Whole-genome CRISPR screening identifies genetic manipulations to reduce immune rejection of stem cell-derived islets

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SUMMARY

Human embryonic stem cells (hESCs) provide opportunities for cell replacement therapy of insulin-dependent diabetes. Therapeutic quantities of human stem cell-derived islets (SC-islets) can be produced by directed differentiation. However, preventing allo-rejection and recurring autoimmunity, without the use of encapsulation or systemic immunosuppressants, remains a challenge. An attractive approach is to transplant SC-islets, genetically modified to reduce the impact of immune rejection. To determine the underlying forces that drive immunogenicity of SC-islets in inflammatory environments, we performed single-cell RNA sequencing (scRNA-seq) and whole-genome CRISPR screen of SC-islets under immune interaction with allogeneic peripheral blood mononuclear cells (PBMCs). Data analysis points to “alarmed” populations of SC-islets that upregulate genes in the interferon (IFN) pathway. The CRISPR screen in vivo confirms that targeting IFNγ-induced mediators has beneficial effects on SC-islet survival under immune attack. Manipulating the IFN response by depleting chemokine ligand 10 (CXCL10) in SC-islet grafts confers improved survival against allo-rejection compared with wild-type grafts in humanized mice. These results offer insights into the nature of immune destruction of SC-islets during allogeneic responses and provide targets for gene editing.

INTRODUCTION

Nearly 100 years ago the first type 1 diabetes (T1D) patient was treated with a “pancreatic extract,” which led to the discovery of insulin (Banting et al., 1922). Since then, the basis of T1D has been shown to be an autoimmune elimination of pancreatic insulin-producing β cells. While acknowledging the impressive technological advances to manage T1D (Kovatchev, 2019), exogenous insulin administration with regular monitoring remains the primary treatment for T1D. In parallel, cadaveric islet or pancreas transplants (Shapiro et al., 2000), have proved to be effective in controlling blood glucose levels, but this treatment is limited by the lack of a consistent and readily available supply of organs/islets and the requirement for systemic immunosuppressants (Shapiro et al., 2017). The prospect of using human pluripotent stem cells (hPSCs) as an unlimited source for β cell differentiation and replacement has been advanced by developing methods to differentiate human stem cells into functional human islets (Helman and Melton, 2021; Nostro et al., 2015; Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015). The first reports of human clinical trials using progenitor cells (Ramzy et al., 2021) or fully differentiated and functional SC-islets (Businesswire, 2021) speak directly to this possibility.

In the light of these encouraging, albeit initial, clinical reports, a major challenge remains of protecting SC-islets from an immune response. The use of immunosuppressants can lead to complications as well as graft impairment in the long term (Lehmann et al., 2008). Encapsulation methods can provide immune protection and graft extraction advantage, but have not yet been determined to be effective (Henry et al., 2018).

Beyond encapsulation, efforts to modify the patient’s immune system have been pursued to blunt or modify the immune response. This includes the use of antibodies to block co-stimulation and amplifying regulatory T cells (Herold et al., 2019; Orban et al., 2011; Raffin et al., 2020). Complementing this approach is genetic modification of the target itself, the SC-islets, to make them opaque or less immunogenic. Strategies include β-2-microglobulin (B2M) or human leukocyte antigen (HLA)-I/II depletions (Castro-Gutierrez et al., 2021; Deuse et al., 2019; Han et al., 2019; Parent et al., 2021; Wang et al., 2015) to prevent donor antigen presentation to T cells, and expression of immune check point inhibitors such as programmed death-ligand 1 (PD-L1) (Castro-Gutierrez et al., 2021; Harding et al., 2019; Yoshihara et al., 2020). Other approaches include expression of CD47 (Deuse et al., 2019, 2021) and HLA-E (Gornalusse et al., 2017) to reduce natural killer (NK) killing when HLA-A, -B, and -C are absent. Another variation is to remove HLA-A and HLA-B but retain one HLA-C allele, requiring only a small number of compatible lines to cover most of recipient populations across the world (Au et al., 2019). All these promising strategies derive from previous knowledge and studies in other contexts; e.g., maternal-fetal immune
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interactions and the ability of cancer cells to avoid immune elimination. Of note, there are few reports of endocrine cell-related targets for immune modulation of β cell survival and function (Cai et al., 2020; Wei et al., 2018).

Here we pursue a complementary approach by first defining the immune interaction with SC-islets, studying the interaction between the human alloimmune immune system and SC-islets with a focus on the transcriptional responses. Using single-cell RNA sequencing (scRNA-seq) and whole-genome CRISPR screening, we find that the JAK/STAT type II interferon (IFN) pathway is a leading modulator of early and late inflammatory response events both in vitro and in vivo. While manipulating the upstream and central mediators of the JAK/STAT pathway provides reduction of SC-islet immunogenicity, the findings indicate that a practical and promising approach is to target downstream components, specifically by depleting the chemokine ligand 10 (CXCL10).

RESULTS

Single-cell transcriptional analysis reveals “alarm” genes that drive immunogenicity of SC-islets

To study immune responses in the context of human alloimmune graft rejection, we chose the Hu-PBL-NSG-MHC<sup>+</sup> class I and II, were transplanted (under the kidney capsule, n = 12) with 5M (Million) SC-islets (HLA-A2 positive), followed by human PBMC injection (termed hPi-mice; 50M/mouse, n = 6) from healthy unmatched donors (HLA-A2 negative). The lack of murine MHC allowed us to monitor the graft function for prolonged durations without the risk of xenogeneic graft-versus-host disease (GVHD). Half of the SC-islet transplanted cohort (n = 6 mice) was used as the control, without PBMC injection (Figure 1A). Graft function failure was determined by human insulin detection in fasting mouse blood 30 min after glucose injection (Figure 1B). Reduction in graft size (Figures S1A and hPi grafts) compared with controls (Figure S1D), as expected for an allogeneic response.

Since graft elimination by PBMCs is incomplete and residual endocrine cells remain in the hPi-mice grafts, we were able to retrieve the SC-islet grafts for single-cell RNA sequencing (scRNA-seq) analysis (Augspornworawat et al., 2020). These samples were used for 10x Genomics mRNA expression library preparation and Illumina sequencing. Datasets were integrated from multiple graft and cell samples (see section “experimental procedures”). As seen in Uniform Manifold Approximation and Projection (UMAP) plots (Figures S1G and S1H), grafted endocrine cells (SC-Endo) from control and hPi-mice maintain their cell identity based on gene markers for SC-α (INS–GCG+),

Figure 1. Single-cell transcriptional profile and whole-genome CRISPR screen of SC-islet grafts in an in vivo humanized model
(A) SC-islets or CRISPR library transduced (LT) SC-islets were transplanted in MHC<sup>+</sup> NSG mice. Half of each mice cohort was injected with human PBMCs, and human insulin was monitored until graft failure was observed. Grafted cells were then extracted (week 10 post PBMCs) and analyzed by scRNA-seq for gene expression, or by qDNA sequencing for gRNA abundance.
(B) n = 6–8 per group of SC-islet transplanted mice.
(C) n = 6 per group of LT SC-islet transplanted mice.
(D) Immunofluorescence (IF) staining of kidney SC-islet grafts sections at week 10 after PBMC injection. Bars represent 100 μm in left (×5) and center (×20) and 20 μm in magnified view (right). Kidney (K) and graft (G) margins are outlined. CHGA, chromogranin A.
(E and F) scRNA-seq analysis of SC-islet grafts.
(G) Analysis of enriched and depleted gene KOs. Rank is plotted against fold changes (hPi versus control) of gRNA counts (×4 integrated per gene) relative to integrated non-targeting (NT) gRNA counts (×941). Significant genes are color coded based on false discovery rate (FDR) as indicated.
(H) Boxplot presenting individual gRNAs counts (full model predictions) from mice replicates (n = 6 per condition times n = 4 targeting gRNAs, n = 85 for NT gRNAs, or n = 50 for intergenic gRNAs) with genes of interest with positive and negative enrichment in screen. Box lines represent median values. Dashed line represents mean of NT gRNA counts in control mice. Error bars or shaded areas are mean ± SD; ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, unpaired two-tailed t test.
SC-β (INS+GCG−), and SC-enterochromaffin cells (SC-EC; TPH1+). hPi grafts had fewer endocrine cells (Figure S1I) compared with controls (~50% reduction), consistent with flow cytometry staining (Figure S1D).

Single-cell technology allows a focus on specific cells populations within heterogeneous SC-islets (Figures S1G–S1I). SC-α, SC-β, and SC-EC exhibited similar patterns of upregulated genes in PBMC infiltrated grafts (Figures 1E, S1J, and Data S1). This suggests that the response in this model system is not specific to a cell population within SC-islets and all transplanted cells are immunogenic.

Among the most upregulated genes are transcripts involved in antigen presentation (B2M; HLA-A, -B, -C, -F; TAP1/2; CD74; PSMB9), inflammatory pathway mediators (STAT1, JAK1/2, IRF1/2) and pro-inflammatory cytokines, including IL32. These genes induce T cell activation and inflammation. In addition, genes that are inhibitory to the immune system are upregulated; e.g. HLA-E, SOCS1, CD274 (PD-L1), and WARS. Upregulation of these genes suggests an induction of IFN type I (IFNα/β) and II (IFNγ) pathways, through JAK/STAT signaling (Platanias, 2005) (Figures 1E, 1F, S1J, and S1K). A key IFN type II upstream component, the IFNγ receptor gene IFNGR1, does not appear to change in hPi-mouse grafts compared with controls (Figure 1F). Pathway analyses confirm the SC-islet response as IFN-driven, one that alarms the immune system through antigen presentation and that can lead to apoptosis of target cells (Tables S1–S3).

Whole-genome CRISPR screen confirms the role of IFN response genes that set the fate for SC-islet survival

Transcriptional responses of SC-islets during the immune interaction described above provide clues to genes that could be manipulated to dampen immune recognition. However, changes in expression per se might represent a pro- or anti-stimulatory response or no effect. To explore this issue, a whole-genome screen using a CRISPR lentivirus library (Doench et al., 2016) was performed.

The Brunello CRISPR library consists of a pool of 76,441 human targeting guide RNAs (gRNAs) and 1,000 control gRNAs (non-targeting [NT] or intergenic) in a lentiviral vector that expresses Cas9. The pooled library targets 19,114 human genes, most of them by four gRNAs per gene. To avoid multiple different gRNAs in cells and a nonspecific effect on the screen results (Doench, 2018), a low infection lentivirus titer (MOI < 1) was used. Library transduced cells (LT SC-islets) were allowed at least 10 days for CRISPR editing, before transplantation to the NSG-MHCnull mouse model, where PBMCs were injected to half of the cohort (hPi-mice, n = 6; control mice, n = 6) (Figure 1A). hPi-mice retained levels of circulating T cells throughout the experiment (Figure S1E). Graft function and subsequent failure due to human PBMC injection was assessed (Figures 1C and S1F). When hPi graft failure was confirmed, 10 weeks after PBMC injection (Figure 1C), both control and hPi grafts were recovered from kidney sites, genomic DNA (gDNA) was extracted, and gRNA regions were amplified by PCR for Illumina sequencing.

