Engineering of immune checkpoints B7-H3 and CD155 enhances immune compatibility of MHC-I−/− iPSCs for β cell replacement

Graphical abstract

Highlights
- MHC-I−/− cells are killed by NK cells via missing-self recognition mechanisms
- Stem cell-derived pancreatic progenitors (PPs) express B7-H3 and CD155 NK ligands
- B7-H3/CD155 knockout (KO) prevents killing of the MHC-I−/− cells by NKs in vitro
- B7-H3/CD155 KO increases immune compatibility of MHC-I−/− PPs in a mouse model

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In brief
Chimienti et al. disrupt via CRISPR-Cas9 the NK-activating ligands B7-H3 and CD155 in human stem cell-derived pancreatic progenitors with an MHC class I-null background, allowing escape from the NK-mediated missing-self recognition and increasing chances of survival after transplantation in NSG mice humanized with donor-derived allogeneic NK cells.
Engineering of immune checkpoints B7-H3 and CD155 enhances immune compatibility of MHC-I−/− iPSCs for β cell replacement

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INTRODUCTION

Over the past decade, induced pluripotent stem cells (iPSCs) have gained considerable attention as a potentially unlimited source of therapeutically relevant cells for regenerative medicine. However, clinical application of iPSCs faces some disease-specific issues. In particular, although patients with type 1 diabetes (T1D) could benefit from therapies based on autologous iPSC-derived insulin-producing cells, the presence of preexisting autoimmune responses against β cell antigens represents a significant hindrance. Likewise, cell therapy with allogeneic iPSCs would result in CD8+ T cell-mediated allograft rejection owing to surface expression of highly polymorphic human leukocyte antigens (HLAs) (Taylor et al., 2011). Several solutions have been proposed to overcome these obstacles, including an HLA-typed stem cell biobank (Taylor et al., 2012), the inactivation of major histocompatibility complex class I (MHC-I) and class II (MHC-II) (Muttapally et al., 2018), and the overexpression of the antiapoptotic CD47 (Deuse et al., 2019) or the CD8+ T cell-inhibitory molecules CTLA4-Ig/PD-L1 (Rong et al., 2014). Among these strategies, the most promising remains the abrogation of MHC-I by genetic inactivation of the β2-microglobulin (B2M) gene (Wang et al., 2015; Norbnop et al., 2020; Petrus-Reurer et al., 2020), the product of which is required for surface expression of classical and non-classical HLA-I molecules. However, MHC-I-deprived iPSCs and their derivatives can trigger natural killer (NK) cytotoxicity via missing-self recognition mechanisms (Bix et al., 1991; Liao et al., 1991). The latest approaches to avoid this drawback have been the ectopic expression of tolerogenic non-classical HLA-I molecules, such as HLA-E (Gornalusse et al., 2017) and HLA-G (Zhao et al., 2014; Shi et al., 2020), since these molecules are high-affinity ligands of the inhibitory CD94:NKG2A and LIR-1 receptors, expressed on most NK cells (Navarro et al., 1999). However, the responsiveness of NK cells depends on a fine balance between cross-antagonizing activating and inhibitory signals, which are differentially integrated by NK cell subsets and may vary according to disease context. For this reason, a single engineering strategy might not be sufficient to fully protect transplanted cells from NK immune attack. To this end, NK-activating signals may represent interesting targets to circumvent missing-self recognition. Moreover, there are no studies showing the
expression pattern of activating ligands in iPSCs and their pancreatic β cell derivatives and how these ligands can affect the outcome of future cell replacement strategies in T1D. In this study, we characterize the expression profile of the main NK-activating ligands on human iPSCs before and during in vitro differentiation into functional β cells. Furthermore, we investigate the impact of the most expressed extracellular activating ligands B7-H3 and CD155 on missing-self recognition mechanisms, assessing the activity of different NK cell subsets against MHC-I-deprived iPSCs and their pancreatic derivatives following the abrogation of such molecules through CRISPR-Cas9 technology. Finally, we corroborate the relevance of activating signal exploitation by in vivo experiments on NK-mediated acute allograft rejection, proposing the knockout of B7-H3 and vating signal exploitation by Cas9 technology. Following the abrogation of such molecules through CRISPR-Cas9 technology. Further, we corroborate the relevance of activating signal exploitation by in vivo experiments on NK-mediated acute allograft rejection, proposing the knockout of B7-H3 and CD155 in iPSC-derived β cells with an MHC-I-null background as an alternative strategy to evade NK cytotoxic activity.

RESULTS

Classical and non-classical HLA class I and HLA class II molecule expression on iPSCs before and during in vitro differentiation into pancreatic β cells

The expression of allogeneic HLA class I molecules can strongly influence the immune response by promoting allograft rejection by CD8+ T lymphocytes and modulating NK cell function via the killer cell immunoglobulin-like receptors (KIRs) and the CD94/NKG2A/C receptors. Analogously, HLA class II recognized by islet-infiltrating CD4+ T cells may play an immunopathogenic role, affecting transplant outcome. We thus evaluated, by flow cytometry, the expression of B2M protein, classical (HLA-A, -B, and -C) and non-classical (HLA-E and HLA-G) HLA-I molecules, and the HLA-DR/-DP/-DQ class II molecules on the CGTRCiB10 iPSC line, both before and during in vitro differentiation to β-like insulin-producing cells, with or without interferon (IFN)-γ, a well-known inducer of MHC expression in many tissues. Undifferentiated cells displayed high levels of B2M (1,652 ± 70 mean fluorescence intensity [MFI]) and HLA-A/B/C (688 ± 183 MFI) (Figures 1A and 1B), whereas we observed significant downregulation of both B2M (10-fold decrease) and HLA-A/B/C (>5-fold decrease) when iPSCs differentiated to posterior foregut (PF), followed by a further reduction in transitioning to pancreatic endoderm (PE; >10-fold decrease). On the other hand, the late stages of differentiation were characterized by re-expression of B2M (431 ± 66 MFI) and HLA-A/B/C (368 ± 91 MFI). No expression of HLA-E/G and HLA-II molecules was detected either on undifferentiated cells or during pancreatic differentiation (Figure 1B). Exposure to IFN-γ increased from 2- to 5-fold the surface levels of B2M and HLA-A/B/C and induced de novo expression of HLA-E/G molecules in undifferentiated iPSCs and during all the steps of differentiation, while no effect was observed on expression levels of HLA class II molecules (Figures 1A and 1B).

