Stem Cell Reports, Volume *17*

Supplemental Information

Genetic manipulation of stress pathways can protect stem-cell-derived islets from apoptosis *in vitro*

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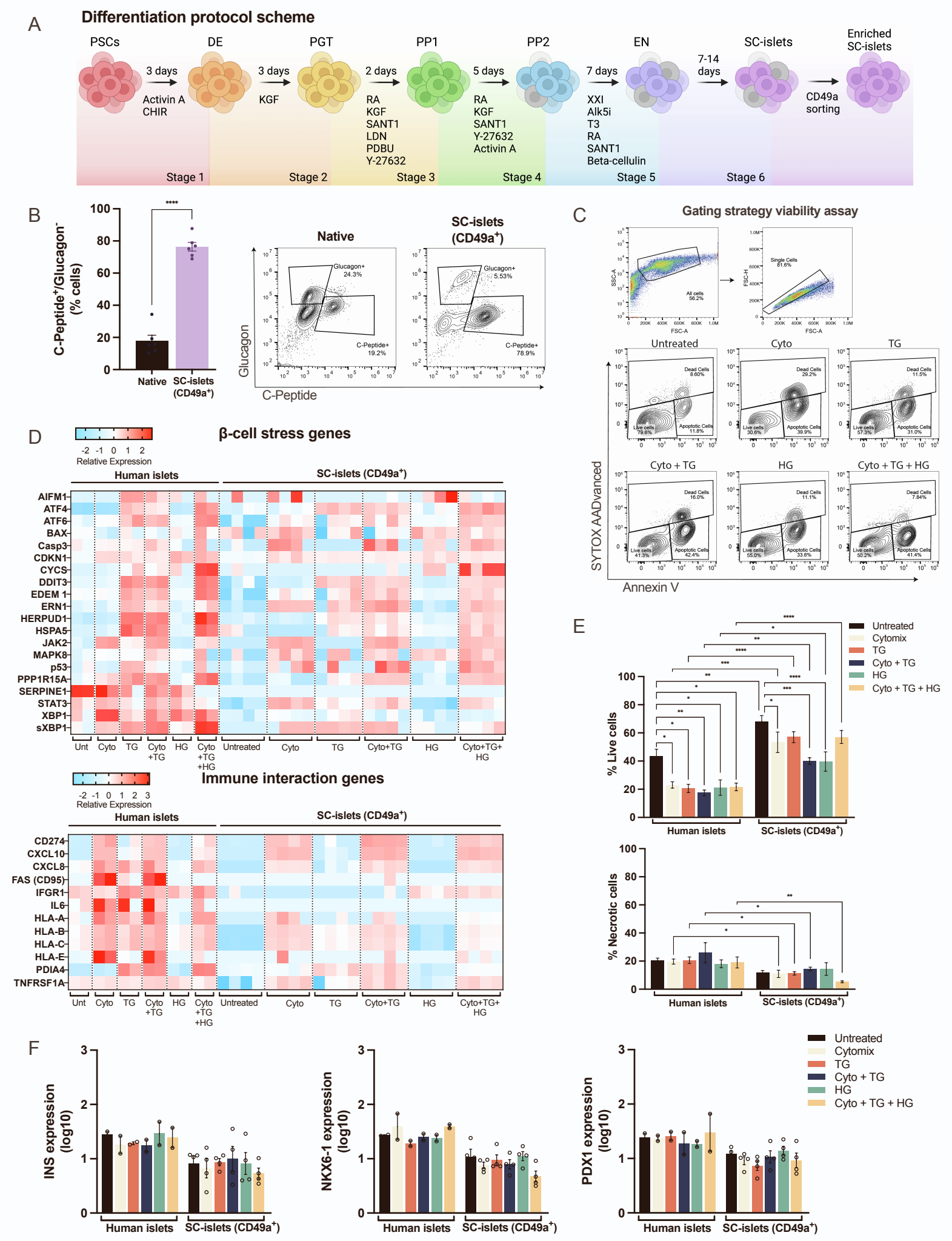
A
Differentiation protocol scheme
PSCs
DE
PGT
PP1
PP2
EN
SC-islets
Enriched SC-islets
3 days
Activin A
CHIR
3 days
KGF
2 days
RA
KGF
SANT1
LDN
PDBU
Y-27632
5 days
RA
KGF
SANT1
Y-27632
Activin A
7 days
XXI
Alk5i
T3
RA
SANT1
Beta-cellulin
7-14 days
CD49a sorting
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Stage 6
B
C-Peptide+/Glucagon- (% cells)
Native
SC-islets (CD49a+)
Glucagon
C-Peptide
Glucagon+ 24.3%
C-Peptide+ 19.2%
Glucagon+ 5.53%
C-Peptide+ 78.9%
C
Gating strategy viability assay
SSC-A
FSC-A
All cells 56.2%
Single Cells 81.6%
FSC-H
Untreated
Cyto
TG
Cyto + TG
HG
Cyto + TG + HG
Dead Cells 8.60%
Live cells 79.6%
Apoptotic Cells 11.8%
Dead Cells 29.2%
Live cells 30.6%
Apoptotic Cells 39.9%
Dead Cells 11.5%
Live cells 57.3%
Apoptotic Cells 31.0%
Dead Cells 16.0%
Live cells 41.3%
Apoptotic Cells 42.4%
Dead Cells 11.1%
Live cells 55.0%
Apoptotic Cells 33.8%
Dead Cells 7.84%
Live cells 50.2%
Apoptotic Cells 41.4%
SYTOX AADvanced
Annexin V
D
β-cell stress genes
Relative Expression
Human islets
SC-islets (CD49a+)
AIFM1
ATF4
ATF6
BAX
Casp3
CDKN1
CYCS
DDIT3
EDEM 1
ERN1
HERPUD1
HSPA5
JAK2
MAPK8
p53
PPP1R15A
SERPINE1
STAT3
XBP1
sXBP1
Unt
Cyto
TG
Cyto +TG
HG
Cyto +TG +HG
Untreated
Cyto
TG
Cyto+TG
HG
Cyto+TG+ HG
Immune interaction genes
CD274
CXCL10
CXCL8
FAS (CD95)
IFGR1
IL6
HLA-A
HLA-B
HLA-C
HLA-E
PDIA4
TNFRSF1A
E
Untreated
Cytomix
TG
Cyto + TG
HG
Cyto + TG + HG
% Live cells
% Necrotic cells
Human islets
SC-islets (CD49a+)
F
INS expression (log10)
NKX6-1 expression (log10)
PDX1 expression (log10)
Human islets
SC-islets (CD49a+)