The response to PBMCs (graft infiltration) was assessed by gRNA counts from hPi LT SC-islet grafts compared with control LT SC-islet grafts, in relation to NT control gRNA counts in the two environments (see section “experimental procedures”). Essential/housekeeping genes are not evaluated because their gRNA transduced cells will have been eliminated shortly after lentiviral infection. This analysis identifies genes that increase or decrease the chance of transplanted SC-islets survival following PBMC injection (Figure 1G). Approximately 12,000 genes that are expressed in SC-islets (by scRNA-seq datasets) were ranked based on enrichment/depletion following PBMC injection. Results show reduction in total and control (NT or intergenic) gRNA reads in all hPi grafts compared with control, confirming cell elimination and graft rejection (Figures 1H left and S1M). Knockout (KO) perturbations that increase survival are positively enriched in hPi (positive values in Figure 1G) and eliminate the difference in gRNA counts between conditions (Figure 1H center). KO perturbations that decrease survival are depleted in hPi (negative values in Figure 1G) and intensify the difference in gRNA counts between conditions (Figure 1H right). We interpret hPi-enriched gene KOs as pro-survival (tolerizing) under immune attack, whereas the opposite occurs with hPi-depleted genes.

Consistent with expressed transcripts (Figures 1E and 1F), the results point to JAK/STAT signaling for antigen processing/presentation and chemokine secretion. Most prominent were the enrichments of B2M, HLA-A, TAP1/2, STAT1, JAK1/2, and CXCL10 gRNAs in LT SC-islet hPi grafts (Figure 1G). KOs of these genes contribute to survival in hPi (Figure 1H).

The observed protective effect of HLA-I KOs is consistent with previous reports (Castro-Gutierrez et al., 2021; Deuse et al., 2019; Han et al., 2019; Parent et al., 2021; Wang et al., 2015). TAPI and TAP2 gRNA enrichments in hPi suggest that immune protection could also be gained by disrupting transport of cytosolic peptides to HLA class I molecules (Scholz and Tampe, 2005).

Interestingly, one of the top hPi-enriched gene perturbations in this screen was for CXCL10 (IP10), an IFN-induced chemokine. Chemokine signaling plays an important role in immune cell recruitment to an inflamed tissue. Other chemokine gRNAs that are hPi-enriched include CXCL5 and CXCL9. CXCL9 is also an IFN-stimulated gene (ISG) that binds the CXCR3 receptor. CXCL5 is known to have chemotactic and activating functions on neutrophils (Chang et al., 1994).
Apart from the canonical mediators of the IFN pathway (STAT1 and JAK1/2), other notable hPi-enriched perturbations are HNRNPAT1P48, GBP2, and TRIM8. hnrNP proteins are involved RNA processing and splicing (Clarke et al., 2021). GBP2 is an IFNγ-induced GTPase involved in protective immunity against microorganisms (Tretina et al., 2019) and is also a marker for an efficient T cell response in breast carcinomas (Godoy et al., 2014). TRIM8 is a RING finger protein that inhibits the JAK/STAT suppressor SOCS1 (Toniato et al., 2002), and therefore might act as a IFNγ pathway inducer.

The bottom of Figure 1G shows gene hits that are beneficial to graft survival under immune infiltration of PBMCs. Artificially expressing these genes may help slow or prevent immune destruction. One example is PTPRA, a negative regulator of JAK/STAT signaling (Gurzov et al., 2015; Stanley et al., 2015). The difference of PTPRA gRNA counts between hPi and control graft is larger than that observed in NT gRNAs, emphasizing the essentiality of PTPRA for graft survival (Figure 1H). Another tyrosine phosphatase, PTPN2, is a T1D risk gene (Barrett et al., 2009; Espino-Paisan et al., 2011) but was ranked lower as a beneficial gene in our screen (Figure 1H). In addition, suppressor of cytokine signaling 1 (SOCS1), also a negative regulator of JAK/STAT (Gallic et al., 2014; Solomon et al., 2011), was upregulated in our scRNA-seq data (Figures 1E and 1F) and exhibited potency as a tolerizing gene (Figure 1H). Other examples that showed a protective effect include small ubiquitin-like modifier 1 (SUMO1), which inhibits STAT1 (Rogers et al., 2003), and the tolerizing surface molecule PD-L1 (CD274) (Castro-Gutierrez et al., 2021; Yoshiihara et al., 2020), IL32, ICAM1, and PRDX1 are known to be pro-inflammatory in other systems (Min et al., 2018; Ribeiro-Dias et al., 2017; Yonekawa and Harlan, 2005) and it is unclear why their gRNAs were hPi depleted.

**SC-islets are responsible for early-stage immune cell activation through alarm genes**

To compensate for limitations of the hPi-mouse model (Shultz et al., 2019) and for unassessed early events (grafts that are retrieved at week 10), we performed an *in vitro* co-culture of allogeneic PBMCs and SC-islet clusters. SC-islet clusters were enriched for β cells (using CD49A magnetic sorting; SC-α and SC-EC still remain at lower numbers) (Veres et al., 2019), dissociated and reaggregated to obtain a more uniform cell count between wells. SC-islets were co-cultured with human allogeneic PBMCs for 24 or 48 h. As controls (time \(t = 0\)), SC-islets remained in culture without PBMC addition. These samples, in addition to PBMCs alone (\(t = 0\)), were used for scRNA-seq (Figure 2A). Prior to co-culture, all SC-islets (controls included) were treated with thapsigargin to enhance and accelerate T cell activation by inducing an ER stress response that was previously shown to recapitulate aspects of autoimmunity (Leite et al., 2020). Differential expression analysis of integrated data from all samples focused on cell populations of interest (Figures S2A–S2C).

CD4, CD8 T cells, and NK cells, at 24- and 48-h co-culture with SC-islets, displayed gene expression profiles of immune activation compared with control (Figure S2D; Tables S1–S3). Transcripts for T cell co-stimulation molecules (including CD28, CD58 [LFA-3], CD40LG, TNFRSF9 [4-1BB], TNFRSF4 [OX40]) and other activation markers (IL2RA [CD25], CD38) are upregulated in T cells as well as inhibitory and exhaustion markers (HAVCR2 [TIM-3], Lag3, PDCD1 [PD-1]) (Figure S2D top). Co-inflammatory cytokines (IFNG and TNF) and chemokines (XCL1/2) are expressed over time in NK and T cells, while anti-inflammatory cytokines (IL10 and TGFβ1) are either undetected or downregulated. T cells and NK sensitization to pro-inflammatory chemokines was increased based on elevated levels.
of CXCR3, a chemokine receptor that binds CXCL9/10/11 (Figure S2D center). Other prominent transcripts are those that play a part in CTL (cytotoxic T lymphocyte) and NK killing functions (Figure S2D bottom: PRF1, GZMB, FASLG), further indications of an allogeneic response in this co-culture system.

We focused on gene expression in SC-α and SC-β cells compared with controls without PBMC addition. Similar to what was observed for the in vivo analysis (Figure 1), upregulated profiles did not differ between co-cultured SC-α and SC-β (Figure 2B) and consisted of clear IFN responses through the JAK/STAT pathway with implications for T cell activation (B2M, HLA-I genes), inflammation (e.g., NFκB1/2), apoptosis signaling (FAS, CASP3), and allo-rejection (Figures 2C, 2D, S2E and Tables S1–S3; pathway analysis, and gene set enrichment analysis (GSEA)).

The in vitro and in vivo experiments described (Figures 1 and 2) point to the conclusion that JAK/STAT signaling in SC-islets is a direct and early consequence of IFN signals received from PBMCs. The unbiased whole-genome screening provides further confirmation of IFN signaling as a critical signaling cascade. We compare readouts from screening provides further confirmation of IFN signaling. In vitro experiments described in the in vitro experiments described, CXCL10 overexpression in SC-islets, compared with NT (Figure S3B). Reduced CXCL10 secretion in CXCL10 KO co-culture with SC-islet reduced T cell activation (CD25 b). Further evidence for the pathway importance in SC-islet immunogenicity comes from co-culture and external IFN stimuli, wherein STAT1 is phosphorylated and translocated to the nuclei of SC-islet cells, and transcription of IFN response elements are induced (Moore et al., 2011) (Figures 2G, 2I, S2G, and S2H).

CXCL10 affects SC-islet immunogenicity

To assess CXCL10 as a target for genetic manipulation compared with other known tolerizing perturbations (β2M KO and PD-L1 overexpression), we co-cultured human allogeneic PBMCs with SC-islets that had been transduced with lentivirus vectors (Figure 3A). For gene KO, vectors expressed Cas9 and gRNAs to CXCL10 and B2M. Overexpression (OE) vectors expressed either CXCL10 or PD-L1 (CD274). All perturbations of target protein expression were assessed compared with NT gRNA or eGFP OE under IFNγ stimuli (Figure S3A). At 48 h after co-culture, SC-islets were stained for apoptotic markers with the focus on SC-β viability (C-peptide staining) (Figure 3B). CXCL10 and β2M deletions improved viability of SC-β under immune attack by PBMCs (Figure 3B) by more than 2-fold. In addition, a destructive effect of CXCL10 overexpression in SC-β cells under immune attack can be seen by the 50% increase of apoptosis in SC-β over-expressing CXCL10, compared with eGFP overexpression (and comparable with PD-L1) (Figure 3B). PBMCs, pre-labeled with cell trace violet to measure proliferation rates, showed reduced T cell proliferation when co-cultured with CXCL10-depleted SC-islets, compared with NT (Figure S3B). Reduced CXCL10 secretion in CXCL10 KO co-cultures was observed (Figure S3C).

CXCR3 is a chemokine receptor expressed on T helper cells, CD8 T cells, NK cells, and monocytes that react with IFN-inducible chemokines, CXCL9/10/11. CXCR3 has a role in chemotaxis and cell proliferation signals (Loetscher et al., 1996) and can also influence T cell polarization to a specific effector lineage (Wildbaum et al., 2002). To evaluate the CXCL10-CXCR3 interaction in SC-islet immunogenicity, PBMC and SC-islet co-culture experiments were performed with a blocking antibody to CXCR3 (Figure 3C). Anti-CXCR3 Ab treatment prior to co-culture with SC-islet reduced T cell activation (CD25...
and CD69 activation marker staining), proliferation and the subsequent SC-β apoptotic effect (Figures 3D, 3E, and S3D). An anti-CXCL10 neutralizing antibody added during co-culture also improved SC-β viability (Figure 3D). Since CXCL10 is thought to induce apoptosis through binding to Toll-like receptor 4 (TLR4) in β cells (Schulthess et al., 2009), we treated SC-islets (pre-co-culture) with a TLR4 blocking antibody, which did not significantly reduce apoptosis in this assay (Figure 3D). Overall, these results point to T cell-mediated SC-islet killing through CXCR3 induction, led by CXCL10.