Profile of NK-activating ligands on iPSCs and during their in vitro differentiation into pancreatic β cells

DNAM-1 (CD226) (Shibuya et al., 1996), NKG2D (Lanier, 2015), and the members of the natural cytotoxic receptors (NCRs) family NKp30 (CD337) and NKp46 (CD338) are pivotal activating NK receptors (Koch et al., 2013; Kruse et al., 2013). We evaluated mRNA and protein expression on an iPS line of the CD226 ligands PVR (CD155) and Nectin2 (CD112); the NKG2D ligands MICa, MICb, ULBP1, ULBP2, ULBP3, ULBP4 (RAET1E), ULBP5, and ULBP6; the CD337 ligands BAG6/BAT3 and VIM; and the CD338 ligand properdin (CFP). In addition, we assessed the tumor-specific CD337 ligand B7-H6 and its closely related co-stimulatory molecule CD276 (B7-H3) (Brandt et al., 2009). Both mRNA and protein quantification confirmed the expression of some activating NK receptor ligands in undifferentiated iPSCs (Figures 2A–2C). As shown in Figure 2B, 98.9% ± 0.8% and 85% ± 6.4% of iPSCs were positive for B7-H3 and CD155, respectively. MICa+ and RAET1E+ cells represented 65.5% ± 6% and 36.9% ± 9.3% of the overall iPSC population, respectively. Immunofluorescence assay demonstrated the intracellular expression of the CD337 ligands BAG6/BAT3 and VIM (Figure S2A), while no significant expression of B7-H6, MICb, CFP, or other ULBPs was detected (Figures 2A, 2B, and S2A). CD112 displayed transcriptional levels significantly higher than those of other ligands (p < 0.001; Figure 2B), but protein appeared to be totally retained in the cytoplasm (CD112+ cells: intracellular staining >99%, membrane staining 0%; Figure S2B).

We then wondered if the expression pattern of these ligands was a prerequisite of our model or a common feature of stem cell lines, either induced pluripotent or embryonic. Consequently, we evaluated the surface expression of NK ligands in four other iPSC lines (CB4, NeoFiWT, DRI1 clone #11, DRI2 clone #3) and in the H1-hESC human embryonic stem cell line. As shown in Figure 2C, all stem cell lines expressed comparable levels of both CD155 and B7-H3. Interestingly, we found differences in surface expression of CD112, which was exposed at different levels on the plasma membrane of the CB4 (23.54% ± 5.39%), NeoFiWT (12.47% ± 3.38%), DRI1#11 (32.19% ± 3.98%), and H1 (35.54% ± 8.32%) cell lines. Significant variability was observed for MICA and RAET1E levels (ANOVA, p < 0.01; Figure 2C). A small percentage of CB4 and NeoFiWT was also positive for MICb, whereas the embryonic cell line was characterized by 22.42% ± 3.74% of MICb+ cells, according to previously reported findings (Suárez-Alvarez et al., 2010).

Since B7-H3 represented the most expressed surface ligand on iPSCs, we chose to extend our investigations to other B7 family genes. The B7 family consists of structurally related immune-regulatory surface proteins (Collins et al., 2005; Xu et al., 2016). To evaluate in depth the expression profile of B7 family genes, we analyzed a single-cell RNA (scRNA) dataset previously collected by our laboratory (Pellegrini et al., 2020), following validation of the activating (B7-H2 and B7-H6) and the inhibitory (B7-H1 and B7-H4) ligands by quantitative real-time PCR. We confirmed by scRNA sequencing the high expression of B7-H3 in undifferentiated iPSCs, whereas low levels of B7-H2 were detected only by quantitative real-time PCR experiments (Figures S2C and S2D). B7-H6 was detected in few cells of the scRNA dataset, and validation of its transcriptional levels confirmed spurious or null expression of this ligand in our model (Figure S2D).

To evaluate the impact of inflammatory cytokines on NK-activating ligand expression, we exposed iPSCs to IFN-γ and/or tumor necrosis factor (TNF)-α. IFN-γ downregulated the CD314
Figure 1. Classical and non-classical HLA class I and HLA class II molecule expression on iPSCs before and during in vitro differentiation into pancreatic β cells

(A) Representative histograms of surface staining with monoclonal antibodies (mAbs) for B2M, classical/non-classical HLA-I, and HLA-II molecules on undifferentiated iPSCs, under basal culture conditions (red) and after treatment with IFN-γ (orange; 10 ng/mL for 12 h). Staining with the related isotype control is shown in light blue.

