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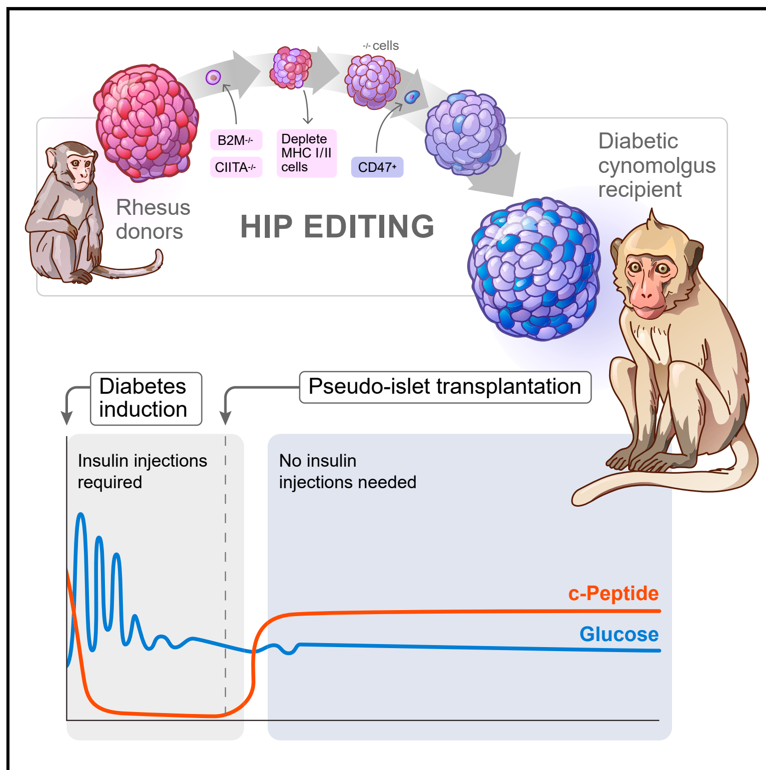
Brief Report

Hypoimmune islets achieve insulin independence after allogeneic transplantation in a fully immunocompetent non-human primate

Graphical abstract



Highlights

- Primary rhesus macaque islets can be edited to become hypoimmune pseudo-islets
- Hypoimmune pseudo-islets retain their morphology and endocrine competence
- They can engraft and achieve insulin independence in a fully allogeneic monkey
- Hypoimmune donor islets may improve clinical islet transplantation outcomes

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In brief

Schrepfer and colleagues engineered hypoimmune (B2M$^{-/-}$, CIITA$^{-/-}$, CD47^{+}) primary rhesus macaque pseudo-islets. They were transplanted into a fully allogeneic, immunocompetent, diabetic, and insulin-dependent cynomolgus monkey without immunosuppression or supportive medication. The islet graft quickly normalized c-peptide and glucose and the monkey became long-term insulin independent without showing any side effects.

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Hypoimmune islets achieve insulin independence after allogeneic transplantation in a fully immunocompetent non-human primate

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SUMMARY

Allogeneic transplantation of pancreatic islets for patients with difficult-to-control diabetes mellitus is severely hampered by the requirement for continuous immunosuppression and its associated morbidity. We report that allogeneic transplantation of genetically engineered (B2M$^{-/-}$, CIITA$^{-/-}$, CD47^{+}), primary, hypoimmune, pseudo-islets (p-islets) results in their engraftment into a fully immunocompetent, diabetic non-human primate wherein they provide stable endocrine function and enable insulin independence without inducing any detectable immune response in the absence of immunosuppression. Hypoimmune primary p-islets may provide a curative cell therapy for type 1 diabetes mellitus.

INTRODUCTION

Patients with type 1 diabetes mellitus (T1DM) and impaired awareness of hypoglycemia lack basic hypoglycemia-induced defense mechanisms and are at increased risk for severe hypoglycemic events.[1,2] Even integrated closed-loop systems face delays in glucose sensing and insulin delivery and cannot fully prevent hypoglycemia in difficult-to-control patients.[3] Islet transplantation has been shown to be superior to intensified insulin therapies[4] as only cell replacement therapy achieves physiological and immediate β cell responses. However, allogeneic donor islet transplantation currently requires systemic, lifelong immunosuppression.[5] In addition, a cumbersome regulatory framework has contributed to the demise of US clinical practice in recent years.[6] To circumvent the need for encapsulation, which has yet to show its usefulness, researchers have attempted immune engineering to engineer hypoimmune islets that escape allogeneic immune rejection (Table S1). Allogeneic, hypoimmune iPSC-derived or donor-derived islet grafts could provide a new, well-tolerated, and much-needed alternative. We have recently established an efficient engineering concept (B2M$^{-/-}$, CIITA$^{-/-}$, CD47^{+}, called HIP) that is applicable to iPSC-derived islet cells and islets isolated from human or non-human primate (NHP) donors. We have shown that human donor islets engineered to HIP pseudo-islets (p-islets) can survive, engraft, and ameliorate diabetes in immunocompetent, allogeneic, diabetic humanized mice in the absence of immunosuppression.[7] Our human HIP iPSC-derived p-islets survived and achieved glycemic control both in immunocompetent, allogeneic, diabetic humanized mice[8] and in a novel autologous autoimmune diabetes model in humanized mice.[7] In rhesus monkeys, we further demonstrated that HIP-engineered primary rhesus macaque p-islets survived for 40 weeks in an allogeneic rhesus macaque recipient without immunosuppression.[8] However, survival was shown in a healthy monkey and the endocrine competence of the transplanted rhesus macaque p-islets remained elusive. We now report the successful and curative treatment of an immunocompetent, diabetic cynomolgus monkey with fully allogeneic, HIP-engineered primary rhesus macaque p-islets without immunosuppression or supportive medication.