**Immunogenicity of CXCL10 and STAT1 KO hESC lines assessed in vitro**

In the light of aforementioned results, two Hues8 hESCs CRISPR KO lines, CXCL10 KO and STAT1 KO, were generated with the rationale of diminishing IFN signaling through a master regulator (STAT1) or by confining the effect to one downstream mediator (CXCL10).

Null mutations were created for CXCL10-GFP (C10G) and STAT1-luciferase (ST1L) lines by homology directed repair (HDR) (see section “experimental procedures;” Figures 4A, 4B, and S4A). KO lines displayed normal karyotypes (Figure S4C). These KO lines were compared with a wild-type (WT) Hues8 line or a luciferase expressing Hues8 line (GAPDH-luciferase [GL]; (Gerace et al., 2021)) as controls. C10G, ST1L, and control lines were differentiated successfully into SC-islets (Pagliuca et al., 2014; Veres et al., 2019) and exhibited glucose-stimulated insulin secretion (GSIS) in transplanted mice (Figures S4D–S4F).

C10G SC-islets had very low levels of intracellular CXCL10 staining and almost undetectable CXCL10 secretion after IFNγ stimulation (figures 4C and 4D). IFNγ
treatment of GL SC-islets induced phosphorylated STAT1 that was impaired in ST1L SC-islets (Figure 4E). The absence of STAT1 in ST1L also led to desensitization to IFNγ, as shown by the downregulation of HLA proteins and CXCL10 as well as inhibitory proteins (HLA-E, PD-L1, and SOCS1) (Figures 4D and 4E).

Gene-modified (GM) and control SC-islets were co-cultured with allogeneic PBMCs. To evaluate the contribution of specific immune populations on SC-islet killing, we also co-cultured GM SC-islets with blood purified T cells (C10G only) and NK cells (Figure 4F). Compared with WT, C10G co-cultures displayed significant protective...
performances against allo-PBMCs, T cells, and NK cells based on improved SC-β (Figure 4G) and SC-islet (Figure S4G) viability and reduced activation and proliferation of T cells in co-cultured PBMCs. In contrast, ST1L did not significantly reduce the response to PBMCs, and more SC-islets were apoptotic after NK cell co-culture (Figures 4Ha and S4G). T cells from ST1L and GL control SC-islets, co-cultured with PBMCs, show the same level of activation and proliferation (Figures 4Ja and S4I). Diminished inhibitory signals such as PD-L1 and SOCS1 (Figure 4E) may explain why ST1L does not reduce the immune response to PBMCs, and the reduced expression of HLA class I may be the cause for increased NK killing (Figures 4Ha and S4G).

**CXCL10-deficient SC-islets are hypoimmunogenic in vivo**

Since full STAT1 depletion (ST1L) shows unimpressive results in reducing the immune response in vitro (Figures 4H and 4J), we focused on C10G for in vivo studies.

Using the in vivo model (Figure 1), C10G or WT SC-islets were transplanted (n = 20), followed by PBMC injection (hPi) from two human donors, leaving three mice in each group without PBMC injection as controls (Figure 5A). Beginning at week 11 after PBMC injection, graft failure was observed in hPi-mice transplanted with WT SC-islets, continuing through week 17, whereas WT control grafts remained functional. Interestingly, C10G SC-islet graft insulin levels remained stable and even increased over time, with no significant difference between hPi and control mice (Figure 5B). At the end of the experiment (week 17 post PBMC), kidney capsule grafts were extracted and stained for endocrine and T cell markers. Consistent with insulin measurements (Figure 5B), we observed a decline in the number of SC-β (and SC-α) in WT hPi grafts, but not in C10G hPi grafts compared with controls (Figure 5C). The improved survival of SC-islets can be attributed to the lower frequency of infiltrating human CD8 T cells, comparing C10G hPi grafts with WT hPi grafts (Figure 5D), while circulating human lymphocyte levels did not change (Figure S5B).
In all, SC-islets with impaired ability to express CXCL10 are not only hypoimmunogenic in vitro (Figure 4) but are also cable of evading immune attack in vivo within an allograft.

**DISCUSSION**

This study used two approaches to reveal genes that drive SC-islet immunogenicity: transcript analysis characterized the responses to immune challenge, and CRISPR genome screening helped assess the cause of those responses.

In responding to allogeneic immune cells, the strongest effect in SC-islets is upregulation of ISGs. The results show that T cells are activated in immune environments and express IFNγ, among many other inflammatory genes. The secreted IFNγ leads to an inflammatory cascade in which ISGs are upregulated in SC-islets. A plausible explanation for T cell activation is by antigen presentation through MHC class I molecules.

The most striking observation was the involvement of chemokines secreted by SC-islets. These results suggest that CXCL10 has a role in the early stage of immune-graft interaction, CXCL10-KO SC-islet cells in an allogeneic in vivo model survived longer compared with surrounding cells with other perturbations. Furthermore, the in vitro and in vivo allogeneic experiments demonstrate that CXCL10-deficient SC-islets are immune evasive compared with WT. CXCL10-deficient SC-islets (C10G) have 2-fold increased survival capability under immune challenge by allogeneic T cells or NK cells. Upon engraftment in a humanized allogeneic in vivo model, C10G avoid immune destruction 7 weeks longer than WT SC-islets. CXCL10 as a secreeted chemokine plays a determining role as a recruiter of immune cells to an SC-islet transplant site, and depleting it keeps those grafts out of the reach of a human immune system.

CXCL10 is one of the most upregulated chemokines in primary human islets (Eizirik et al., 2012) and hPSC-derived islets (Demine et al., 2020; Dettmer et al., 2022) under pro-inflammatory conditions. Islets of recent-onset T1D show CXCL10 expression in regions where infiltrating lymphocytes express CXCR3 (Roep et al., 2010; Uno et al., 2010). Our results show that CXCL10 expression is not exclusive to SC-β cells but is also differentially expressed by other SC-endocrine cells. A recent study also demonstrated the contribution of pancreatic α cells to CXCL10 expression in NOD mice and in recent-onset T1D islets (Nigi et al., 2020).

In our previous study using a T1D autologous in vitro model, CXCL10 was highly secreted from iPSC-islets during co-culture with matched T1D PBMCs (Leite et al., 2020). In this current work, CXCL10 expression was seen in co-cultures but not in late stages of graft rejections, supporting the view of CXCL10 as a first responder or alarm protein at the onset of SC-islet interactions with a hostile immune system. In T1D, islet CXCL10 expression occurs in early stages (Roep et al., 2010; Uno et al., 2010) and serum levels of CXCL10 are elevated in recent-onset compared with long-term T1D individuals (auto-Ab+) (Shimada et al., 2001). Mouse islet isografts expressed high levels of Cxcl10 at day 2 after transplantation into diabetic C57BL/6 mice, but in a lesser degree by day 100 (Bender et al., 2017). Furthermore, analysis of plasma samples from human islet transplant patients revealed that CXCL10 was among the highest released inflammatory mediators and peaked 24 h post transplantation (Yoshimatsu et al., 2017).

In T1D, pancreatic islets react to pro-inflammatory cytokines by inducing the NF-κB and STAT1 signaling that contribute to the immune destruction mechanism of β cells (Cnop et al., 2005; Eizirik et al., 2012). Although our experiments were done in an allogeneic setting, both transcription factors were upregulated in SC-islets, but only STAT1 depletion showed up as a hit in the CRISPR screen. However, when STAT1 KO (ST1L) SC-islets were used, this rescue was not reproduced (Figure 4). The reason might derive from the observation that STAT1-deficient SC-islets lose immune-inducing elements such as HLA molecules and CXCL10, but also suffer from loss of immune-inhibitory functions like PD-L1 and SOCS1.

Downstream to STAT1 is the transcription factor IRF1, which has anti-inflammatory effects in β cells through the induction of SOCS1 (Moore et al., 2011). SOCS1 and PTPN2 are negative regulators of cytokine signaling (Chong et al., 2002; Elvira et al., 2022; Moore et al., 2009) and are both associated with T1D risk loci (Onengut-Gumuscu et al., 2015; Ram and Morahan, 2017). Previous reports have shown that SOCS1 overexpression in NOD mice islets prevent diabetes (Flodstrom-Tullberg et al., 2003), and delays allogeneic islet graft rejection in mouse models (Solomon et al., 2011). Our data show that, under PBMCs + SC-islet interactions, both IRF1 and SOCS1 are differentially up-regulated. SOCS1 KO and PTPN2 KO SC-islets were depleted in hP1 grafts in our CRISPR screen, along with PTPRA KO, another PTP family member (Stanley et al., 2015).

Based on ST1L’s unconvincing results (Figure 4), “pan-JAK/STAT” diminishing strategies should be considered cautiously. These approaches include SOCS1 overexpression and IFNGR1 KO. Transgenic lines of SOCS1 OE or IFNGR1 KO might have consequences of losing the inflammatory negative regulation feedback of JAK/STAT signaling. PD-L1 downregulation under JAK/STAT silencing will expose SC-islets to T cell attack, while HLA downregulation will result in NK cell recognition and killing. It may be useful to co-edit such stem cell lines with additional modification(s) that will address these concerns.
The analyses presented in this paper include many other genes that may be targeted to control the immune response against SC-islets. Modulation of ISGs by identified hits from our in vivo CRISPR screen (e.g., TRIM8, SUMO1), or others of unclear function (e.g., IL32, CAMSAP3), were not considered here but may have the potential to reduce immunogenicity. Nevertheless, this study points to opportunities for future applications of SC-islet as a cell replacement therapy for T1D.

Limitations of the study
An optimal pooled screen would be one that relies on a robust assay with a selection force that separates cells using a phenotype of interest (Doench, 2018). Although we were able to acquire gene hits from the described in vivo CRISPR screen, the assay (hiP model) is not flawless. T cells are the only immune cells that engraft successfully and persist long term, leaving out other immune cells that may also contribute to SC-islet graft destruction, in particular NK cells (Shultz et al., 2019). In addition, pooled screens can benefit from survival selection of cells that could proliferate and amplify the enrichment signal. The enrichment in our screen is based solely on differentiated post-mitotic cells.

EXPERIMENTAL PROCEDURES
Contact for reagent and resource sharing
Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Douglas A. Melton (dmelton@harvard.edu).

Experimental model and subject details
All procedures were performed in accordance with the Institutional Review Board (IRB) guidelines at Harvard University under IRB and Embryonic Stem Cell Research Oversight Committee (ESCR) protocol E00024. All animal experiments were performed in accordance with Harvard University International Animal Care and Use Committee regulations.