(B) B2M, HLA-II, and classical/non-classical HLA-I expression during pancreatic differentiation of iPSCs in the presence or absence of IFN-γ (10 ng/mL). Data are expressed as mean fluorescence intensity (MFI) and are representative of a total of three experiments (n = 3), intended as independent in vitro differentiations of the same iPSC clone (CGTRC1B10) at different passages. iPSC, undifferentiated iPSCs; PF, posterior foregut; PE, pancreatic endoderm; EN, endocrine cells; iBeta, iPSC-derived β cells. Error bars indicate standard deviation. Statistical significances were obtained by one-way ANOVA following Tukey’s post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001.
ligands MICA and RAET1E, whereas TNF-α increased the RAET1E expression only (Figure S3). Expression of NK-activating ligands was also evaluated during in vitro differentiation into insulin-producing cells (Figures 2D–2F, S2F, and S2G). B7-H3 mRNA levels increased over 2-fold during transition to PE, then in the last stages they returned to those observed in undifferentiated cells. Surface expression of B7-H3 does not change significantly, except at the end of differentiation, where we observed a higher variability, likely due to the population heterogeneity of the terminally differentiated cells (Figures 2D and 2E). CD155 displayed a slightly variable, swinging surface expression profile during differentiation, although its mRNA levels grew steadily, increasing over 20-fold in terminally differentiated β cells (iBeta) compared with the undifferentiated ones (Figures 2D and 2E). CD112 was retained in the cytoplasm until the end of differentiation, when surface expression emerged in about 32.17% ± 2.95% of the cells. In contrast, both MICA and RAET1E decreased during differentiation, since only 8.23% ± 1.34% and 2.43% ± 1.01% positive cells were detected, respectively (Figure 2D). A gradual increase during differentiation was observed for the intracellular NCR ligands VIM and CFP (over 10- and 80-fold, respectively), while the CD337 ligand BA9G6/BAT3 expression levels remained essentially stable (Figures S2F–S2H). Commitment to endocrine pancreas showed cells expressing variable levels of B7-H1, B7-H2, B7-H4, B7-H5, and B7-H6 as well (Figure S2I). The scRNA-derived expression data were confirmed by quantitative real-time PCR only for B7-H2 and B7-H4 (p < 0.001) (Figure S2L). No expression of MICB or other ULBPs was detected during differentiation (data not shown).

**Blocking of NK-activating receptor ligands inhibits in vitro killing of MHC-I–/– iPSC lines by an allogeneic CD16+ NK cell subset**

To evaluate the impact of NK-activating ligands on missing-self recognition mechanisms, we generated MHC-I-null iPSCs by CRISPR-Cas9-directed disruption of the B2M gene, preventing HLA-I surface expression. The B2M gene targeting efficiency was about 40%–50%, and MHC-I-null clones were selected by cell sorting based on the expression of B2M and HLA-ABC (Figures S4A and S4B). Moreover, we also engineered B2M–/– iPSC subclones to induce a stable surface expression of HLA-E*0103 molecules bound to the HLA-G-derived leader peptide VMAPRTLFL (Figures S4A and S4B), as in the previously described immune-escape cell model (Gomalusse et al., 2017). Due to the low homologous recombination efficiency of the pepB2M-HLA-E-RES-eGFP construct, we subjected the cells to nocodazole treatment to temporarily block them in G2/M phase, when direct homology-directed repair (HDR) is favored over the non-homologous end-joining (NHEJ) repair system (Lin et al., 2014). Thus, we increased HDR efficiency up to 7%, allowing cell sorting of HLA-E-expressing cells by using GFP as a selection marker (Figure S4B).

B2M–/– and B2M–/–/HLA-E+/+ iPSCs maintained pluripotency, as demonstrated by the OCT4, SOX2, and NANOG expression (Figure S4C), and preserved their ability to differentiate into the three germ layers in vitro (data not shown). Edited cells retained a normal karyotype and remained phenotypically stable over 30 passages after gene manipulation (data not shown). Furthermore, the expression pattern of NK-activating ligands on both B2M–/– and B2M–/–/HLA-E+/+ iPSCs was not affected by gene editing (Figure S4D).

We then tested whether allogeneic CD8+ T lymphocytes and donor-derived CD16+ NK cells recognized in vitro unedited B2M–/– and B2M–/–/HLA-E+/+ iPSCs (Figure 3). Allogeneic CD8+ effector T cells were derived by priming donor-derived peripheral blood mononuclear cells (PBMCs) with gamma-irradiated HLA-expressing target cells (MOLT-4, HLA-A*0101), to boost for T cell recognition of our model of wild-type iPSCs (CGTRCiB10, HLA-A*0101). CD16+ NKs were magnetically sorted from PBMCs of healthy donors, and each donor-derived CD16+ NK primary line was used individually, as a biological replicate, after preactivation by exposure to IL-2 and IL-12. Unedited iPSCs were killed by CD8+ cytolytic T lymphocytes (52.3% ± 5.82% propidium iodide-positive [PI+] cells at 1:1 effector:target [E:T] ratio; 71.67% ± 1.17% PI+ cells at 5:1 E:T ratio), comparable to the positive control cell line MOLT-4 (54.67% ± 2.32% PI+ cells at 1:1 E:T ratio; 72.12% ± 3.32% PI+ cells at 1:5 E:T ratio; 72.12% ± 3.32% PI+ cells at 1:10 E:T ratio).

**Figure 2. Expression pattern of extracellular NK-activating ligands on undifferentiated iPSCs and during in vitro differentiation into pancreatic β cells**

(A) Representative histograms of surface staining with mAbs for NK ligands (red) on undifferentiated iPSCs. Staining with the related isotype control is shown in light blue.
(B) Percentage of positive cells (mean ± SD; n = 6 independent experiments) for surface NK ligands measured by flow cytometry (top) and relative mRNA expression (mean ± SD; n = 3 independent experiments using batches of the same cell line at different passages) of NK ligands assayed by quantitative real-time-PCR on CGTRCiB10 iPSCs. GAPDH was used as a normalizer gene and data are plotted on a logarithmic scale (bottom).
(C) Surface NK ligand expression by flow cytometry in both iPSC (CGTRCiB10, C8, NeoFiWT, DRI1#11, DRI2#3) and embryonic stem cell (H1-hESC) lines (mean ± SD; n = 6 independent experiments). Statistical significances refer to difference between samples in the expression of related markers and were inferred by one-way ANOVA: ***p < 0.001.
(D) Percentage of cells (mean ± SD; n = 3 independent in vitro differentiations) positive for surface NK ligands on iPSCs and during different stages of pancreatic differentiation. Fold change in separation index (mean ± SD; n = 3 independent in vitro differentiations) between the stained population and the negative one (isotype control) of the CGTRCiB10 clone as relative variation in fluorescence intensity during pancreatic differentiation compared with undifferentiated cells. (E) Relative mRNA expression (mean ± SD; n = 3 independent in vitro differentiation experiments on the CGTRCiB10 clone at different passages) of the NK ligands during pancreatic differentiation (CGTRCiB10 clone). GAPDH was used as the normalizer gene and data are reported as fold change over undifferentiated cells.
(F) Representative histograms of surface staining with mAbs for NK ligands (red) or isotype control (light blue) on terminally differentiated CGTRCiB10 cells (iBeta). iPSC, undifferentiated iPSCs; PF, posterior foregut; PE, pancreatic endoderm; EN, endocrine cells; iBeta, iPSC-derived β cells. Error bars indicate standard deviation. Each experiment has been carried out with the indicated cell line at different passages and states of confluence. Statistical significance obtained with two-tailed t test refers to the comparison with iPSC stage: *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 3. Blocking of either NK-activating receptors or their ligands inhibits killing in vitro of MHC-I−/− iPSC lines by allogeneic CD16+ NK cells.

