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

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## SUMMARY

The *in vitro* production of stem-cell-derived islets (SC-islets) has brought forth the potential of transplanting these cells to restore glycemic control in people with diabetes. Nonetheless, alloimmune and autoimmune responses remain considerable challenges for a broad clinical implementation of β-cell replacement therapies. β-cell stress has been implicated in the onset of β-cell immunogenicity and death and is likely to contribute to β-cell failure following transplantation. We show that inducing stress and/or administering cytokines causes SC-islet apoptosis, cellular dysfunction, and an increased expression of β-cell stress- and immune-interaction-related genes. We then demonstrate that manipulating some of these genes results in enhanced protection of SC-islets from apoptosis *in vitro*.

## INTRODUCTION

Type 1 diabetes (T1D) results from autoimmune-mediated destruction of pancreatic β cells (Katsarou et al., 2017). Genetic and environmental factors have been implicated in the emergence of β-cell immunogenicity and dysfunction; however, the initial signals that trigger autoimmunity, the intracellular mediators that result in β-cell destruction, and the cross talk between β cells and immune cells in T1D remain poorly understood (Engin, 2016). Advances in the production of insulin-secreting β cells from human embryonic stem cells (SC-islets) have brought forth the potential of restoring glycemic control in diabetic individuals by transplanting these *in vitro* produced cells (Melton, 2021). While utilizing human pluripotent stem cells to generate SC-islets addresses the issue of islet supply, transplantation will require immune protection with an encapsulation device, manipulation of the host immune response, and/or genetic modification of the transplanted cells (Siehler et al., 2021).

β cells have long been thought to be non-provoking victims of autoimmune destruction. However, recent studies have pointed to the possibility that β-cell stress may contribute to the T1D immune attack, suggesting dysfunction of both the immune system and the β cell (Roep et al., 2020). Thus, approaches to successful cell transplantation for T1D might be expanded beyond calcineurin inhibitor administration, regulatory T cell (Treg) manipulation, and other manipulations of the host immune system (Roep et al., 2020). Therapies that aim to induce selective immune tolerance to islet autoantigens, favoring the engagement of the immune system, are being tested to reverse the immunopathogenesis of T1D (Alhadj Ali et al., 2017). In combination with SC-islet therapy, these could improve β-cell stamina and vitality and protect these cells from metabolic and inflammatory stress.

Several studies on the effects of exposing islets to environmental stressors show reduced expression of key transcription factors as mediators of gluco- and lipotoxicity (Dai et al., 2016). But these experiments, typically done in the context of type 2 diabetes (T2D), may not reflect what happens when SC-islets are transplanted into T1D patients. One can anticipate the induction of β-cell stress by metabolic overload, exposure to the proinflammatory milieu, and/or glucotoxicity. As part of the goal to mitigate the responses to transplanted SC-islets, we characterized the stress response of SC-islets upon exposure to environmental triggers and use that information to protect SC-islets from stress-induced apoptosis.

## RESULTS

### SC-islets and human islets are vulnerable to stress *in vitro*

To examine the stress response of SC-islets and compare it with that of human islets (HIs), we investigated the level of apoptosis of SC-islets and HIs following treatment with stress-inducing factors. Embryonic stem cells were differentiated *in vitro* to SC-islets, which generates a mixed population of endocrine and non-endocrine cells (Figure S1A). To examine the β-cell effects, we used anti-CD49a to enrich β cells in the SC-islets for all experiments performed, unless otherwise indicated, and this enriches the β-cell population from ~20% to ~80% purity (Veres et al., 2019) (Figure S1B). Following enrichment, SC-islets and HI controls were treated with three different stress conditions, as previously

 