Quantification and statistical analyses
Statistical analysis was performed by unpaired Student’s t tests as indicated, using Prism v9. All data are presented mean ± SD. p < 0.05 was considered statistically significant. Sufficient sample size was estimated without the use of a power calculation. Data analysis was not blinded.

Graphic illustrations
Graphic illustrations in the manuscript were created with BioRender.com under BioRender’s academic license terms.

Data and code availability
scRNA-seq and pooled CRISPR screen data generated during this study are available at NCBI (GEO: GSE200104) and are composed of listed sub-series related to specific experiments described in this paper.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.08.002.

AUTHOR CONTRIBUTIONS
E.S. conceived the study. E.S., J.H.R.K., A.S.A., and K.B. performed the experiments. D.G. was involved in the experimental design and provided technical support. I.N., V.B., and S.H.S. analyzed the scRNA-seq data. I.N. and E.S. analyzed the CRISPR screen data. E.S. and D.A.M. wrote the manuscript. D.A.M. designed and supervised the research.

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CONFLICTS OF INTEREST
D.A.M. is a founder of Semma Therapeutics and an employee of Vertex Pharmaceuticals, which has licensed technologies from Harvard and HHMI. E.S. and K.B. are now Vertex employees. All other authors declare no competing interests. A patent related to this work was filed by Harvard University.

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Supplemental Information

Whole-genome CRISPR screening identifies genetic manipulations to reduce immune rejection of stem cell-derived islets

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Figure S1. Single cell transcriptional profile and whole genome CRISPR screen of SC-islet grafts in an in vivo humanized model. Related to Figure 1.

(A) Representative images of transplanted kidneys after 10 weeks with or without PBMC injection. Bar=2mm.

(B) Gating strategy used for flow cytometry of hPi/control mice blood to detect human T lymphocytes. %Human CD45+ are gated from mouse CD45 negative population. %CD4+ and %CD8+ are gated from hCD45+/hCD3+.

(C) Frequency of human T-lymphocytes in hPi mouse tissues, by flow cytometry.

(D) Frequency of SC-α (Glucagon+/C-peptide-) and SC-β (Glucagon-/C-peptide+) in recovered from SC-islet grafts, by flow cytometry.

(E) Flow cytometry of human T-lymphocytes in mice blood throughout the experiment, transplanted with library-transduced SC-islets (LT-SC-islets) ±PBMC injections (Control/hPi). %human CD45+ are gated from mouse CD45 negative population. %CD4+ and %CD8+ (only in hPi mice) are gated from hCD45+/hCD3+.

(F) Human Insulin detected by ELISA at the 10th week end point of the experiment, in non-fasted mice. Error bars are mean±SD. *p<0.05, **p<0.01, unpaired two-tailed t-test, LT SC-islets Tx+PBMCs (hPi) compared to the control.

(G) UMAP plots of human graft cells extracted from mice after 10 weeks with or without PBMC injection. Integration of n=6 mice per group. SC-endocrine cell clusters are indicated.

(H) Cluster identification (integration of n=12 mice) of specific SC-endocrine cells by gene markers (SC-β:INS+GCG-, SC-α: INS-GCG+, SC-EC: TPH1+).

(I) Cell counts of endocrine cell populations from scRNA-seq integrations.

(J-L) scRNA-seq analysis of SC-islet grafts. (J) Volcano plot of differential expressed genes in SC-EC in hPi vs. control grafts. (K) Differential expression of selected genes in SC-EC, presented as a heatmap. Each row specifies a z-score of the specified gene in all graft samples, in the indicated endocrine population. (L) Violin plots of selected genes, associated with the IFNγ response, expressed in SC-Endocrine cells.

(M) Total or control gRNA reads in CRISPR screen of mice replicates, compared between conditions (±PBMC; n=6 per condition). Box lines represent median values.
A B

C

PBMCs t=0 SC-islets t=0

PBMCs+SC-islets t=24hrs

PBMCs+SC-islets t=48hrs

UMAP_2

UMAP_1

PBMC 0hr SC-islet 0hr CC 24hr CC 48hr

CD4 T-cells 2737 0 2288 1977

CD8 T-cells 2391 0 477 866

B-cells 1140 0 729 859

Monocytes 7725 0 808 681

NK-cells 918 0 152 357

Treg 1217 0 711 654

SC-α 0 1618 877 593

SC-β 0 4723 2672 2006

SC-EC 0 875 573 468

Other 369 0 711 654

Table: Cell numbers - scRNA-seq

Immune cell gene expression

Figure S2
Figure S2. Early response of immune challenged SC-islets profiled by single cell transcription analysis after co-culture with human allogeneic PBMCs. Related to Figure 2.

(A) UMAP plots of PBMC+SC-islet co-cultured cells, immune/SC-islet cell clusters are indicated.

(B) Cluster identification (integration of all time points) of specific cells by gene markers.

(C) Cell counts of cell populations from scRNA-seq integrations. CC=co-culture.

(D) Dot plots representing expression of activation/inhibitory genes in specific immune populations, in response to timed SC-islet stimulation.

(E) Selected GSEA plots for interferon response and TF motifs, FDR values and normalized enrichment scores (NES) indicated.

(F) Violin plots of SC-β timed expression of selected genes.

(G) CXCL10 and phosphorylated STAT1 in SC-islet clusters 48hrs after treatment with 20ng/ml rhlIFNγ. C-peptide staining for SC-β. Bars are 100μm in main panels and 20μm in magnified panels.

(H) IF staining of SC-islet clusters after 48hrs co-culture with PBMC. C-peptide staining (green) for SC-β and DAPI (blue) for nuclei. Bars are 100μm.
Figure S3
**Figure S3. Immunogenicity of CXCL10 expressing SC-islets. Related to Figure 3.**

(A) Flow cytometry analysis of protein expression of indicated perturbation in SC-islets or specifically in C-peptide+ SC-β 48hrs after rhIFNγ treatment. Where indicated CXCL10 secretion was measured by ELISA.

(B) Transduced SC-islets were co-cultured with Cell Trace Violet (CTV) labeled PBMC for 48hrs. PBMCs were then separated and allowed to grow in culture for an additional 7 days, followed by CD3 staining for flow cytometry. CD3+ were gated for the CTV negative fraction of divided cells. PBMCs treated with anti-CD3/CD28 activation beads served as positive control. n=12 for x5 PBMC donors (n=3 for controls).

(C) ELISA for human CXCL10, from supernatant of co-culture of SC-islets transduced with NT/CXCL10 gRNA ±PBMCs, n=2-3 for x2 donors. Dashed line is the lower detection limit.

(D) Antibody treated (as indicated) SC-islets were co-cultured with PBMC for 48hrs. PBMCs were then separated and analyzed by flow cytometry for CD3+ T cell activation marker expression (CD25 or CD69). n=12 for x2 donors.

NT=Non-targeting, OE=overexpression. Error bars are mean±SD. ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 unpaired two-tailed t-test.
Figure S4
Figure S4. Generation and performance of CXCL10 KO and STAT1 KO hESC lines. Related to Figure 4.

(A) Clonal genotyping of endogenous or targeted alleles. Endogenous amplified PCR bands were isolated and sequenced for detection of indels, shown in dashed blue frames. Some lanes were cropped to show only relevant clones.

(B) Karyotyping analysis of G10G and ST1L hESC cell lines.

(C) Pluripotent marker expression by flow cytometry in all 4 lines: wild type (WT), C10G, GAPDH-luc (GL) and ST1L.

(D) Flow cytometry analysis to assess %SC-β (%C-peptide+/NKX6.1+ or %C-peptide+/glucagon) in C10G and ST1L hESC at stage 6 of the β-cell differentiation protocol.

(E) %SC-β in multiple batches of C10G and ST1L differentiations compared to control WT and GL lines. n=2-3 differentiations.

(F) SC-islet GSIS function assay of different lines, 12-15 weeks after transplantation into NSG-mice. Results presented as stimulation ratios of blood human insulin (ELISA) before and 30 min after glucose injection (2g/kg).

(G) Flow cytometry was used to assess %TUNEL+ SC-islets. Apoptosis was calculated by fraction from baseline (%TUNEL without PBMC). Left) WT or C10G SC-β cells (n=4 for x6 PBMC donors, n=2-3 x2 T-cell donors, n=4 x2 NK cell donors); Right) GL vs. ST1L SC-β cells (n=4 for x2 PBMC or NK cell donors).

(H,I) GM SC-islets were co-cultured with PBMC for 48hrs. PBMCs were then separated and analyzed by flow cytometry for CD3+ T cell activation marker expression (CD25 or CD69). (H) n=9 for x3 PBMC donors (I) n=9 for x2 PBMC donors

Error bars are mean±SD. ns=not significant, *p<0.05, **p<0.01, ****p<0.0001 unpaired two-tailed t-test.
**Figure S5**

(A) Post-Glucose

Human insulin (Fold from week 0)

- WT SC-islets Tx
- C10G SC-islets Tx
- WT SC-islets Tx+PBMCs
- C10G SC-islets Tx+PBMCs

(B) Human CD45+ cells

- % from mCD45 negative

Time (weeks) post PBMC injection

Human CD4+ cells

- % from hCD3+ cells

Time (weeks) post PBMC injection

Human CD8+ cells

- % from hCD3+ cells

Time (weeks) post PBMC injection

ns ns ns ns

WT SC-islets Tx+PBMCs

C10G SC-islets Tx+PBMCs
Figure S5. CXCL10 KO SC-islet transplantation in humanized mice. Related to Figure 5.

(A) Graft failure continuously monitored over time after PBMC injections, as measured by human insulin (ELISA) in fasted mice plasma, 30 min after glucose injection to fasted mice. Data presented as fold increase from t=0 before PBMC injections.

(B) Flow cytometry of human T-lymphocytes in mice blood throughout the experiment, transplanted WT/C10G SC-islets +PBMC injections. %human CD45+ are gated from mouse CD45 negative population. %CD4+ and %CD8+ (only in hPi mice) are gated from CD45+/CD3+.