Figure S1: SC-islets and HI are sensitive to stress induction *in vitro*. Related to Figure 1.

(A) Schematic representation of the differentiation protocol used to generate enriched SC-islets.

(B) Average percentage of native and enriched SC-islets across all assays generated using the differentiation protocol (A) and enriched using CD49a magnetic sorting. n=6 SC-islets differentiations. Data are means ± SEMs. Representative flow cytometry plot on the right.

(C) Representative gating strategy for single cells and flow cytometry quantification of Annexin/Sytox in SC-islets untreated or treated with cytokines (IL-1β—50ng/mL, TNF-α—100ng/mL, IFN-γ—500ng/mL, 48h), TG (10μM, 48h), and HG (33mM, 72h). Used in all apoptosis assay gating presented in Figure 1.

(D) Flow cytometry quantification of Annexin/Sytox cells (given as % live and necrotic cells) in SC-islets and HI treated with stress-inducing factors as in (C). n=4 SC-islets differentiations and n=2 HI donors, for 2 independent experiments. Fold-change was computed as the ratio of the treatment value/baseline (untreated HI). Data are means ± SEMs. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, and ****$p < 0.0001$. Ordinary 1-way ANOVA.

(E) Relative mRNA expression of β-cell stress-associated genes (top) and immune interaction-associated genes (bottom) in HI and SC-islets untreated or treated with stress-inducing factors. Each column represents either an HI donor or a SC-islet differentiation and each row represents a gene. The heatmap data was plotted by z-score using the mean expression of all experimental groups.

(F) mRNA expression level of *INS*, *NKX6-1*, and *PDX1* SC-islets untreated or treated with stress-inducing factors as in (C).