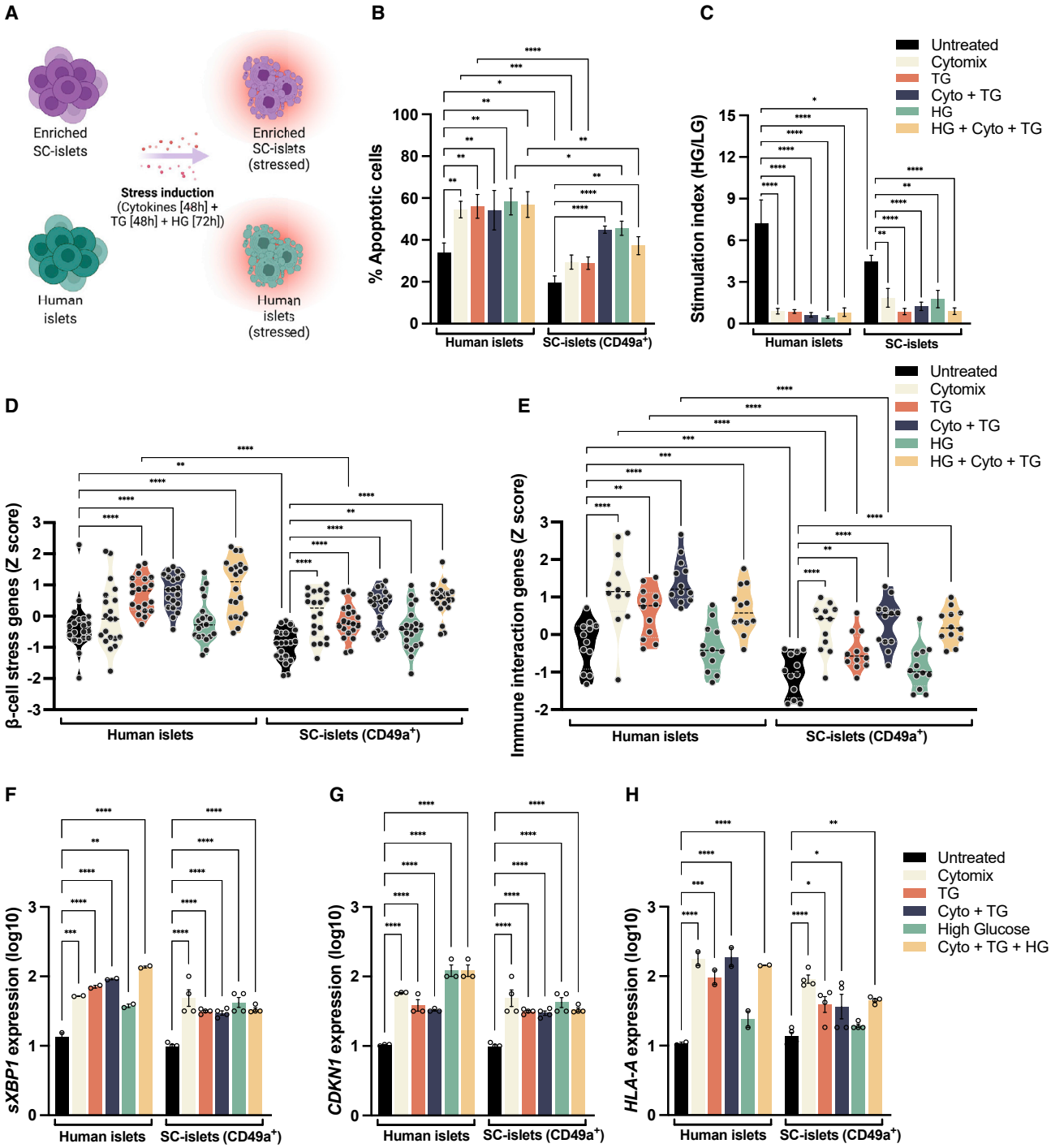


**Figure 1. SC-islets are vulnerable to stress *in vitro***

(A) Experimental design: SC-islets and human islets (HIs) were treated with cytokines (IL-1β—50 ng/mL, TNF-α—100 ng/mL, and IFN-γ—500 ng/mL; 48 h), thapsigargin (TG, 10 μM, 4 8 h) and high glucose (HG, 33 mM, 72 h) to induce inflammatory, ER, and metabolic stress, respectively.

(B) Apoptosis in SC-islets and HIs treated with cytokines, TG, and HG.

(C) Stimulation index of HIs and SC-islets following treatment with stress-inducing factors as in (B).

(D and E) Relative mRNA expression of β-cell stress-associated genes (D) and immune-interaction-associated genes (E) in HIs and SC-islets treated as in (B). Each dot represents one gene from the heatmap in (S1E). Black dashed lines represent the median.

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described (Maxwell et al., 2020) (Figure 1A): cytomix (interleukin [IL]-1β—50 ng/mL, tumor necrosis factor [TNF]-α—100 ng/mL, and interferon [IFN]-γ—500 ng/mL; 48 h), thapsigargin (TG) (10 μM, 48 h), and high glucose (HG) (33 mM, 72 h), mimicking inflammatory, endoplasmic reticulum (ER), and metabolic stress, respectively. The gating strategy for the apoptosis assay is shown in Figure S1C.

HIs exhibited increased levels of apoptosis after treatment with all stressors (cytomix, TG, and HG) compared with untreated controls (Figures 1B and S1D). Similarly, SC-islets exhibited heightened apoptosis when treated with stressors; however, the percentage of apoptotic cells and dead cells in the treated conditions was reduced compared with that of HIs (Figures 1B and S1D). These results indicate that SC-islets are vulnerable to stress and apoptosis *in vitro*, albeit to a lesser degree than HIs in this assay.

An *in vitro* functional assay measuring glucose-stimulated insulin secretion (GSIS) was performed on native, non-enriched SC-islet populations and HI controls following treatment with stressors, as described above. Observed stimulation indices of the HIs and SC-islets treated with stressors revealed irregular patterns following glucose challenges (Figure 1C). These results show an impairment of the coupling of glucose metabolism and insulin secretion in HIs and SC-islets following exposure to environmental stressors. β-cell stress induced by cytokines and nutrients directly impairs insulin secretion and other aspects of β-cell function and survival, indicating the pathological consequences of excess glucose and/or inflammation (Eizirik et al., 2020; Sims et al., 2020).

In addition to cellular death and dysfunction, β-cell stress has been shown to result in reduced expression of genes related to cellular homeostasis and heightened expression of genes related to inflammation, peptide presentation, and stress (Eizirik et al., 2020). To determine whether similar gene expression changes are induced in SC-islets after stress, multiplex gene expression analysis was performed on stressed SC-islets and HIs. Both HIs and SC-islets exhibited increased expression of genes related to β-cell stress (Figures 1D and S1E) and immune interaction (Figures 1E and S1E). Expression of genes upregulated after stress seems to increase most when HIs and SC-islets are exposed to cytokines, TG, and HG combined. Stress induction did not impair SC-islet identity (Figure S1F). In conclusion, stress treatment causes SC-islet gene upregulation *in vitro*, albeit to a lesser level than HI.

## Genetic manipulation is associated with protection of SC-islets from stress-mediated apoptosis and dysfunction

Considering the possible role of β-cell stress in instigating an immune rejection, we hypothesized that targeting genes related to β-cell stress, in addition to those related to immune interaction, might result in enhanced protection of SC-islets from apoptosis. The rationale for the dual approach, targeting stress and immune recognition genes, is that we believe both β-cell stress and islet autoimmunity can be harnessed as targets for intervention strategies. Thus, genes that were elevated upon treatment and involved in peptide presentation and stress were selected.

Noting that *XBP1* (Figure 1F), *CDKN1A* (Figure 1G), and HLA class I expression (Figure 1H) (regulated by the genes *β2M* and *NLRC5*) are upregulated in most stress conditions, we targeted the genes *β2M*, *CDKN1A*, *NLRC5*, and *XBP1* to study resistance to apoptosis. *XBP1* is directly involved in the ER stress response and plays differing roles depending on the nature of the stress. During acute ER stress, as in our study, *XBP1* is involved in the unfolded protein response (Eizirik et al., 2020). *CDKN1A* promotes apoptosis of β cells in response to various stressors (Kaneto et al., 1999). *NLRC5* is a transcriptional regulator of *HLA-ABC* and *β2M*, responsible for orchestrating the expression of critical components in the HLA class I pathway (Meissner et al., 2010). NLRC5 deficiency impairs killing by cytotoxic T cells (Staehli et al., 2012), and knockdown in mesangial cells in HG conditions is associated with reduced inflammation (Luan et al., 2018). Lastly, *β2M* knockout results in the reduction of HLA class I expression and reduces T-cell-mediated immune responses in endothelial cells (Han et al., 2019) and HIs (Wang et al., 2012).