Error bars are mean±SD. ns=not significant, *p<0.05, **p<0.01, unpaired two-tailed t-test.
## SUPPLEMENTARY TABLES

Table S1 - Panther pathway analysis of upregulated genes in a given cell population. Related to results presented in Figures 1 and 2.

| Term                                                                 | Enrichment Ratio | False discovery rate (FDR) |
|----------------------------------------------------------------------|------------------|---------------------------|
| **SC-α in vivo SCRNA-seq**                                            |                  |                           |
| JAK/STAT signaling pathway                                           | 5.419432         | 7.69E-04                  |
| FAS signaling pathway                                                | 2.950094         | 0.026945                  |
| Apoptosis signaling pathway                                          | 2.916708         | 1.20E-06                  |
| T cell activation                                                    | 2.845202         | 2.69E-04                  |
| Toll receptor signaling pathway                                      | 2.438744         | 0.028295                  |
| Inflammation mediated by chemokine and cytokine signaling pathway    | 1.879865         | 0.00133                   |
| **SC-β in vivo SCRNA-seq**                                            |                  |                           |
| JAK/STAT signaling pathway                                           | 15.10667         | 0.09678                   |
| Oxidative stress response                                            | 6.568116         | 0.156495                  |
| T cell activation                                                    | 4.028444         | 0.594957                  |
| PDGF signaling pathway                                               | 3.021333         | 0.623899                  |
| Ras Pathway                                                          | 3.237143         | 1                         |
| Toll receptor signaling pathway                                      | 3.021333         | 1                         |
| **SC-EC in vivo SCRNA-seq**                                           |                  |                           |
| Interferon alpha/beta signaling                                      | 14.13884         | 0                         |
| Antigen Presentation: Folding, assembly and peptide loading of class I MHC | 13.00773         | 3.19E-08                  |
| Interferon gamma signaling                                           | 11.2468          | 0.00E+00                  |
| Cytokine Signaling in Immune system                                  | 4.468829         | 0                         |
| Adaptive Immune System                                               | 3.089258         | 0.00E+00                  |
| Immune System                                                        | 2.916332         | 0.00E+00                  |
| **SC-α in vitro SCRNA-seq**                                           |                  |                           |
| T cell activation                                                    | 6.972308         | 0.501353                  |
| Apoptosis signaling pathway                                          | 4.84188          | 0.599738                  |
| Interleukin signaling pathway                                        | 4.469428         | 1                         |
| JAK/STAT signaling pathway                                           | 11.62051         | 1                         |
| Interferon-gamma signaling pathway                                   | 6.45584          | 1                         |
| Toll receptor signaling pathway                                      | 3.486154         | 1                         |
| **SC-β in vitro SCRNA-seq**                                           |                  |                           |
| JAK/STAT signaling pathway                                           | 19.70435         | 0.043412                  |
| Oxidative stress response                                            | 6.425331         | 0.589624                  |
| Interferon-gamma signaling pathway                                   | 7.297907         | 0.85533                   |
| T cell activation                                                    | 3.94087          | 0.85533                   |
| Apoptosis signaling pathway                                          | 2.736715         | 1                         |
| Inflammation mediated by chemokine and cytokine signaling pathway    | 1.970435         | 1                         |
| **CD4 T cells in vitro SCRNA-seq**                                   |                  |                           |
| T cell activation                                                    | 4.028444         | 0.071289                  |
| Inflammation mediated by chemokine and cytokine signaling pathway    | 2.454833         | 0.08497                   |
| Cytoskeletal regulation by Rho GTPase                                | 3.723474         | 0.08497                   |
| PDGF signaling pathway                                               | 2.7192           | 0.134924                  |
| JAK/STAT signaling pathway                                           | 7.553333         | 0.144705                  |
| Integrin signalling pathway                                          | 2.2751           | 0.153289                  |
Table S2 - Reactome pathway analysis of upregulated genes in a given cell population. Related to results presented in Figures 1 and 2.

| Term | Enrichment Ratio | FDR |
|------|------------------|-----|
| **SC-α in vivo** SCRNA-seq | Interferon alpha/beta signaling | 7.109533 | 0.00E+00 |
| | Interferon gamma signaling | 6.246232 | 0.00E+00 |
| | Interferon Signaling | 4.33996 | 0 |
| | Programmed Cell Death | 3.341008 | 6.27E-10 |
| | Apoptosis | 3.151512 | 2.09E-08 |
| | Cytokine Signaling in Immune system | 2.892824 | 0 |
| **SC-β in vivo** SCRNA-seq | Interferon alpha/beta signaling | 26.14641 | 0 |
| | Interferon gamma signaling | 19.60981 | 0 |
| | Antigen processing-Cross presentation | 14.57861 | 2.75E-12 |
| | Interferon Signaling | 13.73682 | 0 |
| | Cytokine Signaling in Immune system | 5.637821 | 0 |
| | Immune System | 3.252263 | 0 |
| **SC-EC in vivo** SCRNA-seq | Interferon alpha/beta signaling | 14.13844 | 0 |
| | Antigen Presentation: Folding, assembly and peptide loading of class I MHC | 13.00773 | 3.19E-08 |
| | Interferon gamma signaling | 11.2468 | 0.00E+00 |
| | Cytokine Signaling in Immune system | 4.468829 | 0 |
| | Adaptive Immune System | 3.089258 | 0.00E+00 |
| | Immune System | 2.916332 | 0.00E+00 |
| **SC-α in vitro** SCRNA-seq | Cytokine Signaling in Immune system | 10.06695 | 0 |
| | Antigen processing-Cross presentation | 24.9858 | 0 |
| | Interferon gamma signaling | 28.67935 | 0 |
| | Interferon alpha/beta signaling | 45.40897 | 0 |
| | Antigen Presentation: Folding, assembly and peptide loading of class I MHC | 52.77 | 2.51E-10 |
| | Class I MHC mediated antigen processing & presentation | 8.000842 | 7.03E-10 |
| **SC-β in vitro** SCRNA-seq | Cytokine Signaling in Immune system | 7.912271 | 0 |
| | Interferon gamma signaling | 20.52838 | 0 |
| SCRNA-seq                         | Term                                                                 | Enrichment Ratio | FDR       |
|----------------------------------|----------------------------------------------------------------------|------------------|-----------|
| SCRNA-seq                        | Interferon alpha/beta signaling                                      | 37.03158         | 0         |
|                                  | Adaptive Immune System                                               | 3.673768         | 2.10E-06  |
|                                  | Antigen processing-Cross presentation                               | 11.22169         | 4.29E-06  |
|                                  | Signaling by Interleukins                                            | 4.328366         | 2.35E-05  |
| CD4 T cells in vitro SCRNA-seq   | Axon guidance                                                       | 4.14862          | 0         |
|                                  | rRNA processing in the nucleus and cytosol                          | 7.731866         | 0         |
|                                  | Major pathway of rRNA processing in the nucleolus and cytosol       | 8.149807         | 0         |
|                                  | Regulation of expression of SLITs and ROBOs                          | 9.385884         | 0         |
|                                  | Eukaryotic Translation Initiation                                    | 13.07857         | 0         |
|                                  | Formation of a pool of free 40S subunits                             | 14.92786         | 0         |
| CD8 T cells in vitro SCRNA-seq   | Innate Immune System                                                | 3.233159         | 2.43E-06  |
|                                  | Signaling by Interleukins                                            | 3.684541         | 0.004806  |
|                                  | Cytokine Signaling in Immune system                                 | 2.804107         | 0.031589  |
|                                  | Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell | 6.018084         | 0.043057  |
|                                  | CD28 co-stimulation                                                 | 13.75562         | 0.043057  |
|                                  | Cell surface interactions at the vascular wall                       | 5.798446         | 0.043057  |
| NK cells in vitro SCRNA-seq      | Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell | 7.902484         | 3.34E-08  |
|                                  | Cytokine Signaling in Immune system                                 | 3.210722         | 1.60E-07  |
|                                  | Innate Immune System                                                | 2.505698         | 3.71E-06  |
|                                  | Signaling by Interleukins                                            | 3.453186         | 9.57E-06  |
|                                  | Adaptive Immune System                                              | 2.759598         | 1.28E-05  |
|                                  | Interferon Signaling                                                | 3.737693         | 0.015391  |

Table S3 – Gene Ontology (GO) terms of biological processes of upregulated genes in a given cell population. Related to results presented in Figures 1 and 2.
| SC-α in vitro SCRNA-seq | immune response | 6.221897 | 0 |
|------------------------|-----------------|-----------|---|
|                         | defense response| 7.70163   | 0 |
|                         | response to cytokine | 9.497585 | 0 |
|                         | innate immune response | 10.52735 | 0 |
|                         | defense response to other organism | 12.09548 | 0 |
|                         | response to interferon-gamma | 22.02441 | 0 |
| SC-β in vitro SCRNA-seq | immune response | 5.299158 | 0 |
|                         | defense response | 6.792044 | 0 |
|                         | immune effector process | 5.322702 | 0 |
|                         | response to cytokine | 7.575455 | 0 |
|                         | innate immune response | 9.393048 | 0 |
|                         | response to virus | 15.0535 | 0 |
| CD4 T cells in vitro SCRNA-seq | immune response | 2.924152 | 0 |
|                         | regulation of immune system process | 3.126378 | 0 |
|                         | cell activation | 3.026399 | 0 |
|                         | leukocyte activation | 3.222791 | 0 |
|                         | establishment of protein localization to organelle | 4.911323 | 0 |
|                         | protein targeting | 5.584208 | 0 |
| CD8 T cells in vitro SCRNA-seq | immune response | 3.501908 | 2.02E-12 |
|                         | leukocyte activation | 4.200101 | 7.00E-11 |
|                         | cell motility | 3.541587 | 6.17E-10 |
|                         | response to cytokine | 4.154282 | 6.21E-10 |
|                         | cell migration | 3.635175 | 1.93E-09 |
|                         | regulation of immune response | 4.43575 | 2.74E-09 |
| NK cells in vitro SCRNA-seq | cell activation | 3.159824 | 4.04E-13 |
|                         | regulation of immune response | 3.803824 | 4.04E-13 |
|                         | response to cytokine | 3.39481 | 1.51E-12 |
|                         | cellular response to cytokine stimulus | 3.474709 | 3.75E-12 |
|                         | cytokine-mediated signaling pathway | 3.923604 | 1.08E-10 |
|                         | lymphocyte activation | 3.960806 | 6.91E-10 |