(A) CD8+ T cells and NK killing against unedited and edited iPSCs with two target:effector ratios. Jurkat and MOLT-4 were used as positive and negative controls, respectively.

(B) Cytotoxicity test performed on wild-type, B2M−/−, and B2M−/−/HLA-E+/+ iPSCs after preincubation of NK cells with blocking antibodies against NK receptors. Irrelevant IgG antibody was used as mock control. Assays were performed with two target:effector ratios.

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5:1 E:T ratio). As expected, B2M$^{−/−}$ and B2M$^{−/−}$/HLA-E$^{−/−}$ iPSCs escaped CD8$^+$-mediated killing (95% CI 0.41%–1.14% and 0.98%–5.63% PI$^+$ cells, respectively), showing cell death levels equal to those of the negative control cell line Jurkat (95% CI 1.21%–2.29% PI$^+$ cells). On the other hand, B2M$^{−/−}$ iPSCs triggered CD16$^+$ NK cell killing by missing-self recognition mechanisms (46.21% ± 2.82% PI$^+$ cells at 1:1 E:T ratio; 63.24% ± 2.41% PI$^+$ cells at 5:1 E:T ratio). As expected, surface expression of HLA-E molecules protected the B2M$^{−/−}$ iPSCs from NK immune attack (2.95% ± 1.37% PI$^+$ cells at 1:1 E:T ratio; 2.85% ± 0.36% PI$^+$ cells at 5:1 E:T ratio), bringing the levels of cell death even below those of the unedited cells (3.12% ± 0.58% PI$^+$ cells at 1:1 E:T ratio; 11.91% ± 2.88% PI$^+$ cells at 5:1 E:T ratio).

By using blocking monoclonal antibodies, we investigated which specific activating receptors, if any, mediated NK killing of B2M$^{−/−}$ iPSCs (Figure 3B). Concordant with the expression pattern of activating ligands on target cells, preincubation of CD16$^+$ NK cells with anti-CD226, anti-NKG2D, and anti-CD337 blocking antibodies prevented killing of B2M$^{−/−}$ iPSCs (ANOVA, p < 0.001). Furthermore, by using blocking antibodies against CD94/NKG2A, we observed a decrease in NK inhibition by B2M$^{−/−}$/HLA-E$^{−/−}$ iPSCs, while by antagonizing the CD94/NKG2C receptor we highlighted a significant (p < 0.05) reduction in CD16$^+$ NK cell efficacy (95% CI 1.21%–2.29% PI$^+$ cells). On the other hand, B2M$^{−/−}$ iPSCs escaped CD8$^+$-mediated killing (95% CI 0.41%–1.14% PI$^+$ cells at 1:1 E:T ratio; 11.91% ± 2.88% PI$^+$ cells at 5:1 E:T ratio).

The percentage of NK-mediated lysis was also assayed by flow cytometry, quantitative real-time PCR, and immunofluorescence showed a profile comparable to that of the unedited line (Figures S5C–S5E). Moreover, Cas9-mediated B7-H3 knockout did not result in off-target events altering the transcription profile of the other B7 family ligands, as confirmed by the quantitative real-time PCR experiments on both iPSCs and iBeta cells (Figure S5F).

Finally, we also excluded that surface abrogation of B7-H3 and CD155 affected the cytotoxic T cell response. We performed Dunnett’s test using CD8$^+$-mediated lysis levels against B2M$^{−/−}$ cells as control, finding no differences among B2M$^{−/−}$ (1.79% ± 0.34%), B2M$^{−/−}$/B7-H3$^{−/−}$ (2.62% ± 1.32%), B2M$^{−/−}$/CD155$^{−/−}$ (1.02% ± 0.874%), and B2M$^{−/−}$/B7-H3$^{−/−}$/CD155$^{−/−}$ (2.82% ± 1.98%) iPSC lysis percentages (p > 0.99), but only between B2M$^{−/−}$ and wild-type cell lysis percentages (1.79% ± 0.34% vs. 42.10% ± 8.12%; p < 0.001) (Figure S5G). To assay NK-mediated killing, we used freshly isolated CD16$^+$ NK cells as effectors (a different donor for each experiment) and wild-type, B2M$^{−/−}$, B2M$^{−/−}$/HLA-E$^{−/−}$, B2M$^{−/−}$/B7-H3$^{−/−}$, B2M$^{−/−}$/CD155$^{−/−}$, and B2M$^{−/−}$/B7-H3$^{−/−}$/CD155$^{−/−}$ iPSCs as target cells. Jurkat and JY cell lines were used as positive and negative controls, respectively (Figure 4A). Knockout of B7-H3 and CD155 in B2M$^{−/−}$ iPSCs conferred protection from CD16$^+$ NK cell-mediated lysis to the same extent of MHC-I-expressing or B2M$^{−/−}$/HLA-E$^{−/−}$ iPSCs. No synergistic effects were displayed by surface abrogation of both B7-H3 and CD155 molecules. Moreover, flow cytometry analysis showed that knockout of these ligands on MHC-I-deprived iPSCs duly resulted in poor degranulation of CD16$^+$ NK cells (less than 25% of CD107a$^+$ cells; p < 0.01) and, generally, in lower expression of both IFN-γ and TNF-α (less than 10% and 5% of positive cells, respectively; p < 0.01), compared with the NK cells engaging the B2M$^{−/−}$ line (Figure 4B).