To study the effect of gene downregulation on SC-islets viability and function, we transduced SC-islets with lentiviral small hairpin RNA (shRNA) plasmids individually targeting each gene of interest (*β2M*, *CDKN1A*, *NLRC5*, and *XBP1)* or targeting all four genes together (all shRNA group). Following transduction, the SC-islets were treated with a combination of stress-inducing factors: cytomix (IL-1β—50 ng/mL, TNF-α—100 ng/mL, and IFN-γ—500 ng/mL; 48 h), TG (10 μM, 48 h), and HG (33 mM, 72 h) (Figure 2A). Efficacy of the shRNA plasmids was evaluated by quantification of GFP (Figures S2A and S2B) and target protein expression in the SC-islets four days after transduction (Figure 2B). Because treatment with stressors

(F–H) mRNA expression level of spliced XBP1 (F), *CDKN1A* (G), and *HLA-A* (H) in HIs and SC-islets treated with stress-inducing factors as in (B).

(B–H) n = 4 SC-islets differentiations and n = 2 HI donors, for two independent experiments. Data are means ± SEMs. *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.0001. Ordinary 1-way ANOVA.

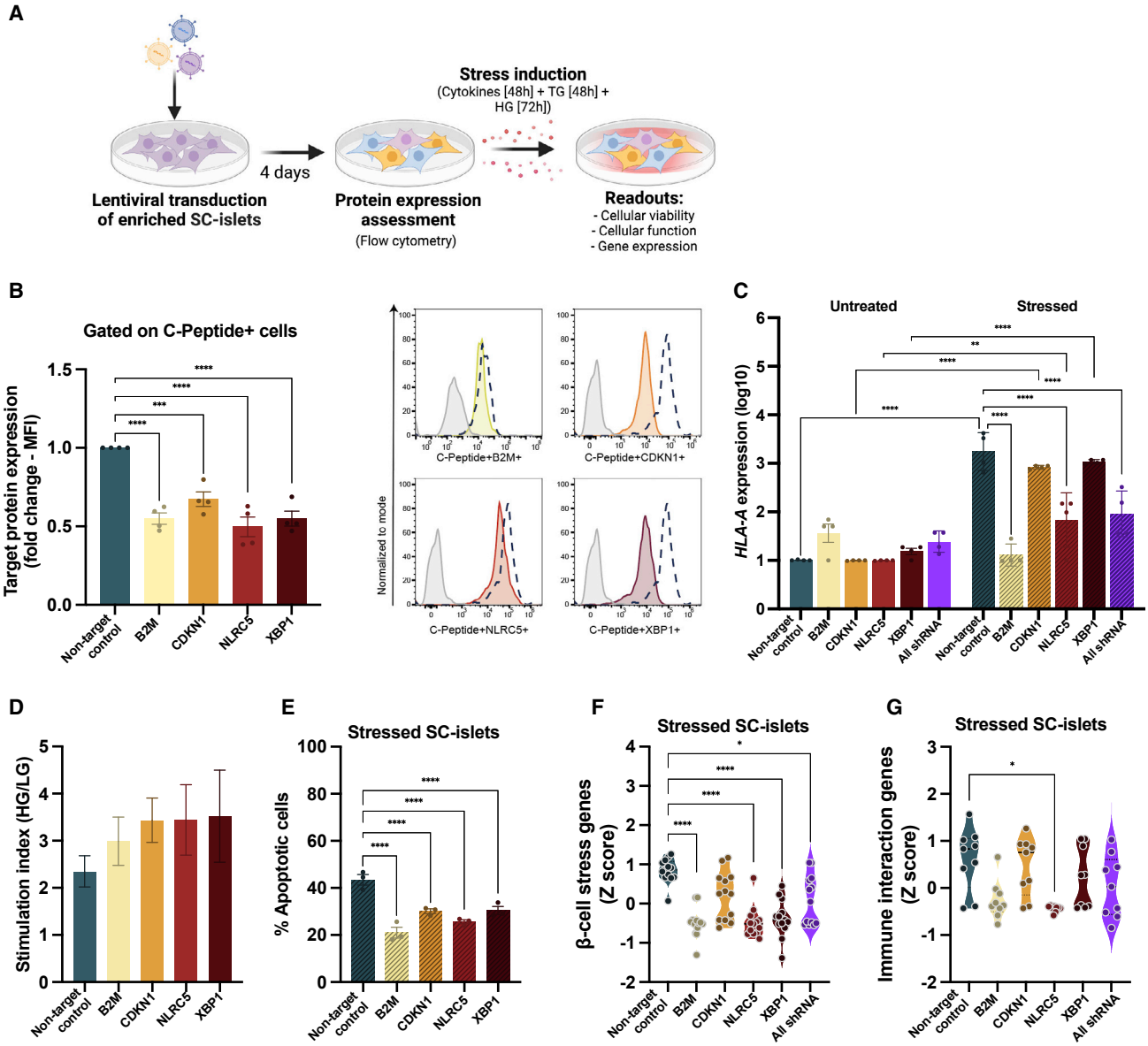


**Figure 2. Genetic manipulation increases protection of SC-islets from stress-mediated apoptosis and dysfunction**

(A) Experimental design: SC-islets were transduced with lentiviral shRNA plasmids individually targeting each gene of interest or targeting all four genes together (all shRNA group). Following transduction, the SC-islets were treated with a combination of stress-inducing factors: cytomix (IL-1β—50 ng/mL, TNF-α—100 ng/mL, and IFN-γ—500 ng/mL; 48 h), TG (10 μM, 48 h), and HG (33 mM, 72 h).