RESULTS

The allogeneic, NHP p-islet transplant setting was purposely designed to maximize the donor-to-recipient major histocompatibility complex (MHC) mismatch. The cynomolgus recipient species was selected because streptozotocin (STZ)-inducible diabetes mellitus can most reliably be induced in this monkey. The rhesus macaque donor was selected as not only is it a different species in the *Macaca* genus and thus considered allogeneic, but it also has major differences in its MHC repertoire. We did not see differences between the naive innate immune

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responses of cynomolgus immune cells and allogeneic cynomolgus or rhesus macaque target cells. To ensure that the overall number of fully edited primary rhesus monkey islets was sufficient for transplant, 4 donor pancreata were used. To prepare them for the engineering, we dissociated islet clusters into single cells and disrupted the *B2M* and *CIITA* genes using CRISPR-Cas9 technology. Cells were allowed to re-cluster into p-islets and were rested. Islet clusters were dissociated again and MHC class I- and II-negative cells were sorted and double-negative cells were re-clustered. Islets were dissociated, transduced to express rhesus CD47 using lentiviral particles, and re-clustered. The islets were then dissociated a fourth time and a total of 11, 15, 11, and 13 million CD47^{+} islet cells were sorted and re-clustered to generate HIP p-islets for the transplantation. HIP p-islets were morphologically similar to the primary unedited rhesus monkey islets and showed the typical HIP phenotype (Figures 1A and 1B). The *in vitro* insulin production and cellular composition (Figures 1C and 1D) of this HIP-modified product was comparable to those of the unedited rhesus macaque islets and also consistent with our previous data.[8]

Diabetes mellitus was induced in a male 13-month-old 1.77 kg cynomolgus monkey with a single injection of 100 mg/kg STZ and daily insulin injections (2 units per day) were started. Blood glucose was monitored twice daily and showed major instability over approximately 2 weeks until a well-controlled steady state was reached (Figures 1E and 1F). The monkey's serum c-peptide dropped dramatically and became barely detectable (Figure 1G). After 78 days, the cynomolgus monkey underwent transplantation of the HIP p-islets into its bilateral quadriceps muscles. After 6 days, the insulin injections were reduced to 1 unit per day; after another 3 days, there were reduced to 0.5 units per day; and insulin was completely discontinued after 3 more days. The monkey did not receive any induction or maintenance immunosuppression during or after HIP p-islet transplantation. As early as 1 week after the transplantation, the monkey's serum c-peptide level had normalized and remained stable throughout the follow-up period of 6 months. The monkey showed tightly controlled blood glucose levels for 6 months, was completely insulin-independent, was continuously healthy, and exhibited no physical or behavioral abnormalities. The animal showed a mild, physiologic weight gain during this time (Figure 1H) and we did not record any hyperglycemia events. C-peptide proved to be a very robust marker for graft performance and could easily be used in a clinical setting.

Repeated immune analyses against transplanted HIP p-islet target cells were performed during the study. After each blood draw, peripheral blood mononuclear cells (PBMC)s, CD3 T cells, and natural killer (NK) cells were isolated, and macrophages were generated from PBMCs. ELISpot assays with PBMCs showed no T cell activation and cytotoxicity assays with T cells showed no killing activity against the allogeneic HIP p-islet cells (Figures 2A and 2B). PBMCs and isolated NK cells or macrophages showed no cytotoxicity (Figures 2C–2E). Total IgM and IgG serum levels were quantified as surrogate markers for induced antibody production, but we did not observe any demonstrable increase (Figures 2F and 2G). No donor-specific antibodies (DSAs) could be detected at any time point during this study (Figures 2H and 2I). There were no antibody-dependent cellular cytotoxicity (ADCC) reactions with NK cell or macrophage effector cells and no complement-dependent cytotoxicity against HIP p-islets (Figures 2J–2L). Overall, despite using a variety of very sensitive screening assays, we found no indication that the allogeneic HIP p-islet graft induced any immune recognition or any type of immune response at any time throughout the duration of the study.

Recently, we showed that an unedited rhesus monkey islet graft transduced to express firefly luciferase was rejected in an allogeneic rhesus monkey recipient within just 1 week.[8] This previous islet graft was very similar to the islet graft of this study in terms of morphology, MHC and CD47 expression, islet composition, and endocrine function. Thorough immune analyses analogous to those presented here showed a strong allorejection response with cellular and antibody-mediated killing of unedited allogeneic islet cells.

To prove that the monkey's insulin independence was fully dependent on the well-functioning HIP p-islets graft and that there was no regeneration or recovery of its endogenous islet cell population, we triggered the destruction of the HIP p-islet transplant using a CD47-targeting strategy. Since the humanized anti-CD47 IgG4 antibody blocks the interaction of rhesus CD47 with cynomolgus immune cell SIRPα, the HIP p-islet cells, which use CD47 to protect from NK cells and macrophages (Figure 1I), get recognized as MHC class I and II double-negative cells and undergo innate immune killing (Figure 1J). The monkey received a total of 9 doses of anti-CD47 IgG4 antibody at 50 mg per dose injected alternately intramuscularly and intraperitoneally starting 196 days after HIP p-islet transplantation. Following antibody ablation of the graft, blood glucose levels increased steadily and after 6 values >127 mg/dL had been recorded, the monkey became hypoactive and lethargic. Insulin injections were resumed 8 days after the start of anti-CD47 IgG4 antibody at the previously established maintenance dose before islet transplantation of 2 units per day. Despite that, we saw widely fluctuating blood glucose levels in the monkey similar to the fluctuations observed in the weeks after the initial STZ-induced diabetes induction and prior to HIP p-islet transplantation (Figure 1F). Measurable c-peptide levels vanished quickly and further support the notion that glucose control had exclusively been achieved by the CD47-overexpressing HIP p-islet graft. Diabetes management was difficult during the remainder of the study and no steady state could ever be re-established. The monkey lost some weight in this period.

On day 234, the monkey was euthanized and underwent necropsy. Histologic analysis of its pancreas showed the near-complete absence of insulin-positive β cells, while islets still stained positive for glucagon and somatostatin (Figures S1A and S1B). This confirmed the effective and selective β cell destruction by STZ and also ruled out any relevant endogenous β cell regeneration in subsequent months. The islet injection sites in both quadriceps muscles were recovered and serial sections were cut. Slides were stained for islet1, a lineage-specific marker for pancreatic neuroendocrine cells not restricted to β cells (Figures S1A and S1C). While islet1 stained endogenous islets well in a healthy cynomolgus pancreas, no traces of islet1 could be detected in any of the serial sections through the recipient monkey quadriceps muscles. However, we found post-inflammatory fibrosis suggestive of recent phagocytic events. We therefore confirmed the complete destruction of HIP p-islets after anti-CD47 IgG4 antibody treatment.