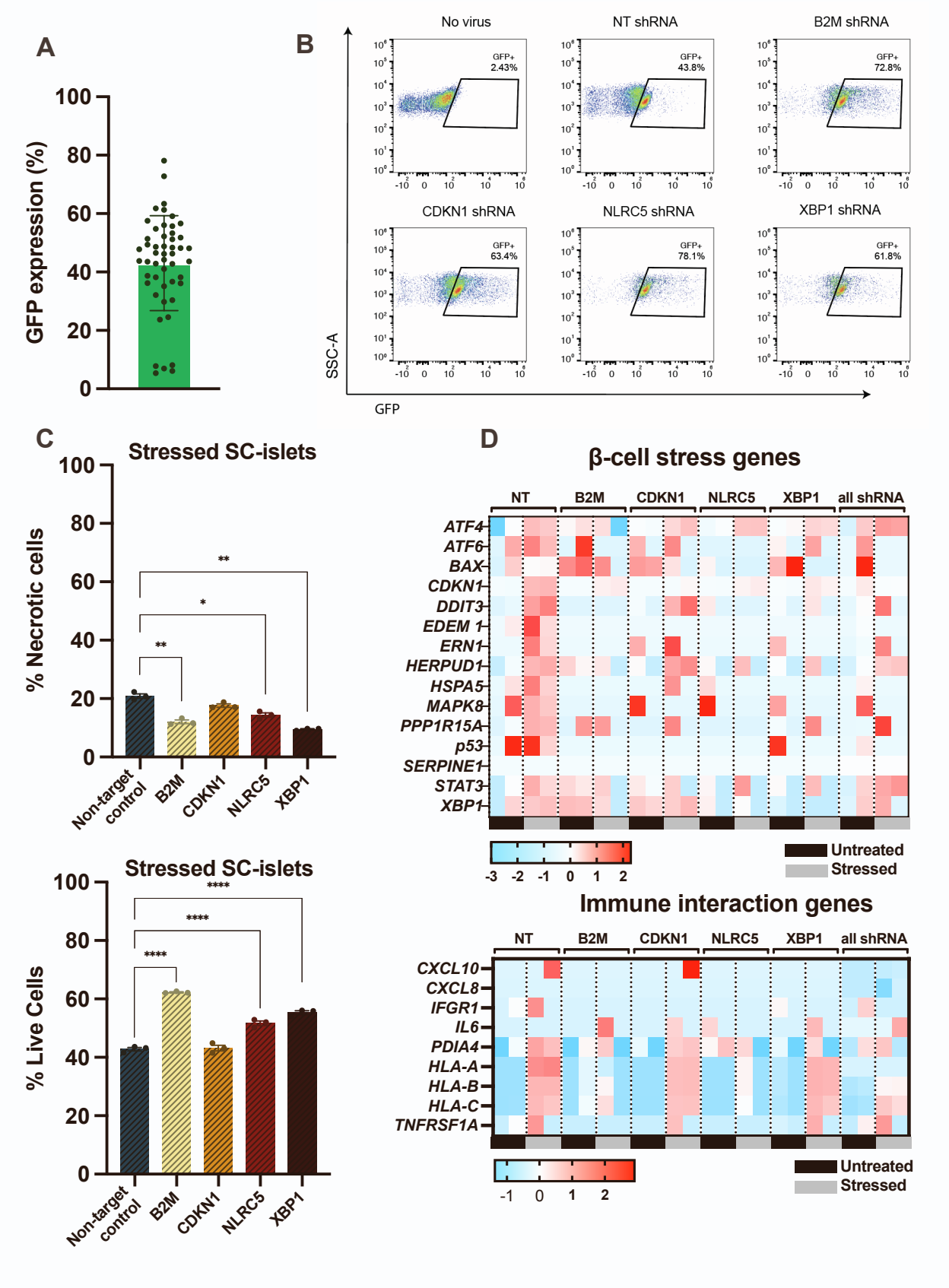
A
GFP expression (%)
100
80
60
40
20
0
B
No virus
NT shRNA
B2M shRNA
CDKN1 shRNA
NLRC5 shRNA
XBP1 shRNA
GFP+
2.43%
GFP+
43.8%
GFP+
72.8%
GFP+
63.4%
GFP+
78.1%
GFP+
61.8%
SSC-A
GFP
C
Stressed SC-islets
% Necrotic cells
**
*
**
Non-target control
B2M
CDKN1
NLRC5
XBP1
Stressed SC-islets
% Live Cells
****
****
****
Non-target control
B2M
CDKN1
NLRC5
XBP1
D
β-cell stress genes
NT
B2M
CDKN1
NLRC5
XBP1
all shRNA
ATF4
ATF6
BAX
CDKN1
DDIT3
EDEM 1
ERN1
HERPUD1
HSPA5
MAPK8
PPP1R15A
p53
SERPINE1
STAT3
XBP1
-3 -2 -1 0 1 2
Untreated
Stressed
Immune interaction genes
NT
B2M
CDKN1
NLRC5
XBP1
all shRNA
CXCL10
CXCL8
IFGR1
IL6
PDIA4
HLA-A
HLA-B
HLA-C
TNFRSF1A
-1 0 1 2
Untreated
Stressed

Figure S2: Genetic manipulation increases protection of SC-islets. **Related to Figure 2.**

(A) Average GFP expression of SC-islets transduced with shRNA.

(B) Representative image of the flow cytometry gating strategy of GFP expression in SC-islets following transduction with shRNAs for each target gene.

(C) Flow cytometry quantification of Annexin/Sytox in SC-islets following genetic modification and treatment with stress-inducing factors. Data are means ± SEMs and representative of three experiments, each with one SC-islets differentiation (n=3). Data are means ± SEMs. Ordinary 1-way ANOVA.

(D) Relative mRNA expression of β-cell stress-associated genes (top) and immune interaction-associated genes (bottom) in SC-islets following genetic modification and treatment with stress-inducing factors. Each column represents a SC-islet differentiation, and each row represents a gene. The heatmap data was plotted by z-score using the mean expression of all experimental groups.