(B) Representative fold-change target protein expression with values represented as adjusted mean fluorescence intensity (MFI). Data are means ± SEMs and representative of four experiments, each with one SC-islet differentiation (n = 4). Representative histogram plots are shown on the right. Dashed line represents the non-target control and solid colored plot represent the gene of interest. Isotype control is shown in gray.

(C) mRNA expression level of *HLA-A* in SC-islets following genetic modification and treated with stress-inducing factors as in (A).

(D) Stimulation index of SC-islets following genetic modification. n = 2 SC-islet differentiations.

(E) Apoptosis in SC-islets following genetic modification and treatment with stress-inducing factors as in (A).

(F and G) Relative mRNA expression of β-cell stress-associated genes (F) and immune-interaction-associated genes (G) in SC-islets stressed as in (A), shown as violin plots. Each dot represents one gene from the heatmap in (S2D). Black dashed lines represent the median.

(C–G) Data are means ± SEMs. n = 3 SC-islet differentiations, for three independent experiments. *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.0001. Ordinary 1-way ANOVA.

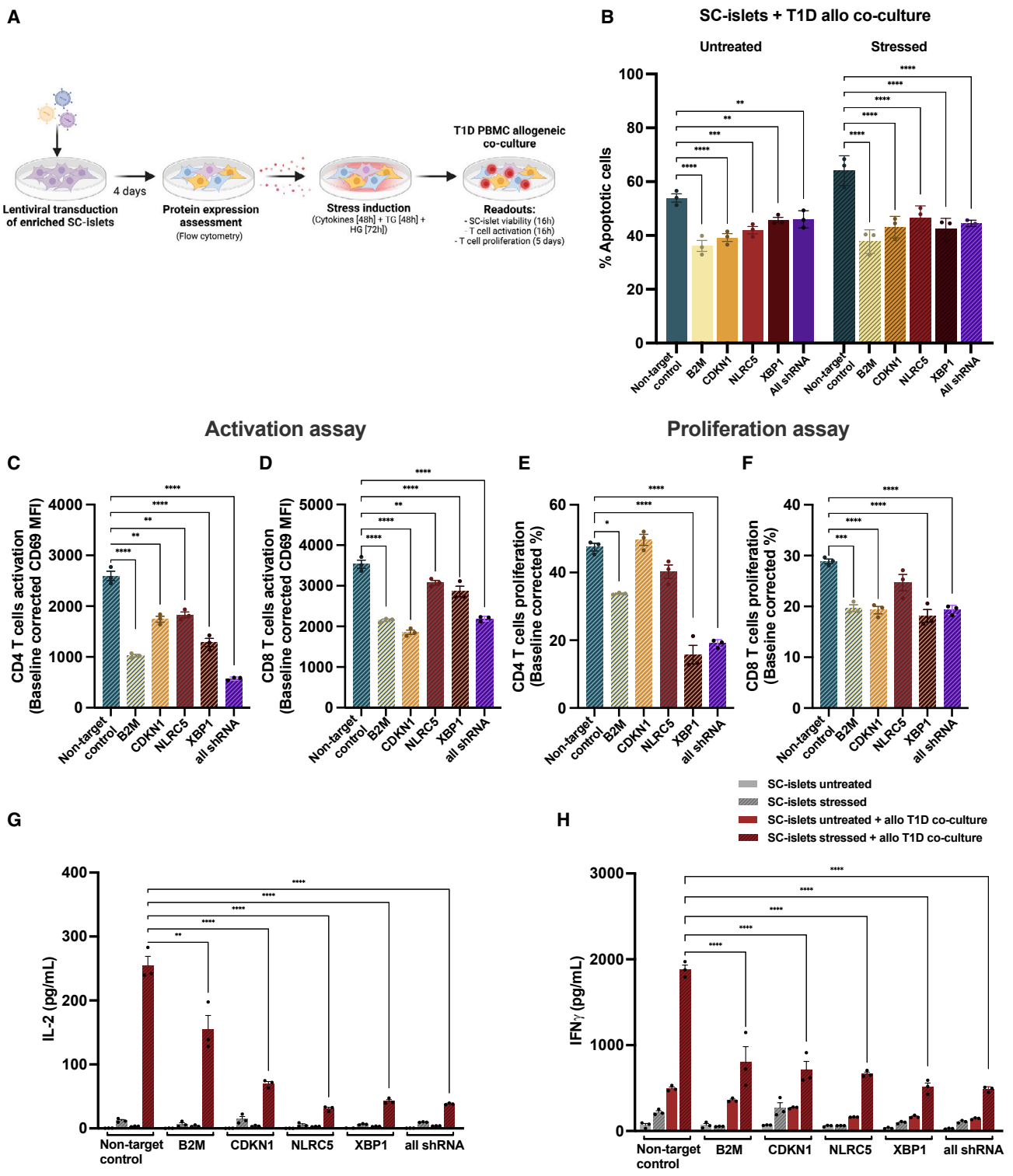


**Figure 3. Genetic manipulation increases protection of SC-islets from apoptosis mediated by allorecognition *in vitro***

(A) Experimental design: following transduction of SC-islets with lentiviral shRNA plasmids individually targeting each gene of interest or targeting all four genes together (all shRNA group) and treatment with stress-inducing factors (cytomix [IL-1β—50 ng/mL, TNF-α—100 ng/mL, and IFN-γ—500 ng/mL; 48 h], TG [10 μM, 48 h], and HG (33 mM, 72 h]), SC-islets were co-cultured with allogeneic T1D PBMCs.

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leads to upregulation of HLA class I in SC-islets (Demine et al., 2020; Leite et al., 2020), following transduction and stress induction of SC-islets, we assessed *HLA-A* expression (Figure 2C). Target protein expression revealed that CDKN1A and *XBP1* shRNAs efficiently reduce individual gene expression in the untreated condition and that *β2M* and *NLRC5* shRNAs inhibit *HLA-A* upregulation upon stressor treatment. Furthermore, genetic modifications did not impair SC-islet function, as measured by a GSIS assay (Figure 2D).