**B7-H3 and CD155 abrogation prevents in vitro missing-self recognition of MHC-I$^{−/−}$ iPSCs by an activated CD16$^+$ NK cell subset**

On the bases of the above-described results we hypothesized that B7-H3 and/or CD155 may play a key role in NK activation in the absence of MHC-I. We then generated iPSC knockout lines for B7-H3 and CD155 genes on the MHC-I-null background by CRISPR-Cas9 technology (Figure S5A). We derived double-knockout B2M$^{−/−}$/B7-H3$^{−/−}$ and B2M$^{−/−}$/CD155$^{−/−}$ iPSC lines, and, to evaluate potential synergic effects, we generated a triple-knockout B2M$^{−/−}$/B7-H3$^{−/−}$/CD155$^{−/−}$ iPSC line. Biallelically edited clones were obtained by cell sorting according to the absence of surface expression of B7-H3 and CD155 (Figure S5B). The three lines remained stable in culture over 30 passages, and the expression of OCT4, SOX2, NANOG, and SSEA4 assayed by flow cytometry, quantitative real-time PCR, and immunofluorescence showed a profile comparable to that of the unedited line. In all cytotoxicity tests, percentage of PI$^+$-positive target cells was measured by flow cytometry after co-incubation with effectors for 4 h. Effector-mediated lysis was normalized on basal cell death percentage occurring in target cell lines after incubation without effectors. Error bars indicate standard deviation. Statistical significance was obtained by two-tailed t test refers to comparison with mock control; n = 3 independent experiments carried out using a different donor each time; *p < 0.05, **p < 0.01, ***p < 0.001.
differences were found for NKG2D, CD226, CD338, or CD96 (Figure 5B). Finally, CD16dim NK cells showed a significant increase in killing activity against wild-type stem cells (23.88% ± 4.55% vs. 14.93% ± 3.30%, ANOVA, p < 0.05, at 1:1 effector:target ratio, and 49.10% ± 6.22% vs. 37.99% ± 6.62%, ANOVA, p < 0.01, at 5:1 effector:target ratio), B2M+/C0+/C0+/HLA-E+/+ (39.74% ± 4.13% vs. 26.66% ± 4.45%, ANOVA, p < 0.001, at 5:1 effector:target ratio), and B2M+/C0+/C0+/CD155+/C0+/C0+ (27.99% ± 5.86% vs. 19.43% ± 4.39%, ANOVA, p < 0.05, at 1:1 effector:target ratio, and 48.70% ± 8.38% vs. 37.21% ± 3.28%, ANOVA, p < 0.01, at 5:1 effector:target ratio) iPSCs compared with the CD16+ subset, whereas B2M+/C0+/C0+ cells appeared less susceptible to CD16dim NK-mediated cytotoxicity (31.16% ± 5.99% vs. 48.07% ± 8.36%, ANOVA, p < 0.001, at 1:1 effector:target ratio, and 50.80% ± 7.29% vs. 78.67% ± 4.39%, ANOVA, p < 0.0001, at 5:1 effector:target ratio) compared with their CD16+ counterparts (Figure 5C). Notably, B7-H3 but not CD155 abrogation further reduced lysis mediated by CD16dim NK cells at a 1:5 target:effector ratio (16.39% ± 5.08% vs. 32.57% ± 3.39%, ANOVA, p < 0.0001, for B2M+/C0+/CD155+/B7-H3+/+ and 25.04% ± 4.38% vs. 37.26% ± 6.85%, ANOVA, p < 0.001, for B2M+/C0+/CD155+/B7-H3+/+), suggesting a putative key role for the CD337/B7-H3 axes in CD16dim NK activation (Figure 5C). On the other hand, no significant differences were found among wild-type, B2M+/C0+/C0+, and B2M+/C0+/C0+/CD155+/C0+/CD155+/C0+ for CD16dim-mediated killing, suggesting that HLA-I-independent killing mechanisms are probably due to a reduced expression of inhibitory KIRs, as reported in the literature (Amand et al., 2017).

Both a previous study (Gornalusse at al., 2017) and the experiments on the blocking of NKG2C receptor suggested that B2M+/C0+/HLA-E+/+ cells could still be lysed by NKG2C+ NK cells. Generally, the expression of the NKG2A and NKG2C receptors is mutually exclusive, identifying two different NK subsets: NKG2A+/NKG2C− and NKG2A−/NKG2C+. Therefore, to directly demonstrate the involvement of NKG2C+ cells in the elimination of B2M+/C0+/HLA-E+/+ cells, we performed cell sorting experiments.
on total NK cells (Figure S6A) to enrich the two NKG2A+/NKG2C- and NKG2A-/NKG2C+ subsets, which were challenged in cytotoxicity assays with B2M-/-/HLA-E+/+ cells (Figure S6B). As expected, HLA-E-expressing iPSCs failed to properly evade the immune attack of NKG2C+ NK cells compared with their NKG2A+ counterparts (46.12% ± 7.50% vs. 11.30% ± 4.48%, ANOVA, p < 0.001, at 1:1 effector:target ratio, and 65.58% ± 8.55% vs. 17.80% ± 4.32%, ANOVA, p < 0.0001, at 5:1 effector:target ratio). Moreover, these results could explain the increased susceptibility of B2M-/-/HLA-E+/+ to the CD16dim NK cell subset, since CD16dim NKs are characterized by a higher number of NKG2C+ cells. Conversely, downregulation of the activating ligands B7-H3 and CD155 were used to prevent the cytotoxic response of both NKG2A+ and NKG2C+ subsets (Figure S6B).