Supplemental Experimental Procedures

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**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rat anti-C-peptide  | Developmental Studies Hybridoma Bank (DHSB) | GN-ID4, RRID: AB_2255626 |
| Mouse anti-NKX6.1   | DHSB   | F55A12, RRID: AB_532379 |
| Goat anti-NANOG     | R&D Systems | AF1997 |
| Goat anti-SOCS1     | Life Technologies | Cat#PA517938 |
| Mouse anti-glucagon Alexa 647 | Santa Cruz Biotech | Cat#SC-514592 |
| Rabbit anti-STAT1   | abcam  | Cat#ab2415 |
| Mouse anti-CD8      | BD Biosciences | HIT8a, Cat#550372, RRID: AB_393643 |
| Mouse anti-NKX6.1 PE-conjugated | BD Biosciences | Cat#563023 |
| Mouse anti-pSTAT1 Alexa 647-conjugated | Biolegend | Cat#666410 |
| Mouse anti-SOCS1 Alexa a88-conjugated | Santa Cruz Biotech | Cat#SC-518028 |
| Mouse anti-IP10 PE-conjugated | Biolegend | Cat#519504 |
| Mouse Anti-HLA-ABC Alexa 647-conjugated | Biolegend | W6/32, Cat#311414 |
| Mouse Anti-HLA-ABC PE-conjugated | Biolegend | W6/32, Cat#311406 |
| Mouse anti-HLA-E PE-conjugated | BD Biosciences | Cat#12995342 |
| Mouse anti-CD119 PE-conjugated | Biolegend | GIR-94, Cat#308704 |
| Rat anti CD16/CD32 (Fc Block) | BD Biosciences | Cat#553141, RRID: AB_394656 |
| Rat anti murine CD45 Alexa 647-conjugated | Biolegend | Cat#03124 |
| Mouse antibody                                      | Manufacturer       | Catalog  |
|----------------------------------------------------|--------------------|----------|
| Mouse anti-CD45 Alexa a88-conjugated               | Biolegend          | Cat#304017 |
| Mouse anti-CD3 APC-conjugated                      | Biolegend          | UCHT1, Cat#300412 |
| Mouse anti-CD3 PE-conjugated                       | Biolegend          | UCHT1, Cat#300408 |
| Mouse anti-PD-L1 APC-conjugated                    | Biolegend          | 29E.2A3, Cat#329708 |
| Mouse anti-CD3 PB-conjugated                       | Biolegend          | UCHT1, Cat#300417 |
| Mouse anti-CD8 PE-conjugated                       | Biolegend          | T8-Leu2, Cat#344706 |
| Mouse anti-CD4 PE/Cy7-conjugated                   | Biolegend          | RPA-T4, Cat#300512 |
| Mouse anti-CD69 Alexa 647-conjugated               | Biolegend          | FN50, Cat#310918 |
| Mouse anti-CD25 Alexa 700-conjugated               | Biolegend          | M-A251, Cat#356118 |
| Mouse anti-CD107a FITC-conjugated                  | ThermoFisher       | H4A3, Cat# BDB555800 |
| Mouse anti-CD56 APC-conjugated                     | Biolegend          | Cat#362504 |
| Mouse anti-CD49a PE-conjugated                     | BD Biosciences     | Cat#559596 |
| Mouse anti-Oct4 Alexa 647-conjugated               | BD Pharmingen      | Cat#653710 |
| Mouse anti-Sox2 PE-conjugated                      | BD Pharmingen      | Cat#560291 |
| Mouse anti-SSEA4 V450-conjugated                   | BD Pharmingen      | Cat#561156 |
| Mouse IgG1 PB-conjugated                           | Biolegend          | Cat#400131 |
| Mouse IgG1 Alexa 488-conjugated                    | Biolegend          | Cat#400132 |
| Mouse IgG3, κ V450- conjugated                     | BD Biosciences     | Cat#561600 |
| Mouse IgG1 PE-conjugated                           | Biolegend          | Cat#400114 |
| Mouse IgG2b, κ PE-conjugated                       | Biolegend          | Cat#400314 |
| Mouse IgG2a, κ PE-conjugated                       | Biolegend          | Cat#400214 |
| Mouse IgG2a, κ Alexa 647-conjugated                | Biolegend          | Cat#400234 |
| Mouse IgG2b, κ Alexa 647-conjugated                | Biolegend          | Cat#400330 |
| Mouse IgG1 Alexa 647-conjugated                    | Biolegend          | Cat#400136 |
| Mouse IgG2a, κ APC-conjugated                      | BD Biosciences     | Cat#17472442 |
| Mouse IgG2b, κ APC-conjugated                      | Biolegend          | Cat#400322 |
| Mouse IgG2b, κ Alexa 700-conjugated                | Biolegend          | Cat#400334 |
| Mouse IgG1 Alexa PE/Cy7-conjugated                 | Biolegend          | Cat#400126 |
| Donkey anti-mouse Alexa 647                        | Life Technologies  | Cat#A31571 |
| Donkey anti-rabbit Alexa 488                       | Life Technologies  | Cat#A21206 |
| Donkey anti-rabbit Alexa 594                       | Life Technologies  | Cat#A21209 |
| Donkey anti-goat Alexa 647                         | Life Technologies  | Cat#A21447 |
| Donkey anti-rat 488                                | Life Technologies  | Cat# A21208 |

**Virus vectors**

| Virus vector | Manufacturer   | RRID: |   |
|--------------|----------------|-------|---|
| lentiCRISPRv2 | Broad institute | Addgene_52961 |   |
| pLX_307      | Broad institute | Addgene_41392 |   |
| Oligonucleotides |  |
|-----------------|-----------------
| **Non targeting gRNA**<br>TTTACGATCTAGCGCGTAG | This paper | N/A |
| **B2M gRNA**<br>GCTACTCTCTCTTTCTGGCC | This paper | N/A |
| **CXCL10 gRNA (for virus and HDR)**<br>GTAATCAACCTGTTAATCCA | Virus: eSpCas9-LentiCRISPR v2 by Genescript<br>HDR: IDT (Alt-R) | N/A |
| **STAT1 gRNA (for virus)**<br>TGCTGGCACCAGAACGAATG | eSpCas9-LentiCRISPR v2 by Genescript | N/A |
| **STAT1 gRNA#1 (for HDR) Alt-R**<br>AAAGCTGGTGAACCTGCTCC | Alt-R IDT | N/A |
| **STAT1 gRNA#2 (for HDR) Alt-R**<br>GCAGCTTGACTCAAAATTCC | Alt-R IDT | N/A |
| **IFNGR1 gRNA** | eSpCas9-LentiCRISPR v2 by Genescript | N/A |

| pHDM-vsvG, -tat, rev, gag/pol | Harvard Medical School DNA Resource Core | N/A |

| Biological Samples |  |
|--------------------|-----------------|
| **Human Peripheral Blood apheresis collars**<br>Kraft Family Blood Donor Center, Brigham and Woman’s Hospital |  | N/A |
| STAT1 Edited Forward                      | IDT | N/A |
|------------------------------------------|-----|-----|
| AGATAAATGCCTGCTCTTTACT                   |     |     |
| STAT1 gRNA cut site Forward              | IDT | N/A |
| GGTCAGATGGTGTTGAATGAC                    |     |     |
| STAT1 gRNA cut site Reverse              | IDT | N/A |
| CCCTTCACCTTTCTATGTCAAT                   |     |     |

**Chemicals, Peptides, and Recombinant Proteins**

| Chemical                          | Supplier          | Catalogue Number |
|----------------------------------|-------------------|------------------|
| Activin A                        | R&D Systems       | Cat#338-AC       |
| Rock Inhibitor Y-27632           | DNSK              | Cat#DNSK-KI15-02 |
| Chir99021                        | Stemgent          | Cat#04-0004-10   |
| KGF                              | Peprotech         | Cat#100-19       |
| Retinoic acid                    | Sigma-Aldrich     | Cat# R2625       |
| LDN193189                        | Sigma-Aldrich     | Cat#SML0559      |
| Sant1                            | Sigma-Aldrich     | Cat#S4572        |
| PBDU                             | EMD Millipore     | Cat#524390       |
| XXI                              | EMD Millipore     | Cat#565790       |
| Alk5i II                         | Axxora            | Cat#ALX-270-445  |
| T3                               | EMD Millipore     | Cat#642511       |
| Betacellulin                     | ThermoFisher Scientific | Cat# 565790     |
| Human IFN gamma                  | R&D Systems       | Cat#285IF        |
| Human IL-2                       | Peprotech         | Cat#200-02       |
| Thapsigargin                     | Sigma-Aldrich     | Cat#T9033        |

**Critical Commercial Assays**

| Test                               | Supplier          | Catalogue Number |
|------------------------------------|-------------------|------------------|
| Chromium Next GEM Single Cell 3' Kit v3.1, 16 rxns | 10X Genomics       | PN-1000268       |
| Chromium Next GEM Chip G Single Cell Kit, 48 rxns | 10X Genomics       | PN-1000120       |
| Dual Index Kit TT Set A, 96 rxns | 10X Genomics       | PN-1000215       |
| Kapa qPCR Library Quantification Kit, Complete Universal Kit | Roche Sequencing Solutions | Cat#07960140001 |
| High Sensitivity D5000 Tape        | Agilent Technologies | 5067-5592        |
| High Sensitivity D5000 Reagents | Agilent Technologies | 5067-5593        |

**Experimental Models: Cell Lines**

| Line                               | Supplier          | Catalogue Number |
|------------------------------------|-------------------|------------------|
| Human ESC line Hues8               | HSCI              | HVRDe008-A       |
| Human ESC line Hues8 GAPDH- luciferase (GL) | HSCI              | HVRDe008-A-1     |
| Lenti-X™ 293T Cell Line           | Takara Bio        | Cat#632180       |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Douglas A. Melton (dmelton@harvard.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All procedures were performed in accordance with the Institutional Review Board (IRB) guidelines at Harvard University under IRB and Embryonic Stem Cell Research Oversight Committee (ESCRO) Protocols E00024. All animal experiments were performed in accordance with Harvard University International Animal Care and Use Committee regulations.

METHOD DETAILS

hESC cell culture and differentiation

Human embryonic stem-cell (hESC) Hues8 maintenance and differentiation was carried out as previously described (Pagliuca et al., 2014). Induced pluripotent stem-cell lines were obtained from stocks maintained by the Melton laboratory. hESC line was maintained in cluster suspension culture format using mTeSR-1 (Stem Cell Technologies, 85850) in 500-ml spinner flasks (Corning, VWR) spinning at 70 r.p.m. in an incubator at 37 °C, 5% CO2 and 100% humidity. Cells were passaged every 72h or 96h: induced human pluripotent stem-cell clusters were dissociated to single cells using gentle cell dissociation reagent (Stem Cell Technologies; 07174) and light mechanical disruption, counted and seeded at 0.6 M cells/ml in mTeSR-1 + 10 μM Y27632 (ROCK inhibitor). Cell lines were authenticated by DNA fingerprinting, karyotyping (Cell Line Genetics) and all lines tested negative on routine mycoplasma contamination verifications. Differentiation flasks were started 72 h after passaging, by replacing mTeSR-1 medium with the appropriate differentiation medium including growth factors and small molecule supplements as previously described (Veres et al., 2019):

SC-β cells protocol
Stage 1: 24 hours in S1 medium supplemented with Activin A (100ng/ml), CHIR99021 (1.4μg/ml) and Rock Inhibitor (10μM), followed by 48 hours Activin A (100ng/ml) only.

Stage 2: 72 hours in S2 medium supplemented with KGF (50ng/ml) and Rock Inhibitor (10μM).

Stage 3: 48 hours in S3 medium supplemented with KGF (50ng/ml), LDN193189 (200nM), Sant1 (0.25μM), retinoic acid (2μM), PBDU (500nM) and Rock Inhibitor (10μM).

