTLA4-Ig and anti-CD154 mAb plus liraglutide. **I**, and **J**, H10C60 transplanted in an omental pouch (2,312 IEQ) and treated with CTLA4-Ig and anti-CD154 mAb plus liraglutide, the arrow indicates a biopsy of the omental pouch on POD 43. **K**, and **L**, H12C109* transplanted in an omental pouch (10,000 IEQ) and treated with CTLA4-Ig and anti-CD154 mAb plus liraglutide, the arrows indicate stopping administration of anti-CD154 mAB on POD 98, stopping administration of CTLA4-Ig on POD 167 and survival omentectomy on POD 203 (elective necropsy on POD 212). **M**, and **N**, NHP *H14C19 transplanted within the bursa omentalis (12,000 IEQ) and given thymoglobulin, Enbrel, FK106, and rapamycin. **O**, and **P**, H14C48 transplanted within the bursa omentalis (12,000 IEQ) and given thymoglobulin, Enbrel, FK106, and rapamycin. **Q**, and **R**, NHP H14C59 transplanted in a biologic scaffold on the omentum (12,000 IEQ) and given basiliximab, Enbrel, FK106, and rapamycin. **S**, and **T**, NHP 14C12 transplanted in a biologic scaffold on the omentum (12,000 IEQ) and given basiliximab, Enbrel, FK106, and rapamycin. **U**, and **V**, NHP 14C72 transplanted in a biologic scaffold on the omentum (11,688 IEQ) and given CTLA4-Ig plus anti-CD154 mAb, autologous MSCs and inspired O2. **W**, and **X**, NHP H14C126 transplanted in a biologic scaffold on the omentum (17,303 IEQ) and given CTLA4-Ig plus anti-CD154 mAb, autologous MSCs and inspired O2. *=Capsules did not contain CXCL12.

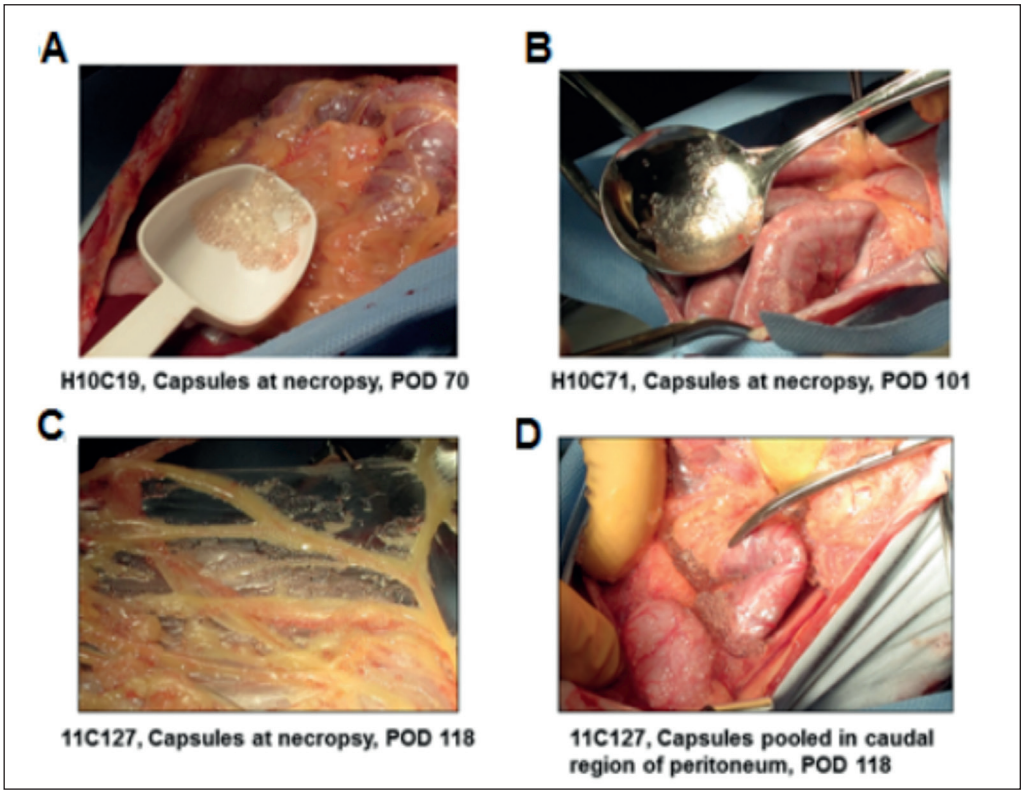

Figure 5. Images of clean, non-fibrotic double alginate capsules containing NHP islets collected from the peritoneal cavity of STZ-diabetic NHPs. **A**, Free-floating, clean capsules retrieved from peritoneal cavity of NHP H10C19, POD 70. **B**, Free-floating, clean capsules retrieved from peritoneal cavity of NHP H10C71, POD 101. **C**, Clean, non-fibrotic capsules resting on the omentum of NHP 11C127, POD 118. **D**, Capsules pooled in the Douglas pouch of NHP 11C127, POD 118.