**Double knockout of B7-H3 and CD155 increases survival of MHC-I-/- iPSC-derived β cells from immune attack mediated by both CD16+ and CD16dim NK cell subsets**

We performed in vitro pancreatic differentiation of all gene-edited cell lines. The ICS gene expression (as well as other endocrine and β cell markers) of terminally differentiated cells and their ability to secrete insulin in response to glucose in dynamic perfusion were not affected (Figures 6A and 6B and data not shown). We performed NK cytotoxicity experiments on both unedited and gene-engineered iPSC-derived insulin-producing cells (iBeta). As reported in Figure 6C, the B7-H3 and CD155 knockout, as well as HLA-E overexpression, significantly reduced CD16+ NK cell-mediated lysis in an MHC-I-null background (p < 0.001). We observed that double knockout exerted more protective effects than abrogation of CD155 or B7-H3, since B2M-/-/B7-H3-/-/CD155-/- iBeta cells displayed lower CD16+ NK-mediated lysis (34.6% ± 7.9%) compared with B2M-/-/CD155-/- (50.9% ± 6.4%, p = 0.012) and B2M-/-/B7-H3-/- (45.1% ± 7.6%, p = 0.209) cells. Again, B7-H3 knockout showed the strongest effect in reducing killing by the CD16dim NK cells (B2M-/-/B7-H3-/-, 37.23% ± 10.33%; two-tailed t test, p < 0.05) compared with B2M-/-/CD155-/- (55.33% ± 9.19%; two-tailed t test, p = 0.9882) or with the HLA-E-expressing iBeta cells (58.31% ± 6.91%). In addition, B2M-/-/B7-H3-/-/CD155-/- iBeta evaded CD16dim NK-mediated immune attack better than all the other lines (21.45% ± 6.28%, ANOVA, p < 0.001), suggesting a potential synergistic effect of the double knockout of B7-H3 and CD155.

**B7-H3 and CD155 abrogation enhances cell survival of MHC-I-/- iPSCs after transplantation into NSG mice infused with donor-derived NK cells**

Finally, we investigated an in vivo model to assess cell engraftment and survival of firefly luciferase (FLuc)-expressing wild-type, B2M-/-, B2M-/-/HLA-E-/-, and B2M-/-/B7-H3-/-/CD155-/- iPSC-derived pancreatic progenitors (PPs) (Figures S7A–S7C). To properly recapitulate in a preclinical model the in vitro experiments performed with both CD16+ and CD16dim NK cell subsets and to achieve the number of cells necessary to efficiently repopulate immunodeficient mice, we used allogeneic donor-derived primary NK cells after 12-days of in vitro expansion that allowed us to increase the NK cell number up to 10-fold of that of the starting material (Figures S7D and S7E). The expansion did not alter NK cell subpopulation distribution (Figure S7F) nor affect activation efficiency and killing capability, as assessed by CD107a expression and the cytotoxicity activity against K562 cell line in comparison with freshly isolated NK cells (Figures S7G–S7I). For in vivo cytotoxicity experiments we divided NSG mice into two experimental groups: NK cell-injected and control group. Expanded NK cells were activated in vitro and infused via retro-orbital injection of the venous sinus in the NK cell-injected group 24 h before and 24 h after allogeneic-embedded PP subcutaneous transplantation (Figure 7A). The control group received PP cells only. About 24 h after PP transplantation, we observed a comparable reduction in graft area in all samples of the two experimental groups, mainly due to the transplant technique itself (Figures 7B and 7C). This reduction reflected a marked decrease in the bioluminescence signal, which we observed after 24 h in the NK cell-injected group (Figure 7C). Fascinatingly, after 72 h, B2M-/-/PPs displayed a significant decrease in bioluminescence (p < 0.05, one-tailed t test), compared with wild-type, B2M-/-/HLA-E-/-, and B2M-/-/B7-H3-/-/CD155-/- cells (Figures 7B and 7C), whereas mice of the control group still showed a very strong signal, as we observed a slight increase in the graft area, too (Figure 7B). At follow-up (10 days after transplantation), when sample collection was performed, PPs lacking surface expression of both B7-H3 and CD155 resulted in a higher survival rate than their MHC-I-null counterparts, measured as a percentage of retained implants over the total of transplants (85% vs. 57%; p < 0.01). Immunofluorescence on cells transplanted into the NK cell-injected mice finally confirmed the co-expression of PDX-1 and INS, suggesting the beginning of PP maturation into functional β cells in vivo (Figures 7D and 7E). As expected, residual B2M-/- graft cells resulted in less than 20% of PDX1+ cells and around 7% ± 2.8% of PDX1+/INS+ cells, supporting the hypothesis that the degree of susceptibility to the immune system could strongly affect the maturation capability of progenitors (Figure 7E).

**DISCUSSION**

Over the past decades, several preclinical experiments using iPSC-derived cells have propelled the field of regenerative medicine. Yet, the translation of these promising therapeutic approaches to the clinic is hindered by the lack of methods to further reduce the immune attack of iPSC-derived pancreatic progenitors (PPs) and other iPSC-derived lineages. Here, we report that the simultaneous abrogation of B7-H3 and CD155 increases survival of iPSC-derived β cells from immune attack mediated by both CD16+ and CD16dim NK cell subsets. These findings are in accordance with our previous report, which demonstrated that B7-H3 and CD155 are highly expressed on iPSC-derived insulin-producing cells (iBeta) and are the targets of NK cells. Furthermore, we showed that the ablation of B7-H3 and CD155 abrogates the activation and cytotoxicity of NK cells, highlighting the importance of these molecules in mediating immune attack of iPSC-derived cells.
Figure 6. Double knockout of B7-H3 and CD155 increases survival of MHC-I<sup>-/-</sup> iPSC-derived β cells from immune attack mediated by both activated CD16<sup>+</sup> and CD16<sup>dim</sup> NK cell subsets

(A) Percentage of insulin-positive cells during different stages of pancreatic differentiation. Insulin positivity was assayed by flow cytometry; n = 3 independent in vitro differentiations. Error bars indicate standard deviation. Each experiment used distinct in vitro differentiations of the same iPSC clone at different passages. One-way ANOVA followed by Dunnett’s test was performed using undifferentiated iPSCs as control. iPSC, undifferentiated iPSCs; PE, pancreatic endoderm; iBeta, iPSC-derived β cells.