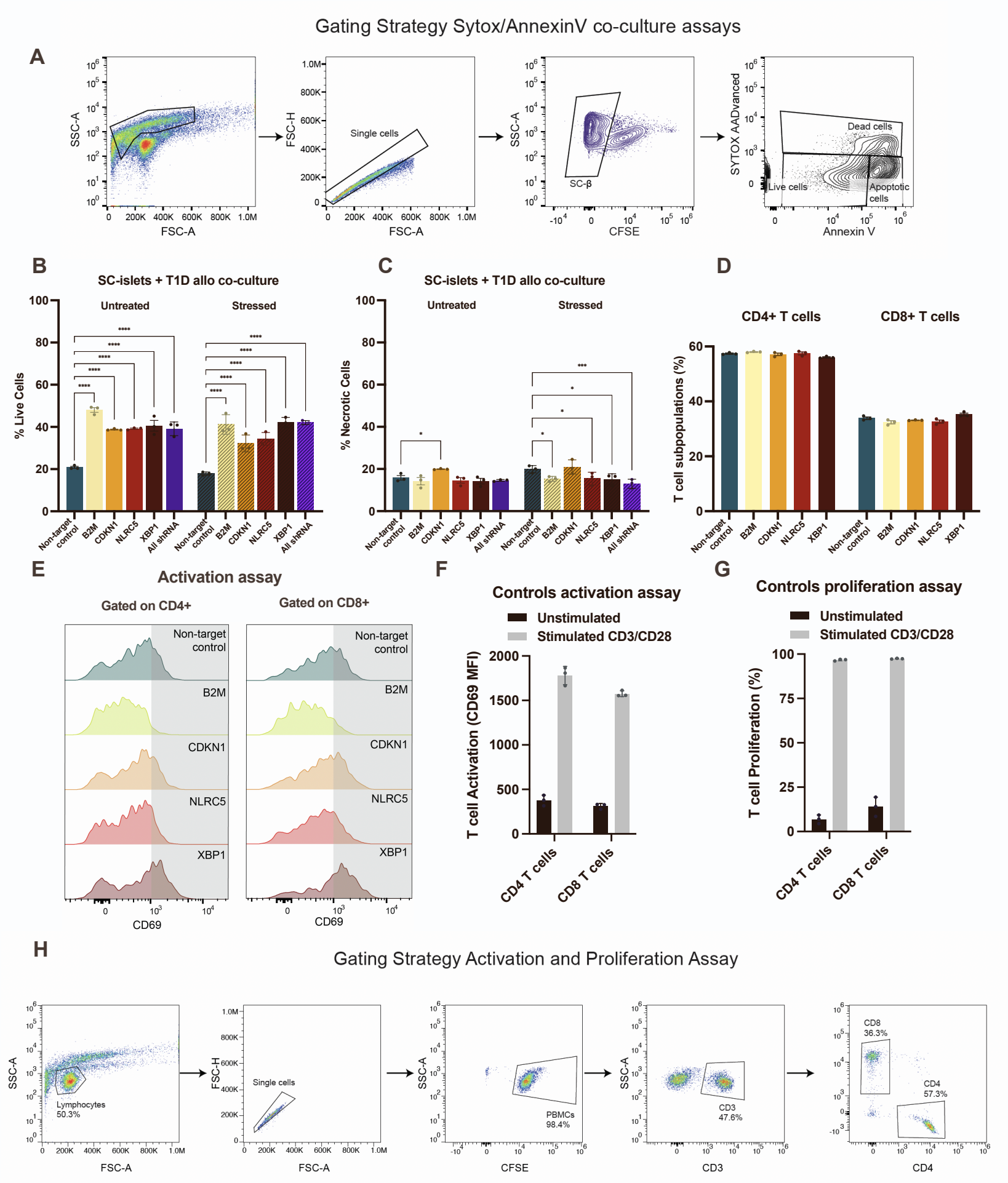
Gating Strategy Sytox/AnnexinV co-culture assays
A
SSC-A
FSC-A
FSC-H
Single cells
SC-β
CFSE
SYTOX AADvanced
Dead cells
Live cells
Apoptotic cells
Annexin V
B
SC-islets + T1D allo co-culture
Untreated
Stressed
% Live Cells
C
% Necrotic Cells
D
CD4+ T cells
CD8+ T cells
T cell subpopulations (%)
Non-target control
B2M
CDKN1
NLRC5
XBP1
All shRNA
E
Activation assay
Gated on CD4+
Gated on CD8+
CD69
F
Controls activation assay
Unstimulated
Stimulated CD3/CD28
T cell Activation (CD69 MFI)
CD4 T cells
CD8 T cells
G
Controls proliferation assay
T cell Proliferation (%)
H
Gating Strategy Activation and Proliferation Assay
Lymphocytes 50.3%
PBMCs 98.4%
CD3 47.6%
CD8 36.3%
CD4 57.3%
CD3
CD4

Figure S3: Activation and killing by T cells is reduced in genetically-modified stressed SC-islets. Related to Figure 3.

(A) Representative gating strategy for SC-islets and flow cytometry quantification of Annexin/Sytox in SC-islets following genetic modification, treatment with stress-inducing factors, and co-culture with allogeneic T1D PBMCs. Used in all apoptosis assay gating presented in Figure 3.

(B-C) Flow cytometry quantification of Annexin/Sytox in SC-islets following genetic modification, treatment with stress-inducing factors, and co-culture with allogeneic T1D PBMCs. Data are means ± SEMs and representative of three experiments, each with one SC-islets differentiation (n=3). Data are means ± SEMs. Ordinary 1-way ANOVA.

Live cell fold-change.