Stage 4: 5 days in S3 medium supplemented with KGF (50ng/ml), Sant1 (0.25μM), retinoic acid (0.1μM) and Rock Inhibitor (10μM).

Stage 5: 7 days in BE5 medium supplemented with Betacellulin (20ng/ml), XXI (1μM), Alk5i-II (10 μM) and T3 (1μM). Sant1 (0.25μM) was added in the first three days, and retinoic acid was added at 0.1μM in the first three days, then at 0.025μM.

Stage 6: 14-21 days in S3 medium, changed every 48 hours.

During feeds, the differentiating clusters were allowed to gravity-settle for 5–10 min, medium was aspirated, and 300 ml of pre-warmed medium was added. All experiments involving human cells were approved by the Harvard University IRB and ESCRO committees.

Cell transplantation

Pre-surgery animals were housed in groups within sterile cages with unrestricted access to food and water. Ambient temperature was maintained between 18 and 25 °C, humidity 30–70% with 12 h light/dark cycles. All animal research was conducted under Harvard IACUC approval. Transplantation of cell clusters was performed as previously described (Pagliuca et al., 2014). Briefly, 5M cells were injected under the kidney capsule of male NSG-(Kb Db)null (IAnull) (DKO) (Jackson Labs; 025216) >8 wk old mice. Post-surgery, mice were single housed and monitored for up to 18 weeks after transplantation. For allograft rejections assays, 50M of human primary peripheral mononuclear cells (PBMCs) were injected intraperitoneally.

Kidney grafts were harvested, processed for sequencing or stained as described below.

For in vivo glucose stimulated insulin secretion (GSIS) and graft function monitoring, Human insulin and C-peptide were quantified from mouse blood plasma collected from the facial vein at fasted (overnight for 16h) and 30 min post-injection of glucose at 2g/kg bodyweight.

Secretion assays

Mouse plasma was used to measure human insulin or human C-peptide by ELISA (ALPCO Diagnostics, 80-INSHUU-E10 and 80-CPTHU-E10 respectively) as described in the manufacturer’s protocol. Supernatant from treated cell cultures was used to measure human CXCL10 by ELISA (BioLegend, 439904) as described in the manufacturer’s protocol.
Flow cytometry (FC)

All stained cells were analyzed using the Attune NxT (Thermo Fischer) flow cytometer. Data analysis was performed with FlowJo (BD) software. For IFN induced protein detection, SC-islets were treated with 20ng/ml recombinant human (rh)IFNγ, 48hr prior to FC staining. For intracellular staining (ICS) of CXCL10, SC-islets were also treated with 2μM monensin for 6hrs (BioLegend, #420701) to block secretion.

Intracellular staining

Differentiated SC-islet clusters, sampled from suspension cultures (1–2 ml), were dissociated using TrypLE Express (Gibco, 12604013) at 37 °C, mechanically disrupted to form single cells, fixed using 1% paraformaldehyde (PFA) overnight and stored 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 (1 h at room temperature), washed three times with Perm/Wash Buffer, incubated with secondary antibodies in Perm/Wash Buffer (1 h at room temperature), washed three times and resuspended in Perm/Wash Buffer.

Surface marker staining

PBS containing 4% 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 at 4 °C), washed three times with blocking buffer, fixed using 1% PFA overnight and stored at 4 °C.

Human lymphocyte staining from mouse whole blood

PBS containing 2% FBS was used as blocking and staining buffer. Cell suspensions from whole blood were washed with blocking buffer and preincubated with Mouse BD Fc Block™ (anti CD16/CD32 Ab, BD Biosciences). Cells were then incubated in blocking buffer with conjugated antibodies (1h at at 4 °C), washed with blocking buffer and fixed with BD FACS lysing solution (BD Biosciences) to lyse red blood cells. For analysis of human immune cells murine cells were identified and excluded by staining with anti-murine CD45 Ab. %CD4+ and %CD8+ were gated from hCD45+/hCD3+

Immunofluorescence Microscopy

Differentiated clusters or tissues were fixed in 4% PFA for overnight at 4 °C, transferred to 30% sucrose overnight, frozen in OCT (Tissue-Tek) and cryostat sectioned. For staining, slides were incubated in CAS block (ThermoFisher, 008120) with primary antibody overnight at 4 °C, washed three times, incubated in secondary antibody for 2 h at room temperature, washed, mounted in ProLong Diamond Antifade Mountant with DAPI, covered with coverslips and sealed with clear nail polish. Representative regions were imaged using Zeiss.Z2 with Apotome microscope.

Magnetic enrichment using CD49a
Stage 6 SC-islet clusters were dissociated using TrypLE Express for 20 min at 37°C. Cells were then quenched with S3 + 10% FBS and spun down. Remaining undissociated cell clusters were mechanically dissociated using a P1000 pipette. The dissociated 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 Biosciences) antibody, covered from light and agitated every 3 minutes. Stained cells were washed twice with 15 mL of sorting buffer by spinning down (5 min, 300 g) and resuspended at their initial density of 10 million cells per 300 μl. To label with microbeads, 40 μL of anti-PE UltraPure MACS microbeads (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. Successful PE enrichment was verified by live-cell flow cytometry on an Attune NxT (Invitrogen) flow cytometer.

**Human primary immune cell isolation and co-culture assays**

We obtained healthy donors’ blood derived apheresis collars from Brigham and Woman’s Hospital. Human PBMCs were isolated using the density gradient medium, Ficoll-Paque Plus (GE health care life sciences, 17144002) and the SepMate tubes (Stem Cell Technologies, 85450); T cells and NK cells were isolated using RosetteSep Human T Cell Isolation Kit (Stem Cell Technologies, 15061 and 15065, respectively). PBMCs or isolated cells were cultured in T-cell media: 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), MEM Non-Essential Amino Acids (ThermoFisher Scientific, 11140050).

For co-culture assays, SC-islets were used as target cells. SC-islet clusters were dissociated using TrypLE Express (Gibco, 12604013) at 37 °C, mechanically disrupted to form single cells, ten thousand cells were plated per well on 96-well V-shaped bottom plates and allowed to reaggregate for 48hrs in S3 media. SC-islets were then treated with antibodies (as described) and/or thapsigargin, 5uM (Sigma Aldrich, T9033) for 5 hours before the co-culture assay. Cells were washed to remove residual thapsigargin and Immune cells (PBMCs/T-cells/NK), pre-labeled with Cell Trace Violet (CTV; ThermoFisher Scientific, C34571) were added at a ratio of 5:1 (immune;target cells) in T-cell media.

**T cell activation and proliferation assays**
After a 48 hours co-culture, the PBMCs/T-cell cell fraction was removed from top cell suspension (SC-islet cluster settle in the bottom). A portion of the cells were taken for flow cytometry staining for CD3+ T cells and activation markers CD69 and CD25. Results are presented as Medial Fluorescent Intensity (MFI), adjusted to baseline MFI of T-cells in an unstimulated PBMC control. The other portion was seeded on 96-well low adherence round bottom plates and allowed to expand for 7-days in T-cell media containing 20 U/ml rhIL-2. Cells were taken for flow cytometry staining and analysis of CD3+ T cells gated cells, while the frequency of CTV negative cells served as a marker for proliferated cells. PBMCs/T cells activated with Dynabeads Human T-Activator CD3/CD28 beads (ThermoFisher Scientific, 111.61) for 48 hours were used as positive control for both assays.

**NK cell activation/degranulation assays**

During co-culture of SC-islets and NK cells, a CD107a antibody was added to bind internalizing degranulation marker CD107a. After a 48 hours co-culture, the NK cell fraction was removed from top cell suspension and taken for flow cytometry staining and analysis of CD56+CD107a+ cells.

**SC-islet and SC-β apoptosis assays**

After a 48 hours co-culture, the SC-islet clusters were dissociated using TrypLE Express at 37 °C, to form single cells, fixed O.N. with 1% formaldehyde (CytoFix; Fisher Scientific, BDB554655) and then stained for flow cytometry using C-peptide antibody and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) for apoptosis with the In Situ Cell Death Detection Kit (Roche Diagnostics, 12156792910). Percent apoptosis was calculated relative to baseline TUNEL staining of SC-islets with no PBMCs.

**Tissue/cell preparation and library preparation for single cell RNA sequencing**

For graft extraction, mice were euthanized, and the transplanted kidney was removed. SC-islets graft was peeled off the kidney, was sliced into small pieces and digested in 2mg/mL collagenase D (Sigma, 11088858001) in RPMI (GIBCO, 11875-085) for 45 min in 37 °C. Additional breaking was used by pipetting and by filtering through a 40μm cell strainer. Cells were then centrifuged and resuspended in 0.5% BSA in PBS and a magnetic mouse depletion kit (Miltenyi, 130-104-694) was used to remove residual mouse cells.

For co-cultured experiment, cells were collected from 96 wells after 48hrs of co-cultured and SC-islets dissociated using TrypLE Express at 37 °C, to form single cells and quenched with S3 media.

Harvested cells from both sources were centrifuged, resuspended in 0.04% BSA in PBS, counted (LUNA-FX7 Automated Cell Counter), adjusted to 1000 cells/μl and sent to the Harvard University Bauer Sequencing Core for 10X Chromium Single Cell 3’ Library preparation and sequencing.
All samples were loaded into Chip G per the user guide from 10x Genomics, no alterations were made at any step of the protocol (Part No. CG000315). GEMs were formed targeting 10,000 cells and reverse transcription completed immediately after. The cDNA was cleaned from the GEM reagents, amplified for a total of 11 cycles and verified via TapeStation (Agilent Technologies). Amplified cDNA was diluted and ran on the 4200 TapeStation instrument using High Sensitivity D5000 tape and reagents (Part No. 5067-5592 & 5067-5593). The amplified cDNA was fragmented, end repaired, and A-tailed followed by adaptor ligation, and PCR amplification for a total of 12 cycles with each sample receiving a unique set of dual indices (Part No. 1000215). Final libraries were diluted and ran using the High Sensitivity D5000 tape and reagents (Part No. 5067-5592 & 5067-5593) on the 4200 TapeStation (Agilent Technologies). Libraries were quantified via Kapa qPCR using the Complete Universal Kit (Part No. 07960140001, Roche Sequencing Solutions) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Libraries were sequenced on an Illumina NovaSeq instrument using the parameters outlined in the user guide (Read1: 28 bp, i7 index: 10 bp, i5 index: 10 bp, Read2: 90 bp).

**In vivo single cell RNA sequencing analysis**

Raw sequencing files were processed using Cell ranger 5.0.0 (10X Genomics). Illumina basecall files were converted to fastq format. Samples were aligned to the GRCh38 genome using STAR aligner (Dobin et al., 2013). Graft samples were also aligned to a GRCh38/mm10 hybrid genome to obtain the human/mouse percentage for each cell. Cells with less than a 50% of aligned reads mapping to the human reference, were discarded.