We then evaluated the protective capacity of the genetic modifications against β-cell death as mediated by stress. Reduction in the expression of all target genes resulted in a decrease in the level of apoptotic SC-islets following treatment with stressors compared with the non-target control shRNA (Figures 2E and S2C). Next, we sought to determine whether our genetic modifications prevent aberrant gene expression following induction of stress. Genetically modified, stressed SC-islets exhibited decreased expression of genes related to β-cell stress (Figures 2F and S2D) and immune interaction (Figures 2G and S2D) compared with the non-target control group. In conclusion, knocking down the selected genes enhanced the protection of SC-islets from apoptosis while maintaining SC-islet identity and function.

### Genetic manipulation increases protection of SC-islets from apoptosis mediated by allorecognition *in vitro*

To study the protective capacity of the genetic modifications on apoptosis mediated by stress and allorecognition, following transduction of SC-islets with lentiviral shRNA plasmids individually targeting each gene of interest or targeting all four genes together (all shRNA group) and treatment with stress-inducing factors (cytomix [IL-1β—50 ng/mL, TNF-α—100 ng/mL, and IFN-γ—500 ng/mL; 48 h], TG [10 μM, 48 h], and HG [33 mM, 72 h]), SC-islets were co-cultured with allogeneic T1D peripheral blood mononuclear cells (PBMCs) (Figure 3A). We then assessed SC-islet viability and T cell activation after 16 h and T cell proliferation after 5 days of co-culture. Individually targeting each gene of interest (*β2M*, *CDKN1A*, *NLRC5*, and *XBP1*) or targeting all four genes together (all shRNA group), resulted in a decrease in the level of apoptotic SC-islets compared with the non-target control (Figures 3B and S3A–S3C). No changes were observed in the quantification of T cell subpopulations within PBMCs after co-culture (Figure S3D). Altogether, these data indicate that reduction in the expression of *β2M*, *CDKN1A, NLRC5*, and *XBP1* provides some protection of SC-islets from apoptosis induced by stress and allorecognition.

To further examine the protective capacity of the genetic modifications against β-cell death by immune-mediated killing, immune activation was assessed by surface staining of T cell activation and proliferation markers as well as quantification of proinflammatory cytokines IL-2 and IFN-γ, following 16 h of co-culture of genetically modified SC-islets with allogeneic T1D PBMCs.

In line with prior studies (Leite et al., 2020), co-culturing SC-islets with allogeneic PBMCs resulted in the upregulation of T cell activation marker CD69. However, reduction in the expression of all target genes within stressed SC-islets resulted in a significant decrease in the level of activation marker expression in both $CD4^+$ and $CD8^+$ T cells after co-culture compared with the non-target control (Figures 3C, 3D, and S3E). Following co-culture with SC-islets, the level of proliferation of both $CD4^+$ and $CD8^+$ T cells was measured (Figures 3E and 3F). Reduction in expression of *β2M, XBP1,* and the all shRNA group condition in stressed SC-islets resulted in a decrease in the level of $CD4^+$ and $CD8^+$ T cell proliferation after co-culture compared with the non-target control. As a positive control, PBMCs were stimulated with anti-CD3/CD28 (Figures S3F and S3G). The gating strategy for the co-culture assay is shown in Figure S3H.

Quantification of proinflammatory cytokines revealed a decrease in IL-2 and IFN-γ upon co-culturing PBMCs with SC-islets containing a reduced expression of the target genes individually or a combination of all shRNAs compared with the non-target control (Figures 3G and 3H). Together these results demonstrate that genetic manipulation of SC-islets results in decreased activation and proliferation of $CD4^+$ and $CD8^+$ T cells as well as reduced expression of proinflammatory cytokines, following co-culture with allogeneic T1D PBMCs.

(B) Apoptosis in SC-islets following genetic modification and treatment with stress-inducing factors as in (A) and co-cultured with allogeneic T1D PBMCs for 16 h.

(C and D) T cell activation after 16 h of co-culture of PBMCs with SC-islets treated with stress-inducing factors as in (A). The values are represented as adjusted MFI and are baseline corrected by the average of each untreated condition.

(E and F) T cell proliferation after 5 days of co-culture of PBMCs with SC-islets treated with stress-inducing factors as in (A). The values are baseline corrected by the average of each untreated condition.

(G and H) Proinflammatory cytokine detection in supernatants collected after 16 h of co-culture of T1D PBMCs with SC-islets treated with stress-inducing factors as in (A).

(B–H) Data are means ± SEMs and representative of three experiments, each with one SC-islet differentiation (n = 3). *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, and ****$p < 0.0001$. Ordinary 1-way ANOVA.

## DISCUSSION

For decades, T1D has been viewed primarily as a T-cell-mediated autoimmune disease in which autoreactive T cells mistakenly destroy healthy, insulin-producing β cells. However, evidence accumulated over the past 15 years points to a role for the β cell as a contributor to the disease rather than an innocent bystander (Eizirik et al., 2020). Although the relative contributions of β-cell pathophysiology and autoimmunity remain unknown, β-cell stress has been shown to play an important role in T1D development. Prior studies on the exposure of HIs to stressors, such as chronic hyperglycemia and proinflammatory cytokines, have been extensively investigated (Brozzi and Eizirik, 2016; Dai et al., 2016; Abdullahi et al., 2017), but the effects of stress on SC-islets function and viability have not been thoroughly explored. As stress-inducing factors are likely to be present following transplantation, an initial understanding of the consequences of stress on SC-islets motivated the present study.