Many capsules had pooled by gravity in the caudal area of the peritoneal cavity (the Douglas pouch) (Figure 5D, representative NHP). The remaining insulin-positive islets were obviously smaller than those originally transplanted on POD 0 (compare Figures 6A and 6B, representative NHP), suggesting islet damage by non-immune mechanisms, such as oxidative stress due to the hypoxic peritoneal environment[10], especially after settling in the Douglas pouch. However, dithizone-positive (Figure 6C), viable (Figure 6D), intact (Figure 6E) islets were present in capsules retrieved from the peritoneum, and substantial concentrations of CP were detected in the PF of all four NHPs (Table I), evidence that some functional islets remained although possibly not enough to normalize BG.

Encapsulated NHP Islets Transplanted in a Biologic Scaffold, Within an Omental Pouch in Immunosuppressed Diabetic NHPs

To prevent free microcapsules from falling into the Douglas pouch, capsules were attached to a biologic scaffold on the omentum using autologous plasma/thrombin clotting and enclosed in an omental pouch[11] (Figure 3B). The rationale for using an omental pouch was three-fold: (1) to prevent capsules from aggregating in the Douglas pouch, where they might be subjected to sub-optimal oxygenation, (2) to increase the oxygenation of islets by implanting them in a highly vascularized site (the omentum), and (3) to place the capsules where they could be retrieved, if necessary (by omentectomy).

To test the feasibility of this transplant site, NHP H10C60 received a minimal islet dose (2,312 IEQ/kg) (Table I) with liraglutide and dual costimulatory blockade. While there was little evidence of graft function (Figures 4I and 4J, Table I), a biopsy (POD 43) showed some capsules located near capillaries in the omentum (Figure 7A), containing viable, insulin-positive islets (Figures 7B and 7C), and surrounded by a thin layer of host cells, predominantly $CD68^+$ macrophages and fibroblasts (Figure 7D). It is likely that the fibrosis resulted from the plasma/thrombin clotting technique used to attach the capsules to the omentum, unlike the free-floating capsules transplanted in the i.p. space in the previous 4 NHPs, which had no fibrosis.