(D) Representative flow cytometry quantification of T cell subpopulations within PBMCs after co-culture, CD4+ (left) and CD8+ (right).

(E) Representative flow cytometry histograms after 16h of co-culture. Related to figure 3E and F.

(F) Representative flow cytometry quantification of T cell activation control measured in unstimulated and stimulated cells co-positive for CD69+, CD3+, and either CD4+ or CD8+ populations (n=2 T1D PBMC donors). Data are means ± SEMs.

(G) Representative flow cytometry quantification of T cell proliferation control measured in unstimulated and stimulated cells co-positive for CD3+ and either CD4+ or CD8+ populations (n=2 T1D PBMC donors). Data are means ± SEMs.

(H) Representative gating strategy for lymphocytes, single cells, PBMCs, CD3+, and CD4+ or CD8+ cell populations; used in all T cell activation and proliferation gating presented in Figure 3.

Table S1. Antibodies used in this study. Related to Experimental Procedures.

ANTIBODIES	SOURCE	IDENTIFIER
Rat anti-C-peptide	Developmental Studies Hybridoma Bank (DHSB)	GN-ID4, RRID:AB_2255626
Mouse anti-glucagon	Santa Cruz Biotech	Cat#SC-514592
Donkey anti-mouse Alexa 647	Life Technologies	Cat#A31571
Donkey anti-rabbit Alexa 488	Life Technologies	Cat#A21206
Donkey anti-rabbit Alexa 647	Life Technologies	Cat#A31573
Donkey anti-goat Alexa 647	Life Technologies	Cat#A21447
Donkey anti-rat 405	Abcam	Cat#ab175670
Mouse anti-CD3 PB-conjugated	Biolegend	UCHT1, Cat#300417
Mouse anti-CD8 PE-conjugated	Biolegend	T8-Leu2, Cat#344705
Mouse anti-CD4 PE/Cy7-conjugated	Biolegend	RPA-T4, Cat#300511
Mouse anti-CD69 Alexa 647-conjugated	Biolegend	FN50, Cat#310918
Mouse anti-CD49a PE-conjugated	BD Biosciences	Cat#559596
Mouse anti-β2M PE-conjugated	Biolegend	2M2, Cat#316305
Goat anti-CDKN1A	R&D Systems	Cat#AF1047
Mouse anti-NLRC5	Santa Cruz Biotechnology	Cat#sc-515668
Rabbit anti-XBP1	Invitrogen	Cat#PA5-25010

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture

Human pluripotent stem-cell maintenance and differentiation was carried out with Harvard University Embryonic Stem Cells 8 (HUES8) as previously described (Veres *et al.*, 2019). All SC-islets used in experimentation were obtained from differentiations performed by the Melton laboratory Foundry. Pluripotent stem-cell lines were maintained in cluster suspension culture format using mTeSR1 (Stem Cell Technologies, 85850) in 500-ml spinner flasks (Corning, VWR) spinning at 70 r.p.m. in an incubator at 37°C, 5% CO_2 and 100% humidity. Cells were passaged every 72 h: human pluripotent stem-cell clusters were dissociated to clumps using gentle cell dissociation reagent (Stem Cell Technologies, 07174) and light mechanical disruption, counted and seeded at 0.5 M cells/ml in mTeSR1 + 10 μM Rock Inhibitor-Y27632 (DNSK International, DNSK-KI-15-02). Cell lines were authenticated by DNA fingerprinting, karyotyping (Cell Line Genetics), and all lines tested negative on routine mycoplasma contamination verifications. The HUES8 lines used throughout the study matched HUES8. Differentiation flasks were started 72 h after passage by removing mTeSR1 medium and replacing with the protocol-appropriate medium and growth factor or small molecule supplements as previously described (Veres *et al.*, 2019). Small molecules and signaling factors were prepared and stored as single-use aliquots. During feeds, differentiating clusters were allowed to

gravity-settle for 5–10 min, medium was aspirated, and ~300 ml of pre-warmed medium was added.

β-cell differentiation protocol

Stage 1: 24 hours in S1 medium supplemented with Activin A (100ng/ml) and CHIR99021 (1.4 µg/ml), followed by 48 hours Activin A (100ng/ml) only.
Stage 2: 72 hours in S2 medium supplemented with KGF (50ng/ml).
Stage 3: 48 hours in S3 medium supplemented with KGF (50ng/ml), LDN193189 (200nM), Sant1 (0.25 µM), retinoic acid (2 µM), PBDU (500nM) and Rock Inhibitor (10 µM).
Stage 4: 5 days in S3 medium supplemented with KGF (50ng/ml), Sant1 (0.25 µM), retinoic acid (0.1 µM) and Rock Inhibitor (10 µM).
Stage 5: 7 days in BE5 medium supplemented with Betacellulin (20ng/ml), XXI (1 µM), Alk5i-II (10 µM) and T3 (1 µM). Sant1 (0.25 µM) was added in days 1 to 3, and retinoic acid was added at 0.1 µM in days 1 to 3, then at 0.025 µM.
Stage 6: 14-21 days in S3 medium, changed every 48h.