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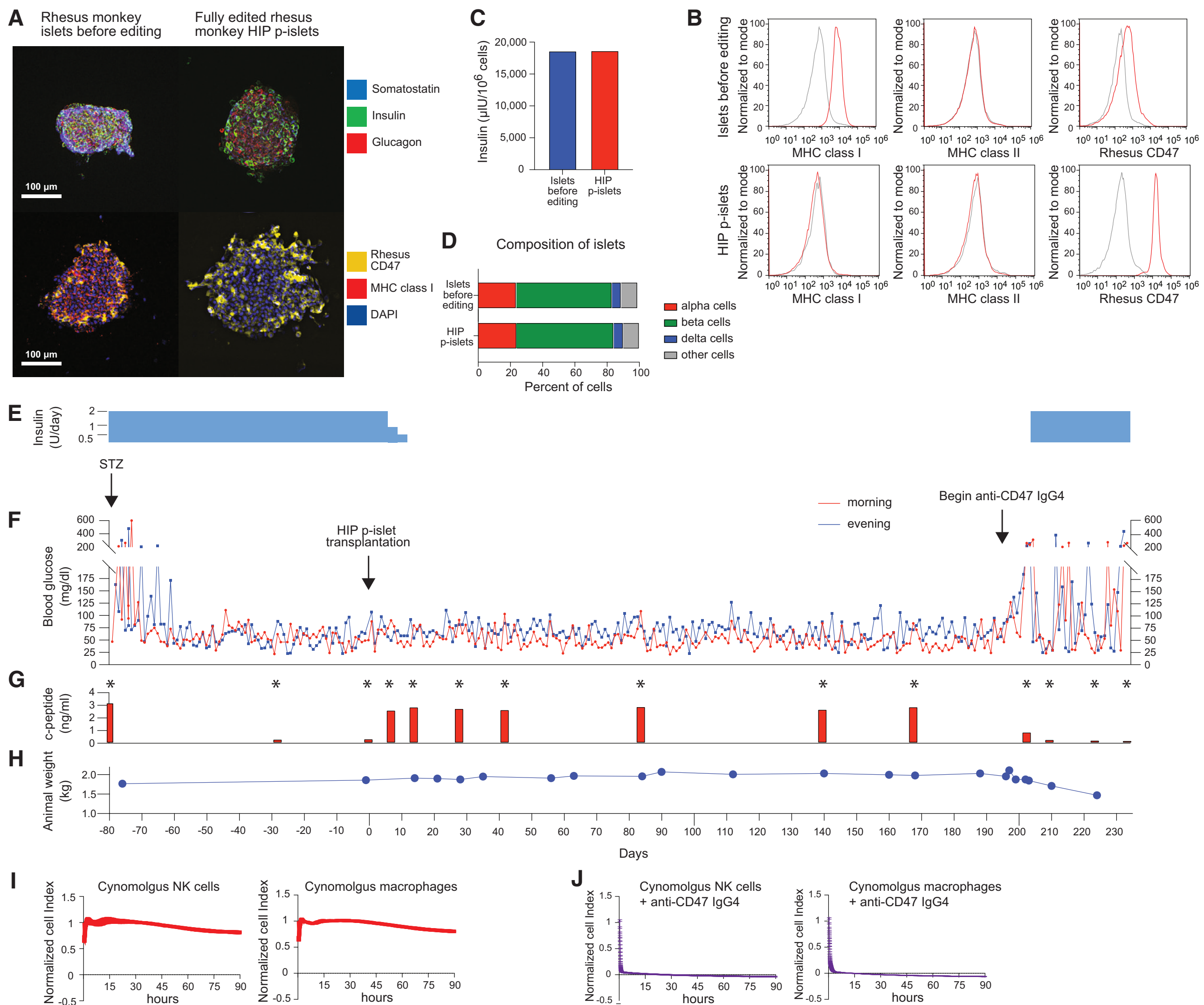

Figure 1. Phenotyping and allogeneic transplantation of rhesus HIP p-islets

(A) Immunofluorescence stainings of somatostatin, insulin, and glucagon (representative pictures of five independent experiments) are shown for primary rhesus monkey islets before editing, and those of fully edited HIP p-islets are also shown. Immunofluorescence phenotyping is for rhesus CD47, MHC class I, and DAPI (representative pictures of five independent experiments).

(B) MHC class I and II and rhesus CD47 expression was assessed by flow cytometry (representative histograms of two independent experiments; gray = isotype control, red = islet cells).

(C) Insulin release of *in vitro* rhesus macaque HIP p-islets is assessed by ELISA (mean of three technical replicates).

(D) The composition of rhesus macaque HIP p-islets of α, β, δ, and other cells (mean of four technical replicates).

(E) The daily dose of exogenous insulin is shown over the time of the study.

(F) Morning (red) and evening (blue) blood glucose levels are shown.

(G) Serum c-peptide levels were assessed in intervals. Asterisks indicate c-peptide measurement time points. Levels were assessed in one cynomolgus monkey.

(H) Animal weight over the course of the study.

(I and J) *In vitro* impedance cytotoxicity assays with HIP p-islet cells as targets and IL-2-stimulated cynomolgus monkey NK cells and macrophages. The assays were performed in the absence of (I) and presence of (J) an anti-CD47 IgG4 antibody. (Mean ± SD; three replicates per group and time point).

DISCUSSION

Systemic immunosuppression to prevent the rejection of allogeneic islet grafts in patients comes with considerable morbidity, including chronic kidney injury, infections, and cancer, and has still only permitted graft survival for a mean of 4.4–5.9 years in clinical studies.[5,9,10] Despite immunosuppression, patients frequently become sensitized, which complicates any subsequent transplants.[5] This concept therefore provides limited benefit for a lifelong disease in patients often diagnosed at young ages and there is a need for better alternatives. Both donor islets and pluripotent stem cell-derived islet organoids have been envisioned as endocrine therapeutics. Given our phylogenetic proximity, NHP models have gained importance for