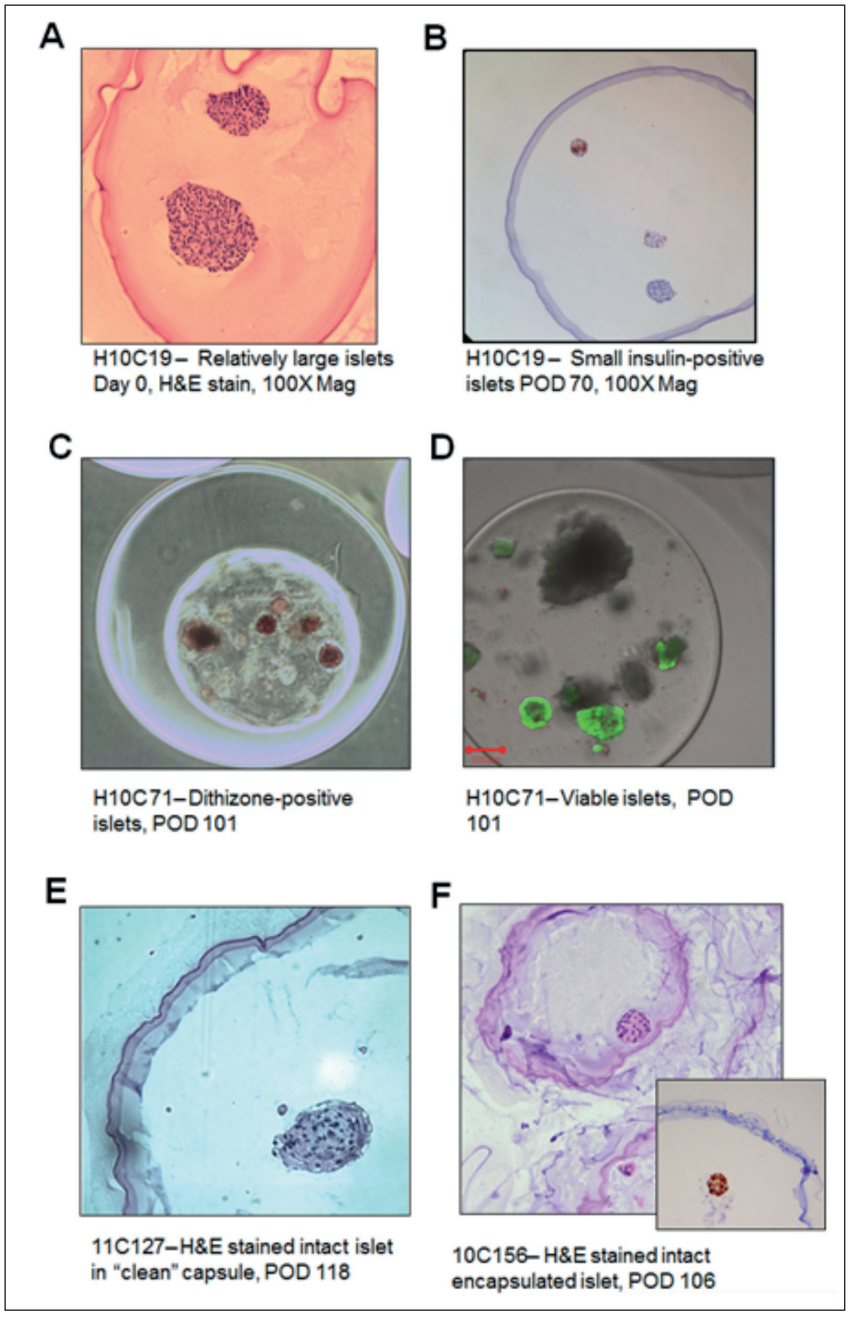

Figure 6. Histological, immunohistochemical, and viability stains of encapsulated NHP islets from the peritoneal cavity of SZN-diabetic NHPs. **A**, Relatively large islets originally transplanted in NHP H10C19, POD 0; 100X Mag. **B**, Relatively small, insulin-positive islets collected from NHP H10C19 at necropsy on POD 70, 100X Mag. **C**, Dithizone-positive islets from the peritoneal cavity of NHP H10C71, POD 101. **D**, Viable (Calcium AM fluorescent, green) islets from the peritoneal cavity of NHP H10C71, POD 101. **E**, H &E-stained intact islet in a free-floating capsule from the peritoneal cavity of NHP 11C127, POD 118. **F**, H &E-stained encapsulated islets from the peritoneal cavity of NHP 10C156, POD 106, Inset: Insulin-positive islet in capsule adherent to the omentum of NHP 10C156, POD 106.

The experiment was repeated in NHP H12C109 using a larger islet dose (10,000 IEQ/kg) (Table I). Because the omentum was relatively small, the entire omental surface was required to accommodate the encapsulated islets (~12 ml), and when folded in half to form a pouch, the two opposite layers of capsules were unavoidably in direct contact. Fasting CP levels were higher than pre-transplant values, although ≤1.0 ng/ml, and starting on POD 20 the graft functioned for 37 days, with 32% decrease in EIR and 23% decrease in FBG (Table I). After POD 98, the average FBG increased to pre-transplant levels despite increasing the insulin dose (Figure 4K). Anti-CD154 mAb was discontinued on POD 98 (***Supplementary Figure 4***), followed by a 26-day reduction in average EIR and FBG (31% and 45% *vs.* pre-transplant levels, Table I). Withdrawal of CTLA4-Ig on POD 167 resulted in erratic glucose metabolic control, notwithstanding increased insulin administration. A survival omentectomy on POD 203 resulted in steep increases in EIR

and sequential negative CP values (Figure 4K and L), demonstrating that the minimal CP had been due to islets in the omental pouch. Encapsulated islets from the omental pouch stained positively for pro-inflammatory cytokines known to damage islets[22-24] (IL-6, TNFα, IL1-β, and IFNγ, ***Supplementary Figure 5***) and for three endogenous markers of hypoxia[25] (osteopontin, GLUT-1, and HIF1α, ***Supplementary Figure 6***).