Magnetic enrichment using CD49a

Following SC-islet differentiation, stage 6 clusters were dissociated using TrypLE Express for 20 min at 37°C. Cells were then quenched with S3 media and spun down. Remaining undissociated cell clusters were mechanically dissociated using a P1000 pipette. Following dissociation, the single cells were resuspended in sorting buffer (PBS + 1% BSA + 2 mM EDTA) and filtered through a 37-µm mesh filter. Cells were counted and resuspended at a density of 10 million cells per 300 µL in 15 mL conical tubes. Cells were stained at room temperature for 20 min using a 1:100 dilution of anti-human CD49a PE-conjugated (BD 559596) antibody, covered from light, and agitated every 3 min. Stained cells were washed twice with 15 mL of sorting buffer by spinning down (5 min, 300*g*) and resuspended to their initial density of 10 million cells per 300 ml. To label with microbeads, 40 mL of anti-PE UltraPure MACS microbreads (Miltenyi 130-105-639) were added for each 10 million cells, and the cell solution was incubated for 15 min at 4°C, agitated every 5 min. The stained cells were washed twice as above and resuspended to a target density of 25–30 million cells per 500 µl. Volumes of 500 µL (containing no more than 30 million cells) were then magnetically separated on LS columns (Miltenyi 130-042-401) in a QuadroMACS separator (Miltenyi 130-090-976) using the recommended protocol. Briefly, 500 µl of cells was added to a pre-washed column, washed with 3 ml of sorting buffer three times, removed from the separator, and washed with a final volume of 5 ml. The final cell fractions from different columns were pooled. Successful PE enrichment was verified by live-cell flow cytometry on an Attune NxT (Invitrogen) flow cytometer. Typical

yields were approximately 10–15 million purified cells when starting with ~150 million total cells. An example purification result is shown in Supplementary Figure 1.

Cellular viability and apoptosis assay

SC-islet clusters were plated at a density of ~150,000 cells per well on 96-well round bottom plates and treated with IFN-γ, 500 ng/ml (Peprotech, 300-02), IL-1β, 50ng/mL (Peprotech, 200-01B), TNF-α, 100ng/mL (Peprotech, 300-01A), thapsigargin, 10 μM (Sigma Aldrich, T9033) for 48h and high glucose, 33mM for 72h. Untreated human islet and SC-islets controls were cultured in the same buffer for the same time period as the treated samples, CMRL medium for human islets and S3 medium for SC-islets. Following treatment, and co-culture in the case of the immune-protection assays, cells were washed to remove residual cytokines, thapsigargin, and glucose. Apoptosis was determined by staining the SC-islets and human islet controls with Annexin, Pacific Blue/SYTOX AADvanced, according to the manufacturer's recommendations (ThermoFisher, A35136). In brief, SC-islet clusters were dissociated, and single cells were stained at room temperature for 30 min using a 1:20 dilution of Annexin V, Pacific Blue conjugate (ThermoFisher, A35122) and a 1:100 dilution of SYTOX AADvanced (ThermoFisher, S10274). After the incubation period, 400 μl of 1X annexin binding buffer was added and mixed gently. The analysis of the stained cells was done by flow cytometry, measuring the fluorescence emission using 405 nm excitation (Pacific Blue™ dye) and with 488 nm excitation (SYTOX® AADvanced™). The samples can contain three populations: live cells showing a low level of violet and red fluorescence, apoptotic cells showing a high level of violet fluorescence and no red fluorescence, and necrotic cells showing a high intensity red and violet fluorescence. Stained cells were analyzed using the Attune NxT (ThermoFisher) flow cytometer.

Flow cytometry

Intracellular Marker Staining

SC-islet clusters, sampled from suspension cultures, were dissociated using TrypLE Express (GIBCO, 12604013) at 37°C, mechanically disrupted into single cells, fixed using 4% PFA for 30 min at room temperature and stored in PBS at 4°C. For staining, fixed single cells were incubated in Perm/Wash Buffer (BD Biosciences, 554723) for 30 min at room temperature, then incubated in Perm/Wash Buffer with primary antibodies (1h at room temperature), washed three times with Perm/Wash Buffer, incubated with secondary antibodies in Perm/Wash Buffer (30 min at room temperature), washed three times and resuspended in Perm/Wash Buffer. Stained cells were analyzed using the Attune NxT (ThermoFisher) flow cytometer.

Surface Marker Staining

PBS containing 2% Fetal Bovine Serum (FBS) was used as blocking and staining buffer. Immune cells or other dissociated single cells were washed and blocked with blocking buffer for 30 min at 4°C, then incubated in blocking buffer with conjugated antibodies (1h at 4°C), washed three times with blocking buffer, fixed using 4% PFA for 30 min at room temperature and stored in PBS at 4°C. Stained cells were analyzed using the Attune NxT (ThermoFisher) flow cytometer.