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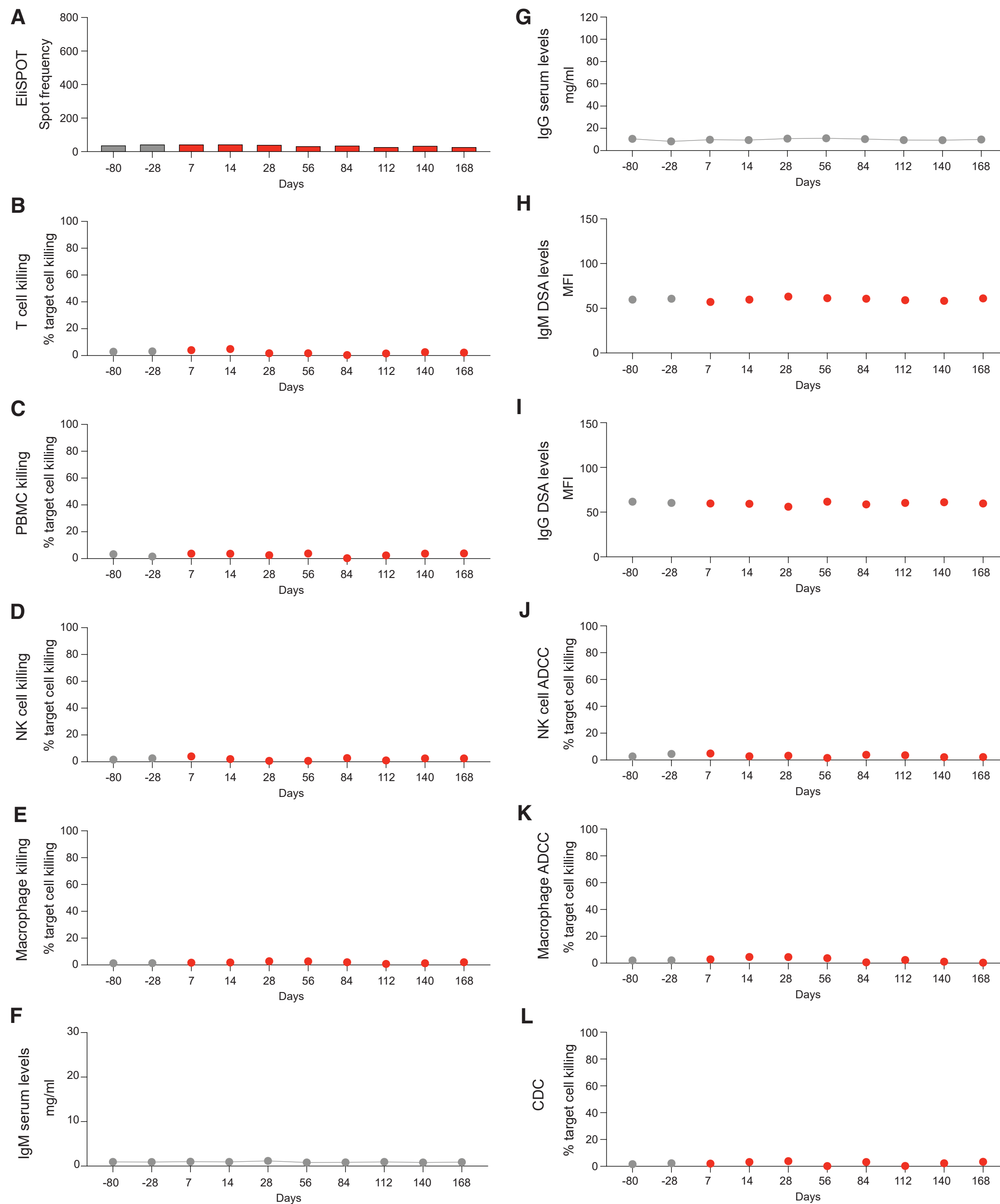

Figure 2. Cellular and antibody-mediated responses against allogeneic rhesus HIP p-islets

(A) ELISpot assays with recipient monkey PBMCs drawn at scheduled timepoints.

(B–E) Killing assays with recipient monkey T cells (B), PBMCs (C), NK cells (D), and macrophages (E). Percent target cell killing is shown on the y axis.

(F–I) Total serum IgM (F) and IgG (G) levels and DSA IgM (H) and IgG (I) levels are shown.

(J–L) ADCC assays with de-complemented recipient monkey serum and NK cells (J) or macrophages (K) and CDC assays with complete recipient monkey serum (L) are shown. Percent target cell killing is shown on the y axis. Assays are from one cynomolgus monkey.

translational studies of new cell therapy concepts. Recently, the transplantation of human iPSC-derived islets into four heavily immunosuppressed rhesus macaques has been reported.[11] All animals were euthanized within half a year because of islet graft failure or lethal complications from immunosuppression. Attrition of iPSC-derived islets from chronic inflammation was seen in the animals that lost graft function and has similarly been observed in T1DM patients implanted with stem cell-derived pancreatic endoderm cells in a clinical study. A macro-encapsulation device that does not offer immunoprotection was used and histology

after retrieval by 12 weeks showed mainly host-derived fibroblasts with some endocrine cells and host immune cells.[12] Allotransplantation of primary cynomolgus islet grafts into diabetic monkeys was performed via portal vein infusion and different immunosuppression regimens were tested.[13] Over-immunosuppressed animals treated with rapamycin with or without tacrolimus experienced severe complications and half of them died early. In the group with milder, better tolerated immunosuppression, only 1 out of 4 animals achieved insulin independence for 180 days despite getting over 20 injections of an anti-CD40L antibody. These data illustrate that the level of immunosuppression required to achieve long-term islet allograft survival is substantial and typically becomes intolerable. Therefore, to shield the cells from the host immune system, micro-encapsulated human islets have been transplanted into a diabetic cynomolgus monkey.[14] Despite additional immunosuppression with co-stimulation blockade, only minimal endocrine function could be achieved. Co-transplantation of cynomolgus islets and streptavidin-FasL-presenting microgels to the greater omentum of four allogeneic monkeys was performed with 3 months of rapamycin immunosuppression.[15] The animals showed reduced insulin dependance after islet transplantation but did not reach insulin independence.

In this study, we demonstrate that an HIP-engineered rhesus macaque p-islet graft provided lasting endocrine function in a fully immunocompetent cynomolgus monkey that achieved insulin independence without any immunosuppression. In macaques, evolutionary orthologs of human leukocyte antigens (HLAs) have undergone several rounds of duplication, which has led to a complex MHC system with significant copy number variation.[16] With our rhesus macaque donors and the cynomolgus macaque recipient being different species[17] in the *Macaca* genus, we aimed to maximize the MHC mismatch to set the bar for success high. This strategy does not utilize allogeneic transplants within pedigreed colonies that are often Mafa-A or Mafa-B haploidentical or MHC haploidentical[18] and that generate more favorable transplant results.[15] In addition, other typical tolerance-inducing strategies require careful MHC class II matching.[19] All rhesus donors and the cynomolgus recipient were blood type B to avoid blood group incompatibility, as it is standard in clinical allotransplantation. Our repeated and thorough immune analyses showed no immune activation by the allogeneic HIP p-islets and no immune response was detected at any time point during the follow-up. These data demonstrate that our hypoimmune engineering completely circumvents allograft rejection, does not build immune memory, and thus achieves markedly better protection than systemic immunosuppression, and it is associated with no observed side effects.