Next, an MHC-matched pair of animals, 14C12 and H14C59, received equal doses of encapsulated allogeneic islets (12,000 IEQ/kg each) from the same islet preparation in an omental pouch, but using steroid-free systemic immunosuppression (Table I). NHP 14C12 had marginal CP on POD 3 (0.24 ng/ml); subsequent CP values >0.2 ng/ml were associated with high FBG. There was no post-transplant improvement in glycemic control. At necropsy, capsules were coated with host cells (***Supplementary Figure 7A***) including $CD68^+$ macrophages (***Supplementary Figure 7B***) plus $CD3^+$ lymphocytes (***Supplementary Figure 7C***) and $CD4^+$ T cells (***Supplementary Figure 7D***), suggesting that the islets had been immunologically rejected, in spite of the immunosuppression. The companion NHP H14C59 had a maximum fasting CP on POD 21 (0.81 ng/ml), insufficient to improve FBG, and a steep decrease in CP after POD 28. Necropsy revealed that because of a technical failure, most of the islets did not remain in the omental pouch as intended but had pelleted into the Douglas pouch.

Encapsulated NHP Islets Transplanted in the Bursa Omentalis in Immunosuppressed Diabetic NHPs

In a preliminary experiment with cell-free double capsules infused into the bursa omentalis of a healthy NHP (***Supplementary Figure 8***), the capsules dispersed evenly between the two layers

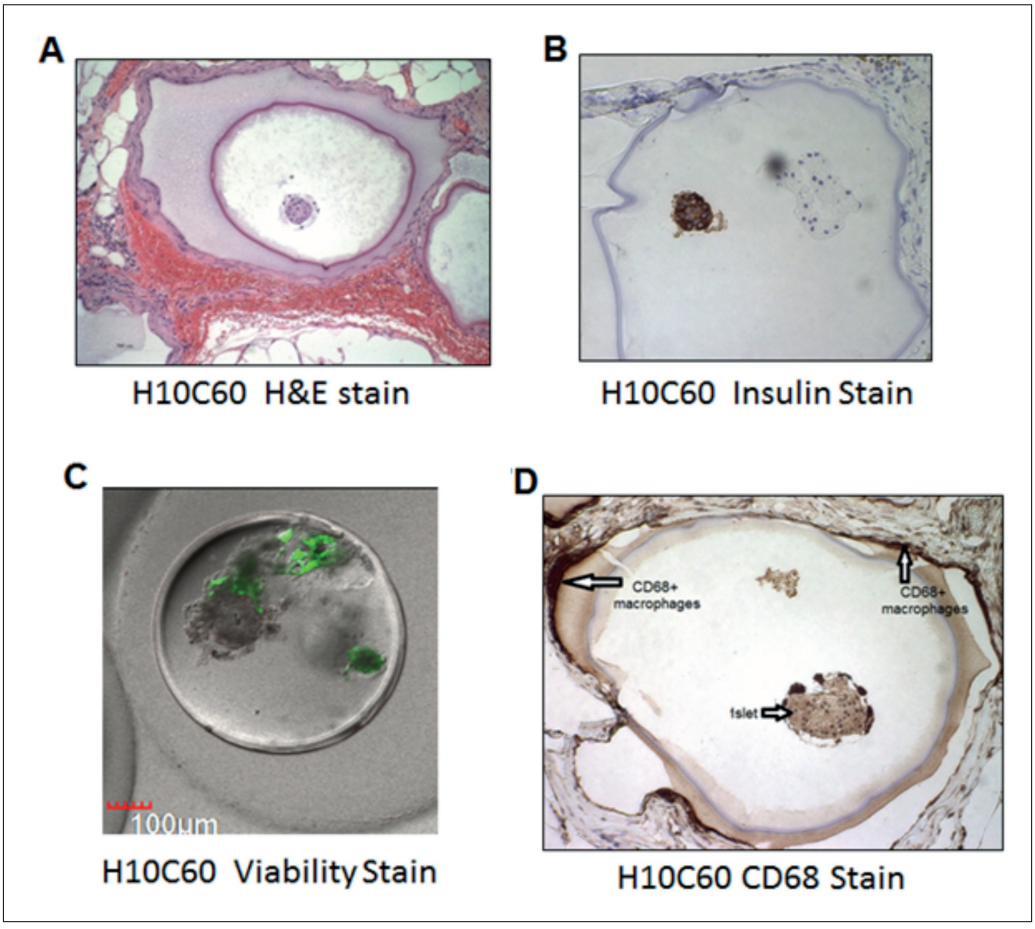

Figure 7. Immunohistochemical staining of encapsulated NHP islets in a biologic scaffold in the omental pouch of NHP H10C60 at necropsy on POD 43. A, H&E stained capsules. B, Insulin-stained encapsulated islets. C, Viability-stained encapsulated islets. D, CD68+ macrophages in a layer of host cells surrounding capsule.