Processed scRNASeq data was analyzed in R version 4.0.3 using the Seurat suite version 4.0.6 (Hao et al., 2021). Count matrices were loaded into a Seurat object filtering out genes detected in less than 3 cells and cells with less than 200 genes. Quality control filtering was adjusted for each sample as indicated in **Supplementary File 2**. A total of 35,647 cells from 14 samples passed these quality control steps (**Supplementary File 2**). Data normalization and scaling were performed using Seurat’s SCTransform function adding the mitochondrial percentage as an additional regressed variable.

Samples were integrated in two ways. Grafts injected with PBMCs (hPi) and graft samples without injection (control) were integrated to assess the graft component. hPi samples and control PBMC samples were integrated to explore differences in the immune component. In both cases, samples were integrated following the default integration guidelines for SCT transformed datasets from [https://satijalab.org/seurat/articles/integration_introduction.html](https://satijalab.org/seurat/articles/integration_introduction.html), with 3000 variable features.

To explore transcriptional heterogeneity and to perform initial cell clustering, principal component analysis and nonlinear dimensional reduction using Uniform Manifold Approximation and Projection (UMAP) (McInnes et al., 2018) were applied using 40
dimensions and resolution 1.0 for the integrated hPi and control samples, and 30 dimensions and 0.8 resolution for the integrated hPi and PBMCs. Cell types were assigned to clusters using known marker genes.

Differential expression at the gene level between sample types was performed with DESeq2 1.30.1 (Love et al., 2014) (https://bioconductor.org/packages/DESeq2/) using a pseudobulk approach where counts are aggregated for each cluster at the sample level.

Seurat’s plotting functions were used to obtain violin plots. Tidyverse packages (Hadley Wickham (2017). tidyverse: Easily Install and Load the 'Tidyverse'. R package version 1.2.1. https://CRAN.R-project.org/package=tidyverse) were used for data processing.

The distribution of the SC alpha, SC_beta and SC_EC clusters were further analyzed. By selecting the cells from these clusters and reclustering them. Principal component analysis and nonlinear dimensional reduction using UMAP (McInnes et al., 2018) were applied using 10 dimensions and resolution 0.1.

**In vitro single cell RNA sequencing analysis**

Raw sequencing files were processed using Cell ranger 5.0.0 (10X Genomics). Illumina basecall files were converted to fastq format. Samples were aligned to the GRCh38 genome using STAR aligner (Dobin et al., 2013).

Processed scRNAsSeq data was analyzed in R version 4.0.3 using the Seurat suite version 4.0.6 (Hao et al., 2021). Count matrices were loaded into a Seurat object filtering out genes detected in less than 3 cells and cells with less than 200 genes. During quality additional filtering was used. Cells with less than 1000 genes, less than 3000 UMIs or with a higher mitochondrial percentage than 15%, were discarded. A total of 42,922 cells from 8 samples passed these quality control steps. Data normalization and scaling were performed using Seurat’s SCTransform function adding the mitochondrial percentage as an additional regressed variable. All samples were integrated using Harmony (Korsunsky et al., 2019) regressing the time point variable.

To explore transcriptional heterogeneity and perform initial cell clustering, principal component analysis and nonlinear dimensional reduction using UMAP (McInnes et al., 2018) were applied using 20 dimensions and resolution 0.3. Cell types were assigned to clusters using known marker genes. An evaluation of the UMAP plots showed that 61 cells had been overcorrected (i.e: endocrine cells in the PBMCs only samples or vice versa) during the integration and were removed from the analysis.

For differential expression and dot plot generation, data was also processed using Scanpy (version 1.8.1) (Wolf et al., 2018) to annotate the cell types. Cell ranger output was filtered to retain cells with no more than 15% MT transcripts and 4000 highly variable genes were identified using the highly_variable_genes function with the Seurat v3 option.
Cell types were assigned to clusters using known marker genes. Results of identified cell types were then visualized using dotplot plotting functions.

Using the annotations, differential expression was measured using fold change between treatment conditions and the Mann-Whitney hypothesis test p-values after correction for multiple comparisons using the FDR procedure implemented in the multipletests function from the statsmodels library.

**Lentivirus Preparation and Transduction**

Lentiviral particles were produced by transfecting 293T cells (Takara Bio) with the packaging vectors pHDM-vsvg, pHDM-tat, pHDM-rev, and pHDM-gag/pol along with lentiviral backbone vectors using the TransIT-293 transfection reagent (Mirus). Lentiviral particles were collected 48 h and 72 h post transfection and concentrated 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.

The lentiviral vector lentiCRISPRv2 [a gift from Feng Zhang (Addgene plasmid # 52961 ; http://n2t.net/addgene:52961; RRID:Addgene_52961 (Sanjana et al., 2014)] was used to clone guide RNAs (gRNA sequences described in Key resource table; custom clone service from GensScript). For overexpression, lentiviral vectors containing open reading frame (ORF) sequences of eGFP, CD274, CXCL10 and SOCS1, cloned into pLX_307, were obtained from the Broad institute inventory.

For transduction, cell clusters collected from spinner flask suspension cultures were dissociated in TrypLE Express (Life Technologies) for 7 min, followed by mechanical dissociation and centrifugation at 300 g for 5 min at room temperature (RT). Cell pellets were resuspended at a density of 2.5 million cells/mL in the stage-matched medium with polybrene reagent (Santa Cruz) at 10 μg/mL. Single-cell suspensions were combined with concentrated lentiviral particles and allowed to reaggregate in spinner flasks, in a humid 37 °C incubator and 5% CO₂.

**Whole genome CRISPR screen in vivo and analysis**

Brunello pooled library pooled plasmid DNA in a 1 vector system (lentiCRISPRv2 backbone; Addgene # 73179) was obtained from the Broad institute Genetic Perturbation Platform (GPP), to generate pooled lentivirus. Lentivirus and SC-islet transduction was as described above. To determine a titer that will lead to a multiplicity of infection (MOI) that is less than 1, SC-islets were seeded in 6 well plates and treated with different virus volumes per cell number. After 2-3 days transduced SC-islets were treated with puromycin (9μg/ml) and cell counts were taken after 4 days to evaluate cell death ratios compared to a control well with no selection.

Library transduced (LT) SC-islets were transplanted under the kidney capsule of NSG-MHCnull mice and PBMCs were injected at week 4 after transplantation. Full experiment layout is described in the results section and in Figure 1. Retrieved graft tissue were homogenized (Polytron PT 1200E, KINEMATICA) and a Quick-DNA™ Midiprep Plus Kit.
(Zymo Research, D4075) was used to extract genomic DNA (gDNA). gDNA was submitted to GPP for PCR amplification of the integrated construct containing a barcode sequence, and Illumina sequencing to determine the abundance of each gRNA in each sample. Sequencing resulted in 132,183,231 matching reads which consists of a 82% of total reads. PoolQ v3 software was used to deconvolute sequencing files and quantitate gRNA barcodes counts in each sample (https://portals.broadinstitute.org/gpp/public/software/poolq).

To identify genes that may influence graft depletion, we estimate the gene by environment interactions of KO allele targets and PBMC graft environments (hPi), which can be interpreted as a difference in PBMC depletion between the KO and WT alleles. A separate model is fit for each of the target genes using observed sequenced read counts as the outcome and all available data across mouse and guide replicates. Read counts are modeled as negative binomial, with additive random effects for targeting guide and mouse, and fixed effects representing the graft allele and condition. The full model is given by:

$$\log(\text{counts}) \sim 1+\text{KO}+\text{PBMC}+\text{KO} \times \text{PBMC}+(1|\text{Mouse})+(1|\text{Guide})$$

Significance of the KO*PBMC interaction is evaluated using a likelihood ratio test comparing with a reduced model that only includes the additive effects. All of the models are fit using the glmer.nb function from the lme4 R package. To correct for multiple comparisons, p-values are adjusted using the Benjamini-Hochberg FDR procedure, implemented in the R function p.adjust with option ‘fdr’. A filter was applied to remove any gene hits that were not expressed in at least 10% of either SC-α or SC-β cells in at least one experimental condition across in vivo and in vitro scRNA-seq experiments (Figures 1 and 2). To visualize the results from a subset of selected CRISPR targets, we produced boxplots of the full model predictions for each allele and treatment combination.

**Generation of hESC knockout lines**

Gene modified (GM) lines were generated by homology directed repair (HDR), via nucleofection of a Cas9/sgRNA ribonucleoprotein complex (RNP) and a targeting vector. The targeting vector (OriGene) was designed to facilitate the in-frame integration of GFP or luciferase cassettes with puromycin resistance into exon 2 or exon 3 of the CXCL10 or STAT1 loci, respectively (Figures 4A and 4B). Culture and expansion were performed on Matrigel® (Corning) coated plates with mTeSR™ Plus media (Stem Cell Technologies). Cells were clump passaged every 72h or 96h. For nucleofection Hues8 monolayers were dissociated into single cells with Accutase (Stem Cell Technologies), and 1x10^6 cells were nucleofected using the 4D-Nucleofector (Lonza) with 5 μg targeting plasmid and RNP (120 pmol targeting sgRNA and 104 pmol Alt-R Cas9 (IDT), according to the manufacturer's instructions. Nucleofected cells were then plated in a matrigel-coated tissue culture plate containing mTeSR™ Plus, cloneR (Stem Cell Technologies) and 7.5 μM RS-1 (Xcessbio). After 48h, puromycin (0.5μg/ml) was added and surviving colonies were transferred to 96-well plates for PCR and expansion. Genomic DNA was
extracted and purified using Quick-DNA™ (Zymo Research), and target cassette knock-in was confirmed by PCR analysis (Figure S4A) using Phusion® Hot Start Flex 2x master mix and primer sets (see key resource table) that amplify the wild type and targeted genomic alleles (blue and red arrows, respectively in Figure 4A and 4B). Several heterozygous clones were acquired from each knockout, and we selected a CXCL10-GFP (C10G) clone and a STAT1-luciferase (ST1L) clone that contained the integrated transgene in one allele along with a nonhomologous end-joining (NHEJ) mutation in the intact endogenous allele, determined by PCR and Sanger (GENEWIZ) sequencing (Figure S4A). Overall, C10G and ST1L contained null mutations in both alleles.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed by unpaired Student’s t-tests as indicated, using the Prism v9. All data are presented mean ± SD. p<0.05 was considered statistically significant. Sufficient sample size was estimated without the use of a power calculation. Data analysis was not blinded.

**GRAPHIC ILLUSTRATIONS**

Graphic illustrations in the manuscript were created with BioRender.com under BioRender's Academic License Terms.

**DATA AVAILABILITY**

scRNA-seq and pooled CRISPR screen data generated during this study are available at NCBI GEO accession number GSE200104, and composed of listed SubSeries related to specific experiments described in this paper.

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