As had been shown with HIs, stress induction of SC-islets causes increased apoptosis, irregular GSIS, and heightened expression of stress and immune-interaction-related genes. The heightened expression of immune-interaction- and β-cell-stress-related genes upon stress induction in both HIs and SC-islets follows on prior studies that were performed on HIs exposed to proinflammatory cytokines *in vitro* (Eizirik et al., 2009) and studies that found stress-impaired transcription factor expression and insulin secretion in transplanted HIs (Dai et al., 2016). Furthermore, studies on induced pluripotent stem cell (iPSC)-islets showed that cytokine treatment induced a proinflammatory phenotype and stress (Demine et al., 2020). We see a similar pattern in our results in which untreated SC-islets express low levels of genes related to immune interaction and, following stress induction, increase the expression of HLA-ABC chemokines, such as CXCL8 and -10, and β-cell stress genes, *XBP1* and *Caspase 3*. However, compared with HIs, SC-islets have reduced apoptotic cells and expression of genes related to peptide presentation and cytokine signaling in the treated conditions. These results support the idea that SC-islets may be less vulnerable to apoptosis mediated by cellular stress than HIs, although one cannot draw a strong conclusion from this *in vitro* assay.

Downregulation of the target genes with shRNAs was associated with protection of SC-islets from apoptosis mediated by stress while not altering the function of the cells. In addition, we observed unexpected results, namely the same level of protection from all target genes combined compared with targeting a single gene. This outcome demonstrates that protection against apoptosis is limited by methods of gene modification, such as shRNA-mediated gene silencing. Further examination of the protective capacities of the genetic modifications against immune-mediated β-cell death was determined through quantification of immune activation, proliferation, and SC-islets apoptosis. In line with previous studies (Leite et al., 2020), co-culturing stressed SC-islets with allogeneic PBMCs resulted in the upregulation of T cell activation and proliferation compared with co-culture with unstressed SC-islets. Reduction in the expression of target genes within stressed SC-islets, however, resulted in decreased levels of T cell activation and proliferation after co-culture compared with the non-target control. It is important to note that in these studies we quantified activation through the presence of only one activation marker (CD69) and one time point; therefore, it is possible that quantifying other activation markers that act through different pathways, at multiple time points, may lead to different levels of protection. Altogether, these results demonstrate that reduction in the expression of genes related to immune recognition and β-cell stress confers a modest level of protection of SC-islets from apoptosis mediated by stress and allorecognition *in vitro* and reduces the expression of other genes related to stress and immune recognition.

The findings of this research should be interpreted considering three significant limitations. First, we did not want to interfere with the homeostasis of β cells by deleting genes (Zhang et al., 2020; Bilekova et al., 2021) and instead performed a gene knockdown instead of knockout strategy. This approach would be strengthened by using techniques in which the expression of specific genes that do not interfere with β-cell homeostasis is permanently prevented. Second, we selected the target genes based on prior findings in the literature. An unbiased screening approach focusing on β-cell protection might provide novel targets that may have a more protective outcome. And third, we observed modest effects with our approach, and the pooled gene assays did not confer greater protection. To have complete protection of SC-islets, a different gene combination or approach might be necessary.

This is an initial study of a difficult problem. One genetic change is unlikely to solve the problem of protection from stress or immune rejection. The data presented here begin the understanding of the effects of stress on SC-islets function and viability *in vitro*. This information is a step forward toward the possibility of using SC-islets in long-term transplantation therapies without the need for lifelong and general immunosuppression.

## EXPERIMENTAL PROCEDURES

An expanded section is available in the supplemental experimental procedures. All procedures were performed in accordance with the IRB guidelines at Harvard University under IRB and ESCRO protocols.

### Cell culture

Human pluripotent stem cell maintenance and differentiation were carried out with Harvard University Embryonic Stem Cells 8 (HUES8), as previously described (Veres et al., 2019).

### Magnetic enrichment using CD49a

Following SC-islets differentiation, the β-cell population was enriched using magnetic sorting, as previously described (Leite et al., 2020).

### Apoptosis assay

SC-islets were plated at a density of ~150,000 cells per well on 96-well round bottom plates and treated with IFN-γ, IL-1β, TNF-α, and TG for 48 h, and HG for 72 h. Following treatment, and co-culture in the case of the immune-protection assays, cells were washed to remove residual cytokines, TG, and glucose. Apoptosis was determined by staining with Annexin/SYTOX.

### Glucose-stimulated insulin secretion assay

GSIS assay was performed as previously described (Blum et al., 2012).

### T cell activation and proliferation assays

Transduced SC-islets were used as target cells. Approximately 200,000 target cells were plated on 96-well round bottom plates and treated with IFN-γ, IL-1β, TNF-α, and TG for 48 h and HG for 72 h. PBMCs (200,000 per well) were then added to SC-islets, with and without pre-treatment. After 16 h of co-culture, we analyzed T cell activation and, after 5 days of co-culture, T cell proliferation.

Antibodies used in this study can be found in Table S1.

### Quantification and statistical analysis

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

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.01.018.

## AUTHOR CONTRIBUTIONS

N.C.L. designed and conceived the study. Experiments were performed by N.C.L. and G.C.P. D.A.M gave technical support and conceptual advice and supervised the research.

## CONFLICTS OF INTERESTS

D.A.M. is a founder of Semma Therapeutics and advisor to Vertex Pharmaceuticals, which have licensed technologies developed in the Melton laboratory. All other authors declare no competing interests.


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**Stem Cell Reports, Volume *17***