of the bursa, which had a relatively large surface area. Thus, transplantation within the bursa omentalis might avoid the limitations of i.p. transplants, such as aggregation of capsules in the Douglas pouch and capsule crowding within an omental pouch. Two matched NHP recipients (H14C19 and H14C48) were infused in the omental bursa (Figure 3A) with mismatched encapsulated islets from the same islet pool (12,000 IEQ/kg each) without intracapsular CXCL12 (Table I and ***Supplementary Table I***) and were given a steroid-free systemic immunosuppression, including induction with thymoglobulin (Methods, ***Supplementary Information***). Both recipients had improved fasting CP compared to pre-transplant, although <1 ng/ml (Table I, Figures 4Q, R, S, T).

Unfortunately, NHP H14C19 expired on POD 28 due to intussusception and high levels of CMV were detected in all tissues. At necropsy, most of the capsules had aggregated in the caudal portion of the bursa omentalis (***Supplementary Figure 9A***), and many of the islets within these capsules showed evidence of hypoxic damage (Figure S-9B), but insulin-positive islets were still present (***Supplementary Figure 9C***). The surviving companion, NHP H14C48 experienced a peak of CP (POD 14-21) with a notable reduction in EIR and FBG (average 38% and 53%, respectively, POD 28-38). Based on these results, the dose of FK506 was tapered beginning on POD 38 and rapamycin starting on POD 48. However, in the complete absence of FK506 (by POD 54) and rapamycin (by POD 61), glucose control destabilized with negative CP values on POD 76. Thymoglobulin caused severe depletion of lymphocytes (POD 1- 50), especially $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells (Figure 8 A and 8B). Levels of neutrophils and $CD11b^+$ macrophages were stable (POD 1-30) and then peaked by POD 54 (Figures 8A and 8D). By POD 69, in the absence of maintenance therapy, the levels of $CD3^+CD8^+$T cells and $CD20^+DR^+$B cells rebounded (Figure 8B and 8C), but the $CD3^+CD4^+$ T cell population did not recover (Figure 8B) and the total number of neutrophils and macrophages fell rapidly (Figure 8A). At necropsy on POD 120, pristine free-floating capsules were found containing intact, viable islets (Figures S-10 A, B, C). An *in vitro* diffusion study showed that the outer compartment of explanted double capsules was permeable to IgG, but not the inner PLL-coated