Glucose-stimulated insulin secretion assay

GSIS assay was performed as previously described (Blum et al., 2012). Non-diabetic human islets from Prodo labs (50–150-µm-diameter-sized, n = 2) and native SC-islet clusters (n = 4) were divided to collect technical triplicates of secreted products (assayed for insulin). Krebs buffer (KRB) was prepared: 128 mM NaCl, 5 mM KCl, 2.7 mM CaCl2, 1.2 mM MgSO4, 1 mM Na2HPO4, 1.2 mM KH2PO4, 5 mM NaHCO3, 10 mM HEPES (Life Technologies; 15630080), and 0.1% BSA in deionized water. Clusters were washed twice with low-glucose (2.8 mM) KRB and then loaded into 24-well plate inserts (Millicell Cell Culture Insert; PIXP01250) and fasted in low-glucose KRB for 1 h to remove residual insulin in 37°C incubators. Clusters were then washed once in low-glucose KRB, incubated in low-glucose KRB for 1 h, and the supernatant was collected. Following low-glucose incubation, clusters were transferred to high-glucose (20 mM) KRB for 1 h, and the supernatant was collected. Finally, clusters were incubated in KRB containing 2.8 mM glucose and 30 mM KCl (depolarization challenge) for 1 h, and then the supernatant was collected. Clusters were then dispersed into single cells using TrypLE Express, and cell number was counted automatically by a Vi-Cell (Beckman Coulter) to normalize insulin level by the cell number. Supernatant samples containing secreted insulin were processed using the human ultrasensitive insulin enzyme-linked immunosorbent assay (ELISA) (ALPCO; 80-INSHUU-E01.1). Stimulation index was calculated by the ratio of stimulated to basal insulin secretion rates.

NanoString gene array

Stage 6 cells from the β-cell Differentiation Protocol were enriched as described in the section ‘Magnetic enrichment using CD49a.’ Prior to the NanoString assay, the enriched population, CD49a+ SC-islets, were lysed using the RLT buffer (RNeasy Lysis Buffer, QIAGEN). An nCounter gene expression assay was performed according to the manufacturer’s protocol. The assay utilized a custom-made NanoString codeset designed to measure 24 transcripts, including 3 putative house- keeping transcripts. The data was normalized to the average counts for all housekeeping genes in each sample. The heatmap data was plotted by z-score using the mean expression of all experimental groups. nSolver software was used to analyze the data (NanoString Technologies).

Lentivirus preparation and transduction

Lentiviral particles were produced by transfecting 293T cells (Takara Bio, Mountain View, CA, USA) with the packaging vectors psPAX2 (700 ng/µl) and VSVG (350 ng/µl) (1:1), along with 38.5 µg of the shRNA transfer plasmid. Diluted LipoD293 (SignaGen, SL100668) was then added to the DNA solution. The following shRNA constructs were used: non-target SHC016, SHC008 (*β2M*), TRCN0000057254 (*β2M*), TRCN0000057255 (*β2M*), TRCN0000294421 (*CDKN1A*), TRCN0000287091 (*CDKN1A*), TRCN0000127850 (*NLRC5*), TRCN0000019806 (*XBP1*), TRCN0000019807 (*XBP1*), TRCN0000128097 (*NLRC5*), all shRNA constructs were cloned into the pLKO.1 CMV-TurboGFP + shRNA vector. Lentiviral particles were concentrated 48h and 72h post transfection using the PEG-IT virus precipitation reagent (Fisher Scientific, Waltham, MA, USA) overnight at 4°C followed by centrifugation at 1500 g for 30 min at 4°C and stored at 80°C. For transduction, we collected cell clusters from spinner flask suspension cultures and dissociated them in TrypLE Express (Life Technologies, Carlsbad, CA, USA) for 7 min, followed by mechanical dissociation and centrifugation at 300g for 5 min at room temperature (RT). Cell pellets were then resuspended at a density of 1.3 million cells/mL in the stage-matched medium with 10 µM Rock Inhibitor-Y27632 (DNSK International, DNSK-KI-15-02) and 8 µG/mL of Hexadimethrine bromide (Millipore Sigma, 107689). Single-cell suspensions were combined with concentrated (1:100) lentiviral particles and plated on ultra-low attachment six-well plates on a rocker plate set at 70rpm in a humid 37°C incubator and 5% CO_2.

Human primary immune cell isolation

We obtained blood from a T1D de-identified donor from the University of Massachusetts Medical School. Human primary peripheral mononuclear cells (PBMCs) were isolated using the density gradient medium Ficoll-Paque Plus (GE health care life sciences, 17144002) and SepMate tubes (Stem Cell Technologies, 85450). Isolated PBMCs (~30M) were cultured in T cell medium (TCM) consisting of X-VIVO 10 (Lonza, 04-380Q) media supplemented with 5% Human AB Serum (Valley Biomedical, HP1022HI), 5% Fetal Bovine Serum (ThermoFisher Scientific, A3840101), 1% Penicillin/Streptomycin (ThermoFisher Scientific, 15070063), GlutaMAX (ThermoFisher Scientific, 35050061), and MEM Non-Essential Amino Acids (ThermoFisher Scientific, 11140050).