## Supplemental Information

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

**Nayara C. Leite, Gabriela C. Pelayo, and Douglas A. Melton**

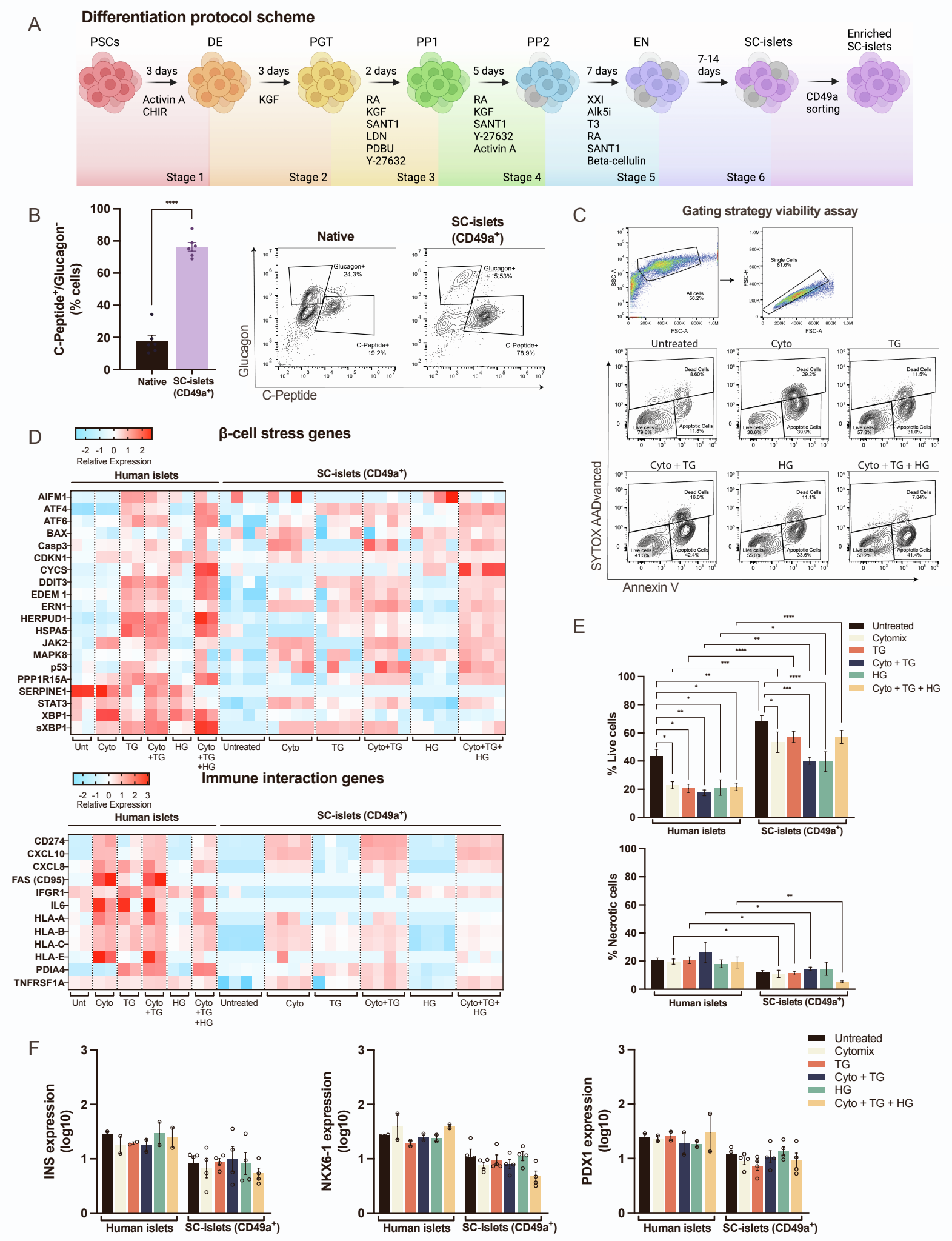
A
Differentiation protocol scheme
PSCs
DE
PGT
PP1
PP2
EN
SC-islets
Enriched SC-islets
3 days
Activin A
CHIR
3 days
KGF
2 days
RA
KGF
SANT1
LDN
PDBU
Y-27632
5 days
RA
KGF
SANT1
Y-27632
Activin A
7 days
XXI
Alk5i
T3
RA
SANT1
Beta-cellulin
7-14 days
CD49a sorting
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
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).

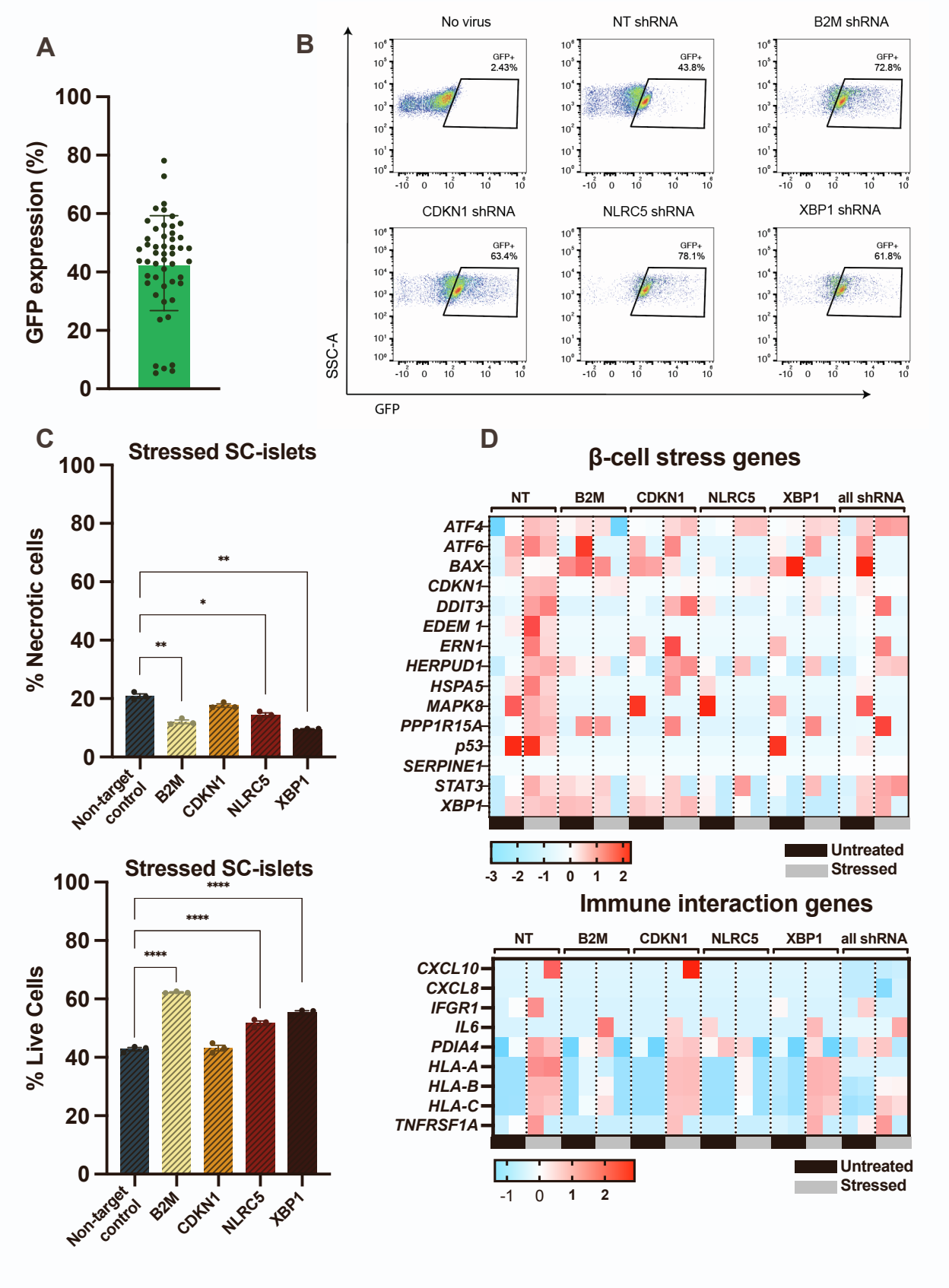
A
GFP expression (%)
100
80
60
40
20
0
B
No virus
NT shRNA
B2M shRNA
CDKN1 shRNA
NLRC5 shRNA
XBP1 shRNA
GFP+
2.43%
GFP+
43.8%
GFP+
72.8%
GFP+
63.4%
GFP+
78.1%
GFP+
61.8%
SSC-A
GFP
C
Stressed SC-islets
% Necrotic cells
**
*
**
Non-target control
B2M
CDKN1
NLRC5
XBP1
Stressed SC-islets
% Live Cells
****
****
****
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.