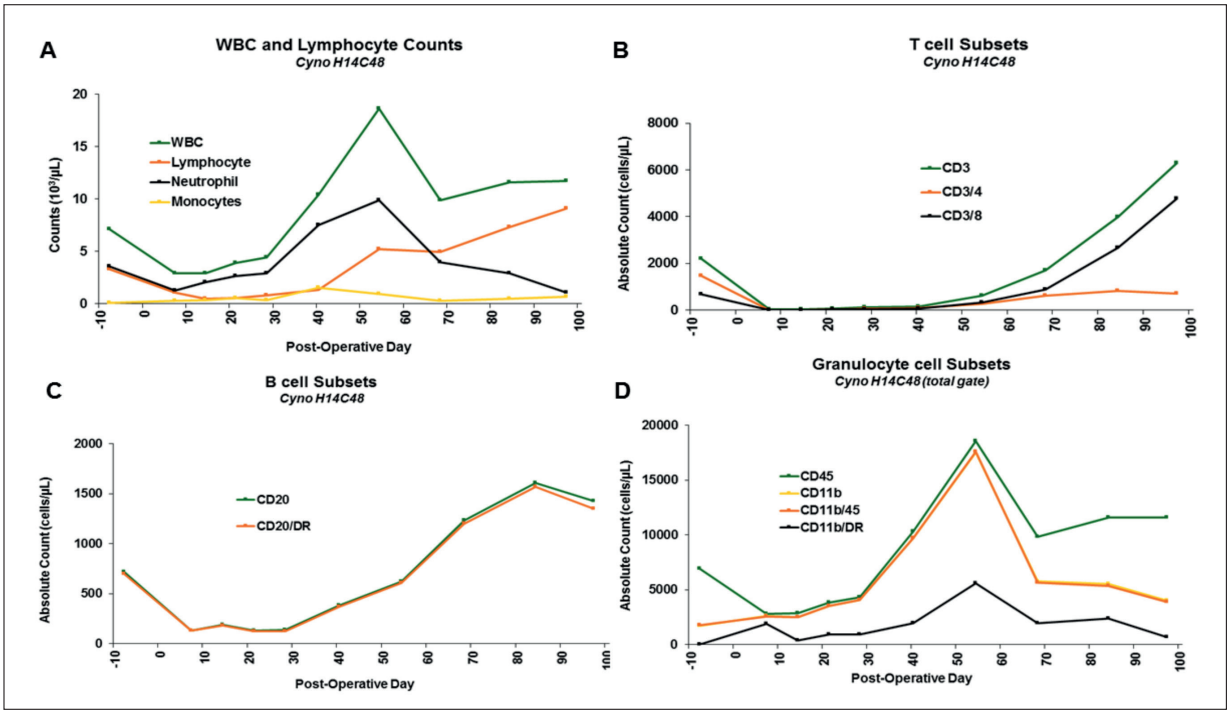

Figure 8. Phenotypes of peripheral blood leukocytes from H14C48 over time post-transplant. **A**, Numbers of white blood cells (green), lymphocytes (orange), neutrophils (black), and monocytes (yellow). **B**, Numbers of CD3 T cells (green), CD3+CD4+ T cells (orange), and CD3+CD8+ T cells (black). **C**, Numbers of CD20+ B cells (green) and activated CD3+DR+ B cells (orange). **D**, Numbers of granulocyte populations, including CD45 cells (green), CD11b macrophages (yellow, under the orange line), CD11b/45 macrophages (orange); and CD11b/DR macrophages (black). NOTE: The Y axis scales are different on each graph.

compartment (***Supplementary Figure 10 D and E***), indicating that the diffusivity characteristics of the capsule remained stable over time in the bursa. However, at necropsy, most of the capsules had aggregated in the caudal portion of the bursa and the islets showed evidence of hypoxic damage (central necrosis), although some insulin-positive islets remained, similar to findings in NHP H14C19 (***Supplementary Figure 9***).

Encapsulated NHP Islets Transplanted in a Biologic Scaffold on the Omental Surface in Immunosuppressed Diabetic NHPs with MSCs and Supplemental Oxygen

Because of the unexpected finding of pooled microcapsules within the omental bursa, the next 2 recipients (14C72, and H14C126, Table I and ***Supplementary Table I***) were transplanted in a biologic scaffold on the omental surface (without a pouch, to utilize a larger omental surface area and maximize islet oxygenation) (Figure 3A; formation of biologic scaffold, ***Supplementary Figure 11***). Since systemic immunosuppression did not prevent host-cell infiltration to the graft-site, we returned to targeted immunosuppression (CTLA4-Ig and anti-CD154 mAb) in the next 2 NHPs. As opposed to all previous transplants, the grafts were almost (14C72) or fully (H14C126) MHC mismatched (***Supplementary Table I***). In Lewis rats, co-transplantation of syngeneic MSCs promoted revascularization and function of intrahepatic syngeneic islets[26], and in NHPs co-transplantation of MSCs enhanced intrahepatic allo-islet engraftment[16]. Therefore, we tested the hypothesis that co-transplantation of autologous MSCs would enhance vascularization near the graft and benefit oxygenation. We also administered peri-transplant oxygen therapy aiming to improve encapsulated allogeneic islet function further (see ***Supplementary Information***).

NHP 14C72 received a lower islet dose (11,688 IEQ/kg) and had fasting CP levels of <1.0 ng/ml, that peaked at POD 14 and decreased slowly thereafter, requiring increasing insulin doses until POD 34 (Figure 4U and 4V). Thereafter, FBG levels decreased as the insulin dose was reduced, reaching minimum average FBG and EIR between POD 90-100 (48% and 42% *vs.* pre-transplant values, respectively, Table I). The average FBG subsequently increased, but EIR remained 32% reduced from pre-transplant levels on POD 170-190. There was partial islet function until elective necropsy (see meal-stimulated CP secretion, ***Supplementary Table III***). At necropsy, non-fibrotic capsules were found unexpectedly free-floating in the peritoneal cavity (***Supplementary Figure 12A***), not attached to the biologic scaffold.Some capsules contained viable islets (***Supplementary Figure 12B***), while others contained non-viable islets only. Capsules remaining on the omentum at necropsy were intact with very mild, if any, fibrosis, and viable islets with some evidence for central necrosis (***Supplementary Figure 12C***). In addition, a small omental mass was present, composed of a mixture of mesenchymal tissues including skeletal muscle, fibroplasia, bone, and adipose cells.