(B) Dynamic insulin secretion assayed by perifusion on iPSC-derived β cells, upon stimulation with 20 mM glucose + 3-isobutyl-1-methylxanthine (IBMX) and 30 mM KCl. Insulin levels are expressed as fold change over basal secretion after 1 h of acclimatation at 0.5 mM glucose; n = 3 independent in vitro differentiations. Error bars indicate standard deviation. Each experiment used a distinct in vitro differentiation of the same iPSC clone at different passages.

(legend continued on next page)
medicine forward, increasing the prospects to create improved platforms for the replacement of damaged or dysfunctional tissues in patients. At the same time, human clinical trials involving iPSC-derived cells have been performed, aiming at the evaluation of the long-term safety and efficacy of transplantation of iPSC-derived retinal pigment epithelial (RPE) cells (Fritsche et al., 2014), dopaminergic progenitors (Stoddard-Bennett and Reijo Pera, 2019), NK cells (Hu et al., 2019), and cardiomyocytes (Cyranoiski, 2018). However, immune reaction to iPSC derivatives may not be completely avoided by using autologous (Zhao et al., 2011) or fully matched donor (Korula et al., 2018) stem cell replacement, making the engineering of “off-the-shelf” hypoimmunogenic cells a sine qua non condition to overcome current engraftment limitations.

In the scenario of cell therapy for T1D treatment, a comprehensive characterization of the molecular determinants that elicit immune responses against iPSC-derived β cells could be the key point for the generation of a cell product invisible to the immune system that can be safely used in the clinical setting. In the present study, we defined the expression profile of the main intra- and extracellular NK-activating receptor ligands on both iPSCs and iPSC-derived insulin-secreting β cells, drawing special attention to the mechanisms following the loss of inhibition and the amplification of activating signals that leads to NK-mediated immune response. Among others, B7-H3 and CD155 emerged as the most expressed surface ligands in our stem cell model. Moreover, we confirmed that expression of both these molecules represents a common feature of both induced pluripotent and embryonic stem cell lines. Interestingly, CD112 was actively transcribed and translated in iPSC lines as well as an embryonic H1 cell line, although in some of them, such as the CGTRC1B10 clone used for gene engineering experiments, it was retained in the cytoplasm, whereas in others (CB4, NeoFiWT, and DRI1#11), was only partially exposed on the plasma membrane.

It has been reported that the ubiquitin-proteasome system (UPS) is detrimental for surface expression of CD112 (Molfetta et al., 2019). The UPS directly influences the pluripotency and commitment of stem cells (Schroër and Adajye, 2014; Saez et al., 2018), and therefore different degrees of UPS activation could explain the observed variability in the surface expression of CD112 in both undifferentiated and terminally differentiated cells.

The expression of both CD155 and CD112 in stem cells has been previously reported (Kruse et al., 2015) and, in accordance with Kruse and colleagues, we found that killing of iPSCs may be mediated by the CD226-activating receptor. We also showed, using blocking antibodies, that NK missing-self recognition mechanisms may underlie the signals mediated by CD337 and NKG2D as well. Specifically, NKG2D-mediated cytotoxicity against stem cells was explained by the expression of MICA, and such response could be likely involved in the outcome of transplantation of the iPSC derivatives. As a matter of fact, it has been recently reported that NKG2D ligands are responsible for the engraftment failure of iPSC-derived cardiomyocytes in a syngeneic mouse model (Nakamura et al., 2019). Finally, we observed that in our model the blockade of B7–H3 determines an increase in the NK response in HLA class I-expressing cells, similar to what has been reported in the literature regarding T lymphocytes (Lu et al., 2020). As a matter of fact, it is used as an immune checkpoint in the treatment of tumors (Lee et al., 2017). However, the results we have observed represent only first evidence, which requires further and future investigations, which may be aimed at clarifying the contrasting role of this molecule (Hofmeyer et al., 2008) in association with class I molecules and antigen-presentation mechanisms.

All current engineering strategies to generate immunotolerant iPSCs converge on the abrogation of class I and class II HLA proteins, followed by knockin of tolerance-promoting immunomodulatory molecules, such as HLA-E (Gornalussae et al., 2017), HLA-G (Shi et al., 2020), CTLA4-Ig/PD-L1 (Rong et al., 2014), and CD47 (Deuse et al., 2019). However, single knockins were only partially effective, and further studies proposed to combine the above strategies, knocking in HLA-G, PD-L1, and CD47 in an MHC-I-null stem cell line (Han et al., 2019). However, the susceptibility profile of a graft may reflect the complexity of a wide range of interactions between target and effector cells by a plethora of other molecules acting as stimulatory signals. In particular, the expression on human iPSCs and their derivatives, such as the pancreatic β cells, of molecular ligands binding the activating counterreceptors on NK cell subsets can play a relevant role. The exploitation of their mechanisms of action is needed to finely manipulate NK missing-self recognition and allograft rejection processes, offering a potentially less invasive alternative to that of inserting exogenous molecules.

We demonstrated that gene manipulation of a protein involved in the activating checkpoints of different NK cell subsets could represent a valid strategy to increase immune evasion of MHC-I-deprived iPSC derivatives. By the generation via CRISPR-Cas9 technology of B2M−/−/B7-H3+/−, B2M−/−/CD155−/−, and B2M−/−/B7-H3−/−/CD155−/− cell lines, we could quantify the effects of knocking out both B7-H3 and CD155 on cytotoxic activity exerted by NK cells on an MHC-I-null cell line. We observed a significant reduction of NK-mediated lysis in all three NK ligand-engineered cell lines compared with B2M−/− cells, suggesting that the downregulation of one of these ligands prevented the stimulation threshold from being reached. This is in line with the hypothesis that proper NK cell activation requires the integration, or the sum, of signals propagated by multiple counterreceptors (Long et al., 2013). NK cells have both a critical physiological role in immune defense, surveillance, and homeostasis and emerging functions in tolerance, autoimmune disease, and immunotherapy, thus providing a link between the innate and the adaptive immune systems (Trinchieri, 1989; Xie et al., 2017; Giancchecchi et al., 2018). Their greatly diversified

(C) Cytotoxicity test on iPSC-derived β cell clusters after co-incubation with CD16+ or CD16dim NK subsets for 4 h. Percentage of PI-positive target cells was measured by flow cytometry and effector-mediated lysis was normalized to basal cell death percentage occurring in target cells after incubation without effectors. Error bars indicate standard deviation; n = 6 independent experiments. All experiments were conducted using six different donors (one for each experiment). Each cytotoxicity experiment was carried out using CD16+ and CD16dim from the same donor. One-way ANOVA followed by Tukey’s post hoc test was performed; *p < 0.05, **p < 0.01, ***p < 0.001.