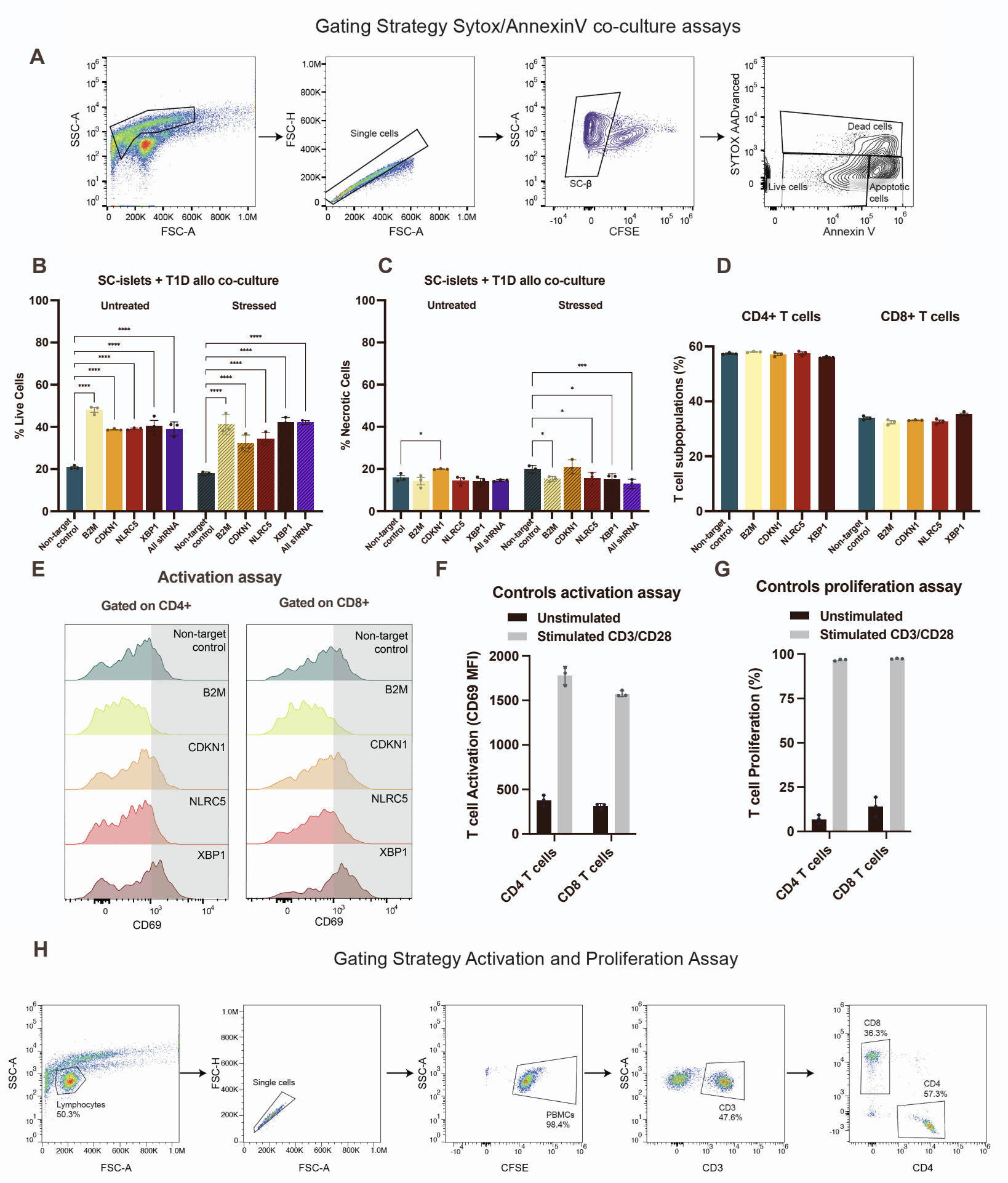
Gating Strategy Sytox/AnnexinV co-culture assays
A
SSC-A
FSC-A
FSC-H
Single cells
SC-β
CFSE
SYTOX AADvanced
Dead cells
Live cells
Apoptotic cells
Annexin V
B
SC-islets + T1D allo co-culture
Untreated
Stressed
% Live Cells
C
% Necrotic Cells
D
CD4+ T cells
CD8+ T cells
T cell subpopulations (%)
Non-target 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.