NHP H14C126 received a larger islet dose (17,303 IEQ/kg) in smaller capsules (900 µm *vs.* 1,200 µm standard diameter), to facilitate adherence to the scaffold and oxygen diffusion to the islets. Having a CP >1 ng/ml that peaked at POD 28 (1.87 ng/ml), this NHP exhibited remarkable glycemic control during the first month with a sharp, 56% decrease in FBG and 91% decrease in EIR (POD 23-34, Figures 4W and 4X, Table I). Thereafter, CP levels slowly decreased and FBG control destabilized with increased EIR. At elective necropsy (POD 140), there was partial islet function (CP levels >0.3 ng/ml, Figure 4X) but similar to NHP 14C72, only a minimal amount of capsules remained attached to the scaffold. Numerous viable islets were present in free-floating pristine, non-fibrotic capsules, but the majority of capsules were in the Douglas pouch. Capsules in the omentum were clean without fibrosis and contained viable islets (***Supplementary Figure 12D***). A small omental mass similar to the one in the previous NHP was present as well. Omental tissues from both animals showed very mild fibrosis and chronic inflammation (***Supplementary Figure 12C***). In both animals, viable islets remaining at necropsy were predominantly small (<200-250 µm diameter) compared to pre-transplant islet sizes (Table II), similar to our findings in NHP H10C19 (Figures 4A and 4B).

Discussion

Our goal was to assess the functional survival of encapsulated islet allografts in diabetic NOD mice and NHPs, hypothesizing that donor islet immunoisolation would reduce or eliminate the necessity for recipient immunosuppression.

Previously, we investigated the efficacy of encapsulated porcine islets in both spontaneously

Table II. Size distribution of encapsulated islets before transplant and at necropsy.

NHP ID	Time	Islet Diameter (μm)						
		50-100	100-150	150-200	200-250	250-300	300-350	350-400
14C72	Pre-Txpl	62%	19%	10%	3%	2%	2%	2%
	Necropsy	67%	31%	2%	0%	0%	0%	0%
H14C126	Pre-Txpl	50%	25%	14%	4%	3%	2%	2%
	Necropsy	72%	24%	4%	0%	0%	0%	0%

Aliquots of dithizone-stained islets were counted pre-transplant and at necropsy, and the percentage of islets in each size category with respect to the total number of IEQ was calculated.

diabetic NOD mice[27] and SZN-diabetic NHPs[17]. We chose that approach because of the potentially unlimited numbers of donor xenogeneic porcine islets, which would be available for eventual clinical therapy for patients with Type 1 diabetes. Although we found that porcine islet xenografts would function long-term in NOD mice given targeted immunosuppression, we were unable to duplicate these results in the NHPs, and we concluded that the xenoislets were vulnerable to destruction when implanted i.p. in NHPs. While we found scant evidence for immune destruction of donor porcine islets and ample evidence for hypoxic injury of donor islets, data from appropriate controls was lacking, namely islet autografts and implants of cell-free capsules.

In the current study we considered performing true islet autografts, re-implanting islets from a pancreatectomized recipient. However, this would have been a challenging approach, fraught with many potential complications for the recipient. Therefore, we chose to implant allogeneic islets, wherein donor and recipients were MHC-matched as closely as possible, as a surrogate for true islet autografts. This model allowed us to minimize any possible host immune reactivity toward encapsulated donor allo-islets in the NHPs. In addition, our current study included the implantation of cell-free capsules as controls and evaluation of four separate graft sites, procedures we had not carried out previously in our porcine to NHP islet study.