Our cynomolgus recipient had an estimated pancreas mass of 5 g with about 50 million β cells.[20,21] The yield of HIP islet cells from each of the 4 pancreata was approximately 11% and the overall transplanted number of HIP β cells was 30 million, representing approximately 60% of the original endogenous β cell mass. It was reported previously that approximately 50% of the β cell mass in cynomolgus monkeys is sufficient to maintain euglycemia.[22] The fact that insulin independence was achieved in this study speaks to the endocrine competence of the transplanted HIP p-islets, which may have been facilitated by the intramuscular injection site. Injections into the muscle circumvent early islet loss through an instant blood-mediated inflammatory reaction (IBMIR) that is known to occur after portal vein injections.[23] The muscle is well vascularized and islet transplantations into striated muscle have been successful clinically.[24,25]

The monkey's morning and evening glucose levels remained narrowly controlled and the maintenance of insulin independence and stable c-peptide levels for over half a year underline the steady endocrine function of the allograft. There were no local irritations from HIP p-islet transplantation, we observed no behavioral or laboratory abnormalities, and the monkey was healthy. The quick relapse of diabetes requiring daily insulin injections after the CD47-targeted, antibody-driven destruction of the HIP p-islet graft showed the dependence of the animal on its graft function. Histology reaffirmed that STZ can be used to selectively destroy β cells and that lasting diabetes can be induced in cynomolgus monkeys[11,26,27] without evidence of reactive β cell regeneration in the pancreas of the animals.[28] The complete destruction of HIP-engineered cells with the anti-CD47 treatment supports the reliability of this safety strategy, which has previously been proven to be effective to ablate human HIP p-islets[7] and HIP CAR T cells.[29] A shortcoming of chemically induced diabetes is that it does not simulate the associated autoimmune component of T1DM. Our prior studies, however, showed that human HIP p-islet grafts are resistant against autoimmune damage in a humanized mouse model of autoimmune diabetes.[7] This successful NHP study provides proof of concept for an upcoming clinical trial using allogeneic, HIP-edited primary islets in patients with type 1 diabetes.

Limitations of the study

The results support our notion that HIP editing can protect allogeneic grafts from rejection while maintaining graft function, but this should be interpreted carefully based on one case. We did not include a control animal receiving unedited allogeneic islets because the vigorous rejection of allogeneic rhesus monkey cells had been documented thoroughly in a recent study.[8] The concept of treating T1DM with HIP-edited donor islets remains limited by the number of pancreas donors, but primary cell transplants do not have the risk of retained pluripotency that is inherit to iPSC-derived therapeutics and therefore, HIP-edited primary p-islets have a favorable safety profile for a first-in-human safety study.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stem.2024.02.001.

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AUTHOR CONTRIBUTIONS

X.H. performed all immunobiology experiments, molecular biology experiments, islet and glucose studies, islet cell culture, and editing work and analyzed the data. K.W. harvested pancreata from NHPs and performed *in vivo* injections into NHP. C.Y. performed *in vitro* immunofluorescence staining and imaging. A.G.O. and A.J.C. performed *in vivo* histopathology and analyzed the data. P.K. isolated the primary islets from donor NHPs. T.D. evaluated the data, developed and produced the figures, and co-wrote the manuscript with S.S. S.S. conceptualized and designed the experiments, supervised the project, and co-wrote the manuscript with T.D. All authors helped edit the manuscript.

DECLARATION OF INTERESTS

All experiments were conducted by or on behalf of Sana Biotechnology, Inc. and no data from UCSF or OHSU were used. A.J.C. and T.D. performed the work in this manuscript as consultants to Sana Biotechnology, Inc. T.D. owns stock in Sana Biotechnology, Inc. P.K. has no financial disclosures. All other authors are employees of and own stock in Sana Biotechnology, Inc. X.H. and S.S. are inventors on a patent (International Application No: PCT/US2022/074878; Title "Genetically modified primary cells for allogeneic cell therapy").

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-HLA-A,B,C antibody (clone G46_2.6)	BD Biosciences	Cat# 555555
IgG1 isotype-matched control antibody (clone MOPC-21)	BD Biosciences	Cat# 554681
anti-HLA-DR,DP,DQ antibody (clone TU39)	BD Biosciences	Cat# 563591
IgG2a isotype-matched control antibody (clone G155–178)	BD Biosciences	Cat# 565357
anti-CD47 antibody (clone CC2C6)	Biolegend	Cat# 323106
IgG1 isotype-matched control antibody (clone MOPC-21)	Biolegend	Cat# 400110
anti-insulin antibody (clone 2D11-H5)	Santa Cruz Biotechnology	Cat# sc-8033
anti-glucagon antibody (clone C-11)	Santa Cruz Biotechnology	Cat# sc-514592
anti-somatostatin antibody (clone G-10)	Santa Cruz Biotechnology	Cat# sc-55565
anti-glucagon (clone 09)	Novus Biologicals	Cat# NBP2-21803AF647
anti-somatostatin (polyclonal)	Novus Biologicals	Cat# NBP2-99309AF350
anti-insulin (clone ICBTACLS)	Life Technologies	Cat# 53-9769-82
anti-human CD47 (clone CC2C6)	Biolegend	Cat# 323108
anti-CD47 (magrolimab, 5F9)	Creative Biolabs	custom order
goat anti-rhesus IgM	BioLegend	Cat# 314506
goat anti-rhesus IgG	Southern Biotech	Cat# 4700-02
anti-CD8 (clone LT8)	Abcam	Cat# ab28010
anti-NKG2A (clone REA 110)	Miltenyi	Cat# 130-114-092
anti-Islet 1 (clone EPR10362)	Abcam	Cat# ab178400
anti-insulin (clone EPR17359)	Abcam	Cat# ab181547
anti-glucagon (clone EP3070)	Abcam	Cat# ab92517
anti-somatostatin (clone EPR3359(2))	Abcam	Cat# ab111912
Bacterial and virus strains		
CAG-CD47 LVV	Thermo Fisher	N/A
Chemicals, peptides, and recombinant proteins		
Bovine DNase I	Sigma-Aldrich	Cat# 11284932001
Cas9 nuclease	IDT	Cat# 1081058
STZ	Sigma-Aldrich	Cat# S0130
Insulin (Lantus)	Sanofi	NDC# 00088-5021
Rhesus monkey IL-2	MyBiosource	Cat# MBS1376561
Critical commercial assays		
ELISA assays for rhesus monkey insulin	MyBiosource	Cat# MBS701773
ELISA assays for cynomolgus monkey c-peptide	Novus Biologicals	Cat# NBP2-59957
ELISA assays for total rhesus IgM	MyBioSource	Cat# MBS742738
ELISA assays for total rhesus IgG	Molecular-Innovations	Cat# IMNRSIGGKTT
Experimental models: Organisms/strains		
Macaca Mulatta	Alpha Genesis Inc.	N/A
Macaca fascicularis	Alpha Genesis Inc.	N/A
Oligonucleotides		
gRNA for macaca mulatta *B2M*: 5′-CGUGAGUAAACCUGAAUCUU-3′	IDT	Custom order
gRNA for macaca mulatta *CIITA*: 5′-GAUAUUGGCAUAAGCCUCCC-3′	IDT	Custom order
Software and algorithms		
Prism 10	GraphPad	N/A