T cell activation assay

Transduced SC-islets were used as target cells. Approximately 200,000 target cells on 96-well round bottom plates and treated the cells with IFN-γ, 500 ng/ml (Peprotech, 300-02), IL-1β, 50ng/mL (Peprotech, 200-01B), TNF-α, 100ng/mL (Peprotech, 300-01A), thapsigargin, 10 µM (Sigma Aldrich, T9033) for 48h and high glucose, 33mM for 72h. Untreated human islet and SC-islet controls were cultured in the same buffer for the same

time period as the treated samples, CMRL medium for human islets and S3 medium for SC-islets. Following treatment, target cells (SC-islets and human islets) were washed to remove residual cytokines, thapsigargin, and glucose. PBMCs (200,000 per well) were then added to re-aggregated SC-islets, with and without pre-treatment. After a 16h co-culture in T cell media as previously described (Leite *et al.*, 2020), we stained CD3+, CD4+, and CD8+ T cells for T cell activation marker CD69 using the following antibodies: CD3 PB-conjugated (Biolegend, 300417), CD4 PE/Cy7-conjugated (Biolegend, 300511), CD8 PE-conjugated (Biolegend, 344705), and CD69 Alexa 647-conjugated (Biolegend, 310918). T cells activated with Dynabeads Human T-Activator CD3/CD28 beads (ThermoFisher Scientific, 111.61) for 48h were used as positive control. Stained cells were analyzed using the Attune NxT (ThermoFisher) flow cytometer. The results of the activation staining are presented as adjusted mean MFI, with the mean MFI of unstimulated PBMCs subtracted from the mean MFI of activation. Data was baseline-corrected to the untreated condition of each shRNA group and a one-way ANOVA with a significance threshold of $p < 0.05$ was used to determine whether T cell activation levels in the treatment conditions of each shRNA group differed significantly from the treatment conditions of the non-target control shRNA group.

T cell proliferation assay

As in the activation assay, approximately 200,000 transduced SC-islets were plated on 96-well round bottom plates and treated with IFN-γ, 500 ng/ml (Peprotech, 300-02), IL-1β, 50ng/mL (Peprotech, 200-01B), TNF-α, 100ng/mL (Peprotech, 300-01A), thapsigargin, 10 µM (Sigma Aldrich, T9033) for 48h and high glucose, 33mM for 72h. Untreated human islet and SC-islet controls were cultured in the same buffer for the same time period as the treated samples, CMRL medium for human islets and S3 media for SC-islets. Following treatment, target cells (SC-islets and human islets) were washed to remove residual cytokines, thapsigargin, and glucose. PBMCs were labeled with CellTrace CFSE (ThermoFisher, C34554) according to the manufacturer's recommendations. Briefly, CellTrace CFSE was diluted 1:1000 in PBS and added to the PBMCs, incubated for 20 min in a water bath, quenched with TCM (as previously described), incubated for 5 min in a water bath, and resuspended in TCM. PBMCs (200,000 per well) were then added to re-aggregated SC-islets, with and without pre-treatment. After a 5-day co-culture, in T cell media, we collected and stained for CD3+, CD4+, and CD8+ T cells using the following antibodies: CD3 PB-conjugated (Biolegend, 300417), CD4 PE/Cy7-conjugated (Biolegend, 300511), and CD8 PE-conjugated (Biolegend, 344705). T cells labeled with CellTrace CFSE and activated with ImmunoCult™ Human CD3/CD28 T Cell Activator (Stem Cell Technologies, 10971) for 5 days were used as positive control. Stained cells were analyzed using the Attune NxT (ThermoFisher) flow cytometer. Data was baseline-corrected to the untreated condition of each shRNA group and a one-way ANOVA with a significance threshold of $p < 0.05$

was used to determine whether T cell proliferation levels in the treatment conditions of each shRNA group differed significantly from the treatment conditions of the non-target control shRNA group.

Cytokines Analysis

Supernatant of co-culture T cell activation experiments were assayed using the MSD proinflammatory panel, a highly sensitive multiplex enzyme-linked immunosorbent assay (ELISA) for quantitatively measuring cytokines including interferon γ (IFN-γ) and interleukin (IL)-2, from the supernatants using an electrochemiluminescent detection method (MesoScale Discovery, Gaithersburg, MD, USA).

Quantification and statistical analysis

Statistical analyses are described in detail where reported. Statistical analyses were carried out using Graphpad Prism software. Statistical assays were performed as described in each figure legend. n represents number of biological replicates in all cases where reported. Biological replicates refer to unique donor-derived batches of human islets or unique differentiations of SC-islets produced from unique suspension cultures.

Supplemental References

Leite, N.C. *et al.* (2020) 'Modeling Type 1 Diabetes In Vitro Using Human Pluripotent Stem Cells', *Cell Reports*, 32(2). doi:10.1016/j.celrep.2020.107894.

Veres, A. *et al.* (2019) 'Charting cellular identity during human in vitro β-cell differentiation', *Nature*, 569(7756), pp. 368–373. doi:10.1038/s41586-019-1168-5.