In this study, encapsulated islet allografts and isografts implanted i.p in diabetic NOD mice survived >1 year without immunosuppression, suggesting that microencapsulation protected against both allo-islet rejection and autoimmune destruction of NOD islets. These positive murine results lead us to initiate encapsulated islet allografts in STZ-diabetic NHPs. Microencapsulated islets were first transplanted i.p. in NHP recipients. Because of the known disparities in immune responsiveness between mice and NHPs, we administered targeted immunomodulatory therapy to NHP recipients. We observed prompt reductions in FBG and EIR and elevations of serum C-peptide. However, islet graft function fell gradually, after 2-8 weeks, depending on the islet mass transplanted. At necropsy, i.p. capsules were free-floating, with no peritoneal inflammatory reaction apparent. Most capsules resided in the Douglas pouch, although some rested on omental surfaces, non-adherent. At necropsy, microcapsules were non-fibrotic and intact with virtually no host cells on their surfaces and contained some viable islets. In this respect, our results are similar to those published recently by Sremac, et al[28]. Cytokine and chemokine levels in serial serum samples and in PF at necropsy were in the range of normal control values, with no significant differences, although we noted an increase in three chemokines after transplant in one recipient; additional study is required. Our double capsules excluded IgG, preventing direct cytotoxic effects of host antibodies on donor islets. However, when the grafts became non-functional, islet volumes were dramatically reduced, compared to pre-transplant measurements; and there was ample immunocytochemical evidence for hypoxic injury of the islets plus a significant decrease in the number of visible islets, islet size, and insulin release over time. We concluded that gravitational descent and aggregation of free-floating i.p. capsules had aggravated the marginal oxygen levels measured previously in the peritoneal space of both mice and NHPs (pO_2=60 mmHg), leading to hypoxic damage to the graft[29,30,10].

We reasoned that the omentum, which is vascularized, might be a more advantageous graft-site than the peritoneal cavity. Furthermore, an additional potential advantage of this site could be reduced exposure to immunosuppressive drugs and the associated diabetogenic effects[31]. When encapsulated islets were placed within a biologic scaffold, with the omentum folded to create a pouch, graft survival was comparable to the i.p. site. However, the space within the scaffold was

too small to accommodate the capsule volume in a single layer, possibly causing capsule crowding and impeding diffusion of oxygen and nutrients to the islets within the capsules.

Next, encapsulated islets were infused directly into the omental bursa, where there is a trend toward higher average pO_2 *vs.* the peritoneal cavity and ample space for an even distribution of encapsulated islets[13]. In addition, the omental bursa is a site that would allow complete capsule removal (*via* omentectomy) if that might ever be required. In the bursa, there was early islet function lasting until maintenance immune suppression was tapered, and then the grafts began to fail, despite minimal levels of $CD3^+CD4^+$T cells in the peripheral circulation, with accumulation of capsules in the caudal area of the bursa and evidence for hypoxic damage to the islets.

Finally, two recipients were transplanted on the anterior surface of the omentum, an adequate surface for distributing capsules in a single layer within a biologic scaffold, thus avoiding the capsule crowding which occurred in a pouch (***Supplementary Figure 11***). In one NHP, capsule diameter was decreased (from 1,200 μm to 900 μm), thereby reducing the graft volume and possibly aiding the diffusion of oxygen to the encapsulated islets. Also, we administered ambient oxygen (45-85 mmHg for 5-7 days) and recipient MSCs (to enhance allogeneic islet engraftment in NHPs[16]). These last experiments, although highly preliminary, have yielded promising graft function (20-28 weeks of elevated C-peptide). Our findings support the continued investigation of supplemental oxygen therapy, as well as co-transplanted autologous bone marrow-derived MSCs, which have repeatedly shown unequivocal safety profiles when infused IV in our STZ-induced diabetic NHP, as well as in several on-going clinical trials[16,32]. We speculate that the confinement of a large number of MSC in a biologic scaffold may have activated signals for MSC differentiation. Obviously, much more work will be required to achieve long-term survival of encapsulated islet grafts in NHPs, and ultimately in patients with T1DM.

Conclusions

We conclude that the limited function of encapsulated islet allografts in NHPs was not due to a host 'foreign-body' reaction to the microcapsules; nor was host immune reactivity a major contributor in NHPs treated with dual costimulatory blockade. Our data strongly suggest that graft-site hypoxia seriously damaged the islets, consistent with the predictions of Korsgren[33]. We believe that the promising approach of donor islet encapsulation will not be successful unless and until methods to deliver more oxygen to the islets are derived, and/or until novel sources of hypoxia-resistant insulin-secreting donor cells are developed.

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Conflict of Interests

The authors declare no competing financial interests.

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