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RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sonja Schrepfer (Sonja.Schrepfer@sana.com).

Materials availability

All the materials will be available upon request to the lead contact under material transfer agreement with Sana Biotechnology.

Data and code availability

This manuscript did not generate any new code. All data reported in this paper will be shared by the lead contact upon request. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

All rhesus macaque and cynomolgus experiments have been approved by the Alpha Genesis Inc. Institutional Animal Care and Use Committee. Four male rhesus macaques (5–10 kg) were used as islet donors. A one-year-old male cynomolgus macaque served as islet transplant recipient.

METHOD DETAILS

Pancreas procurement and islet isolation

Briefly, each of four rhesus macaques, was first exsanguinated. The pancreatic tail was identified and separated from the spleen. Rotating the greater curvature of the stomach anteriorly, the pylorus and duodenum were identified. The duodenum was cut just past the pyloric valve and then distally when it was clear of the pancreatic tissue. The pancreas was then freed from any remaining tissues. The harvested pancreas was preserved with cold UW and shipped to Oregon National Primate Research Center, Beaverton, Oregon, USA, for islet cell isolation.

Pancreata were removed from UW solution and placed in a sterile culture hood. An 18 gauge plastic IV catheter was inserted into the pancreatic duct and the pancreas was inflated with 50–80 mL of RT Media (RPMI 1640 medium (Thermo Fisher), 10% FBS, 100U pen/strep) containing 0.6 mg collagenase P (Sigma Aldrich). After inflation, fat and connective tissue was removed, and the tissue was divided into 12 equal portions and each portion was placed in a 50 mL conical tube containing 11 mL of RT medium with 0.06 mg collagenase P and incubated in a 37°C water bath for 27 min with agitation every 10 min. After digestion, collagenase was poured off, and 10 mL of RT media was added and shaken for 1 min to disrupt remaining connective tissue. Undigested material was removed by straining through a 500 micron filter and islets were washed 3 times with 50 mL of RT media, followed by a 10 min Bovine DNase I (Sigma-Aldrich, 0.8 U/ml) incubation in a 37C water bath. Islets were cultured overnight at 37°C/5%CO_2 in RT media with Bovine DNase I (Sigma-Aldrich, 0.4 U/ml) and shipped to Sana Biotechnology, Inc. the following day.

Genome engineering of primary rhesus macaque islet cells

Primary rhesus macaque cadaveric islets were isolated by the Kievit Lab at OHSU as described. The CRISPR/Cas9 technology was used for the disruption of the *B2M* and *CIITA* genes. Islet clusters were dissociated in single cells using AccuMax (StemCell Technologies) for 10 min at 37°C. The following gRNA sequences were used for the macaca mulatta *B2M* gene 5′-CGUGAGUAAACCU GAAUCUU-3′ and macaca mulatta *CIITA* gene 5′-GAUAUUGGCAUAAGCCUCCC-3’. Lonza P3 Primary Cell 4D-Nucleofector X Kit (cat.no V4XP-3032, Lonza) was used for the transfection of the islet cells. Briefly, cells were transduced with a final concentration of 50 million per mL in P3 buffer. The cell suspensions (20 μL) were pipetted in one well of the 8-strip containing 13 μg Cas9 enzyme and 5 μM sgRNA, respectively. Lonza's 4D-Nucleofector was used for the electroporation with the preset program CA-137. Islet cells were transferred in U-bottom 96-well plates containing 50,000 cells per well in PIM(S) media (Prodo) and rested for 1 h at 37°C and 5% CO_2 before moving the plate on the belly dancer orbital shaker (IBI Scientific, Dubuque, IA) for islet re-clustering. Complete media change was performed after 48 h, and islet clusters were incubated on the belly dancer for another 24 h. Islet clusters were dissociated again in single cells using AccuMax for cell sorting using the anti-HLA-A,B,C antibody (clone G46_2.6,BD Biosciences), which cross-reacts with rhesus MHC class I, or IgG1 isotype-matched control antibody (clone MOPC-21,BD Biosciences) and anti-HLA-DR,DP,DQ antibody (clone TU39, BD Biosciences), which cross-reacts with rhesus MHC class II or IgG2a isotype-matched control antibody (clone G155–178, BD Biosciences). Approximately 60% of cells were double-negative cells and were sorted in the BD FACS Aria II and replated in U-bottom 96-well plates as described above for islet re-clustering on the belly dancer orbital shaker. After 24 h, islets were dissociated in single cells for rhesus CD47 transduction with a CAG-CD47 LVV (custom order, Thermo Fisher) at a MOI of 5. Spinfection was performed with the presence of 10 μg/mL protamine sulfate at 300 g for 15 min. Cells were replated in U-bottom 96-well plates as described above for islet re-clustering on the belly dancer orbital shaker. After 48 h, cells were dissociated in single cells using AccuMax and underwent cell sorting for rhesus CD47 with anti-CD47 antibody (clone CC2C6, BD Biosciences), which cross-reacts with rhesus CD47 or IgG1 isotype-matched control antibody (clone MOPC-21, BD

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Biosciences) on BD FACS Aria II. Approximately 70% of those cells were CD47-high expressing and these islet cells were sorted and replated in low-attachment 6-well plates (product no. 3471, Corning) for islet re-clustering on the belly dancer orbital shaker until transplantation. The 4 pancreata yielded a total of 99, 138, 105, and 115 million islet cells.

Flow cytometry

Rhesus islet cells were dissociated in single cells using AccuMax (StemCell Technologies) for 10 min at 37°C and labeled with APC-conjugated anti-HLA-A,B,C antibody (clone G46_2.6 has shown cross-reactivity with rhesus macaque MHC class I, BD Biosciences) or APC-conjugated IgG1 isotype-matched control antibody (clone MOPC-21,BD Biosciences). Alexa-flour647-labeled anti-HLA-DR,DP,DQ antibody (clone TU39 has shown cross-reactivity with rhesus macaque MHC class II, BD Biosciences) or Alexa-flour647-labeled IgG2a isotype-matched control antibody (clone G155-178, BD Biosciences). FITC-conjugated anti-CD47 antibody (clone CC2C6, BioLegend) and FITC-conjugated mouse IgG1k isotype-matched control antibody (MOPC-21, BioLegend). Representative histograms are shown.

Quantification of islet cluster insulin production

One hundred HIP p-islets were plated in one 6-well and 2 mL of islet media with 5.8 mM glucose (PIM(S) media, Prodo). Supernatants were collected after 24 h and ELISA assays for rhesus insulin (MBS701773, MyBiosource) were performed according to the manufacture's protocol. A microplate reader with an absorbance of optical density (OD) 450 nm (Molecular Devices) was used to measure the insulin level of the standards and study samples. Insulin levels were calculated to uIU per million cells.

ISLET COMPOSITION

Rhesus HIP p-islets were dissociated with AccuMax (StemCell Technologies) for 10 min at 37°C. For the different islet composition, following antibodies were used: anti-insulin antibody (clone 2D11-H5, Santa Cruz Biotechnology), anti-glucagon antibody (clone C-11, Santa Cruz Biotechnology) and anti-somatostatin antibody (clone G-10, Santa Cruz Biotechnology). Results are shown as percentage.

Islet immunofluorescence

Approximately 20 μL of aggregates was collected into a 1.5 mL Eppendorf tube and spun down. The pellet was resuspended in fixation/permeabilization working solution (cat.no. 00-5523-00, eBioscience) and incubated overnight at 4°C. Cells were washed with permeabilization working buffer (cat.no. 00-5523-00, eBioscience) and stained with 5 μL each of glucagon (cat.no. NBP2-21803AF647, Novus Biologicals), somatostatin (cat.no. NBP2-99309AF350, Novus Biologicals), and insulin (cat.no. 53-9769-82, Life Technologies) for 24 h at 4°C. Cells were washed with permeabilization working buffer, mounted to slides with Prolong Gold (cat.no. P36930, Fisher Scientific), and allowed to dry overnight. For MHC and rhesus CD47 immunofluorescence, the same amount of islets were collected and washed with stain buffer (DPBS with 0.1% BSA and 5mM EDTA). Aggregates were stained with 5 μL of PE anti-human CD47 (cat.no. 323108, Biolegend), APC anti-human HLA-ABC (cat.no. 562006, BD Bioscience) for 45 min on ice and washed with stain buffer. Cells were fixed with BD Cytofix on ice for 30 min (cat.no. 554655, Fisher Scientific), washed with stain buffer, mounted on slides with Prolong Gold with DAPI (cat.no. P36931, Fisher Scientific), and allowed to dry overnight. All slides were then imaged on Leica Thunder Imaging System.

STZ induction, insulin treatment, and anti-CD47 antibody

The cynomolgus monkey was fasted for 12 h prior to receiving a single (100 mg/kg) intravenous injection of STZ. The STZ (cat.no S0130, Sigma) was first dissolved in citrate buffer (100 mg STZ and 22 mg citric acid per mL saline) and then diluted to the final concentration in 12 mL cold saline. STZ was administered via the saphenous vein. Blood glucose levels were monitored twice daily using the Precision Xtra Glucometer (Abbott). The diabetic cynomolgus monkey was treated with one injection of insulin (Lantus Sanofi, Paris, France) per day to maintain blood glucose within normal range (30–80 mg/dl). An anti-CD47 antibody was given (magrolimab 5F9 sequence, Creative Biolabs) at a dose of 50mg (diluted in PBS, along with Depo-Medrol 40 mg/kg, Pfizer, New York, NY) and dosed alternately intramuscularly or intraperitoneally for a total of 9 days.

Transplantation of rhesus macaque HIP p-islets

Anesthesia was performed using ketamine (10–20 mg/kg), tiletamine and zolazepam (5–8 mg/kg), and isoflurane (1–4%). Fifty million rhesus HIP p-islets were transplanted into both quadricep muscles of the cynomolgus monkey. Islet clusters were resuspended in 100 μL RPMI-1640 media (Thermo Fisher) including pro-survival cocktail (200 μM ZVAD and 100 nM BcL-xL (both Millipore), 200 ng/mL IGF-1 (PeproTech), 100 μM pinacidil, and 200 nM cyclosporine A (both Sigma-Aldrich)) and loaded into 1 mL syringes without dead volume with a 23 G needle. A 2 cm skin incision was made over the middle anterior side of the quadricep muscles, and the HIP p-islets were injected in a string pattern parallel to the muscle fibers. The incisions were closed with 5-0 absorbable suture using interrupted subcuticular stitches. Buprenorphine SR (0.2 mg/kg) and meloxicam SR (0.3 mg/kg) were given subcutaneously for analgesia and animals were returned to home housing.

Blood collection

Blood was collected from the femoral vein using a 22 G needle, vacutainer sheath, and collection tube. A total of 4 mL blood was drawn for immune cell isolation on days −80, −28, 0, 7, 14, 28, 42, 56, 84, 112, 140, and 168, and 2.5 mL serum was drawn on days −80, −28, 0, 7, 14, 28, 42, 56, 84, 112, 140, 168, 203, 210, 224, and 234. Following venipuncture, manual compression of the vein was maintained until hemostasis was achieved. Blood collection was based on weight of the animal not exceeding AGI maximum bleeds as set forth by the IACUC.

C-peptide ELISA

The cynomolgus c-peptide ELISA kit (Novus Biologicals, Littleton, CO) was used to measure cynomolgus c-peptide in serum. Samples were diluted and pipetted according to manufactures instructions. Briefly, standards and samples were added to pre-coated 96-well ELISA plates and incubated for 1 h. After the removal of unbound proteins by washing, anti-*c*-peptide antibodies conjugated with horseradish peroxidase, were added. These enzyme-labeled antibodies form complexes with the previously bound c-peptide. The enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, tetramethylbenzidine. Samples were analyzed in a microplate reader (PerkinElmer, Waltham, MA).

ELISpot

For uni-directional ELISpot assays, recipient PBMCs were isolated from the cynomolgus recipient at different time points and CD3$^+$ T cells were sorted (BD Aria Fusion). Rhesus HIP p-islet cells were mitomycin-treated (50 μg/mL for 30 min, Sigma) and used as stimulator cells. A total of 1 × 10^5 stimulator cells were incubated with 5 × 10^5 recipient responder T cells for 36 h and IFN-γ spot frequencies were enumerated using an ELISpot plate reader (AID, Strassberg, Germany).

Total IgM and IgG ELISA

The total rhesus IgM ELISA kit (MyBioSource, San Diego, CA) and total rhesus IgG ELISA kit (Molecular-Innovations, Novi, MI) were used to measure total IgM or IgG in the cynomolgus monkey serum. Samples were diluted and pipetted according to manufacturer's instructions. Briefly, standards and samples were added to pre-coated 96-well ELISA plates and incubated for 1 h. After the removal of unbound proteins by washing, anti-IgM or anti-IgG antibodies conjugated with horseradish peroxidase, were added. These enzyme-labeled antibodies form complexes with the previously bound IgM or IgG. The enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, tetramethylbenzidine. Samples were analyzed in a microplate reader (PerkinElmer).

DSA

Sera from recipient animals were de-complemented by heating to 56°C for 30 min. Equal amounts of sera and cell suspensions (5 × 10^6 per mL) were incubated for 45 min at 4°C. Cells were labeled with FITC-conjugated goat anti-rhesus IgM (BioLegend) or FITC-conjugated goat anti-rhesus IgG (Southern Biotech) and mean fluorescence intensity (MFI) was analyzed by flow cytometry (Attune, ThermoFisher).

Cynomolgus macaque NK cell isolation

Cynomolgus PBMCs were sorted on the FACSAria Fusion using FITC-conjugated anti-CD8 (ab28010, 1:5, Abcam) and PE-conjugated anti-NKG2A (130-114-092, Miltenyi) antibodies to select a CD8$^+$ NKG2A$^+$ NK cell population.

Macrophage differentiation from PBMCs

PBMCs were isolated by Ficoll separation from fresh blood and were resuspend in RPMI-1640 with 10% heat-inactivated fetal calf sera (FCS hi) and 1% Pen/Strep (all Gibco). Cells were plated in 24-well plates at a concentration of 1 × 10^6 cells per mL and 10 ng/mL rhesus M-CSF (Biorbyt, Cambridge, Great Britain) was added. Media was changed every other day until day 6. Macrophages were stimulated from day 6 with 1 μg/mL rhesus IL-2 (MyBiosource) for 24 h before the cells were used.

PBMC, T cell, NK cell, and macrophage killing assays on the XCelligence platform

Cynomolgus monkey PBMC, T cell, NK cell, and macrophage killing assays were performed on the XCelligence MP platform (ACEA BioSciences, San Diego, CA.). Specialized 96-well E-plates (ACEA BioSciences) were coated with collagen and fibronectin (Sigma-Aldrich) and 4 × 10^4 target islet cells were plated in 100 μL media. After the Cell Index reached 0.7, the effector cells were added at an effector cell to target cell (E:T) ratio of 1:1. NK cells were stimulated with 1 μg/mL rhesus IL-2 (Peprotech). In some wells, anti-CD47 IgG4 (magrolimab sequence, Creative Biolabs, Shirley, NY) was added in a concentration of 100 μg/mL. As killing control, cells were treated with 2% Triton X-100 in water. Data were standardized and analyzed with the RTCA software (ACEA). Islet cells were collected 90 h after adding the effector cells and were analyzed for % dead cells in flow (Zombie live/dead stain, Biolegend).

CDC and ADCC killing by XCelligence

CDC and ADCC killing assays were also performed on the XCelligence MP platform. For CDC assays, 100 μL of untreated, complement-containing serum (1:1 mixed with media) was added. For ADCC assays, 50 μL heat-inactivated serum with 4 × 10^4 cynomolgus

macaque NK cells or macrophages in 50 μL media were added. As killing control, cells were treated with 2% Triton X-100 in water. As survival control, cells were only incubated with media. Data were standardized and analyzed with the RTCA software (ACEA).

Histology

The islet injection sites in the left and right quadriceps muscles were fixed in 10% neutral buffered formalin, cut into 2–3 mm pieces, and embedded in paraffin. Pieces of a pancreas from a healthy cynomolgus monkey and the pancreas from this study monkey were processed similarly. Blocks were sectioned at 4 μm and stained with hematoxylin and eosin (H&E) and anti-Islet 1 antibody (Abcam, ab178400). Sections that were positive for Islet 1 staining (pancreas) were stained for insulin (Abcam, ab181547), glucagon (Abcam, 92517) and somatostatin (Abcam, ab111912). The healthy pancreas with no STZ treatment was used as a positive control and to compare beta cell mass following STZ treatment. Immunohistochemical staining was performed on the Leica Bond Rxm using the Leica Bond Refine kit (Leica Biosystems - DS9800) for DAB chromogenic staining. Blocking of non-specific binding was done with 1x Animal Free Blocker (SP-5030-250) in 1% NGS (Cell Signaling - 5425S) diluted in TBST (Thermo Fisher - J77500.K2) which also served as the antibody diluent. Slides were dehydrated, cleared, coverslipped, and scanned using a Leica Aperio 200.

Quantification and statistical analysis

Individual data points are presented. GraphPad Prism 10 was used for graphs. No statistical analysis was performed in this study.