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physiopathological behavior is reflected in a substantial number of NK subtypes identified according to the differential expression of the adhesion molecule CD56 and the activating receptor CD16 (Freud et al., 2017; Zimmer, 2020). In particular, the CD56dim/CD16+ NK subset is characterized by lower inhibitory KIR expression, and higher degranulation and cytotoxic activity than CD56dim/CD16dim cells (Amand et al., 2017). By comparing the receptor repertoire of sorted CD16+ and CD16dim NK cells we found that CD16dim cells were characterized by the highest expression of both NKG2C and NKp30 and, in agreement with previous findings (Amand et al., 2017), by a higher percentage of NKG2A+ cells. Wild-type iPSCs are slightly more susceptible to killing by CD16dim NK cells compared with the effects exerted by their CD16+ counterparts, whereas surface abrogation of MHC-I did not significantly increase killing, suggesting that the mechanisms involving HLA class I-mediated inhibition could be partially missing in CD16dim NK cells, probably due to decreased expression of inhibitory KIRs (NCT04678557, NCT03163511, and NCT0229354). By in vivo assessments of wild-type, B2M+/−/HLA-E+/−, and B2M+/−/B7-H3−/+/CD155−/− luciferase-expressing PP cells transplanted in NSG mice infused with allogeneic donor-derived NK cells, we highlighted the role of B7-H3 and CD155 as key ligands for NK-mediated recognition of grafts with an MHC-I-null background, supporting the proposal for a B2M+/−/B7-H3−/+/CD155−/− stem cell line as an alternative cell source for β cell replacement strategies. In conclusion, B7-H3 and CD155 deletion can offer prospects for creating a next-generation platform of cell therapy for the treatment of T1D.

Limitations of the study

The editing of NK-activating molecules on stem cells and their derivatives represents a key tool to elucidate the role played by NK cells in rejection upon allografting of MHC-I-null grafts. In the present work, we edited just a single cell line as a putative cell model recapitulating common features of different human stem cells. However, the immunogenic profile of MHC-I−/− grafts could be affected by line-dependent cell-surface expression of different activating ligands and/or by intrinsic heterogeneity of stem cell derivatives, thus requiring additional editing steps to obtain the desired immune-escape phenotype. Moreover, our in vivo model focused on investigating acute rejection, emphasizing the missing-self recognition mechanism.

Figure 7. B7-H3 and CD155 surface abrogation enhances cell survival of MHC-I−/− iPSC-derived PPs after subcutaneous transplantation into NSG mice injected with allogeneic donor-derived NK cells

(A) Schematic representation of in vivo cytotoxicity protocol using allogeneic donor-derived primary NK cells and allogeneic-embodied Fluc-expressing iPSC-derived PP cells.

(B) Representative mice of the NK cell-injected group (left) and the control group (right), as acquired by IVIS at different time points (0, 24, and 72 h) after PP cell subcutaneous transplantation. 1, wild type; 2, B2M+/−; 3, B2M+/−/HLA-E+/−; 4, B2M+/−/B7-H3−/−/CD155−/−.

(C) Histogram of bioluminescence signals acquired by IVIS and normalized by area of subcutaneous alginate-embedded implants at different time points (0, 24, and 72 h) after transplantation. Data are plotted on a logarithmic scale and are representative of a total of seven mice (n = 7). Each NQS mouse of the NK cell-injected group was infused with a different single NK donor. Error bars indicate standard deviation. One-way ANOVA followed by Dunnett’s post hoc test was performed using B2M+/− as control; *p < 0.05.

(D) Immunofluorescence staining for the endocrine markers PDX1 (red) and INS (green) on grafts explanted from the NK cell-injected group 10 days after transplantation. Cell nuclei are stained with Hoechst (blue). Scale bar, 100 μm (20× original magnification).

(E) Percentage of PDX1+ (progenitors) and PDX1+/INS+ (β cells) assessed by immunofluorescence in grafts from the NK cell-injected group 10 days after transplantation. Data are expressed as the mean of positive cells in at least three explanted grafts for wild-type, B2M+/−/HLA-E+/−, and B2M+/−/B7-H3−/−/CD155−/− and in two explanted grafts for B2M+/−. Error bars indicate standard deviation. One-way ANOVA followed by Tukey’s post hoc test was performed; *p < 0.05, **p < 0.01, ***p < 0.0001.
mediated by NK cells as it occurs in humans. However, such model may not properly recap the fine process that ensues in an autochthonous context, nor can it infer the impact of a complete immune system on graft survival and functions. Finally, since the in vivo follow-up was only 2 weeks long, further investigations and appropriate in vivo assessments will be necessary to fully evaluate the long-term survival and functionality, as well as the safety of gene-engineered iPSC-derived β cells.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.C., M.M., and L.P; methodology, R.C., T.B., P.M., and A.L.; investigation, R.C., T.B., S.T., F.M., S.P., A.C., A.A., and M.T.L.; formal analysis, R.C. and T.B.; resources, A.L. and L.P.; writing – original draft, R.C. and L.P.; writing – review and editing, R.C., T.B., P.M., V.S., A.L., M.M., and L.P.; supervision, A.L., M.M., and L.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| APC anti-human β2-microglobulin antibody [2M2] | BioLegend | Cat#316312; RRID: AB_10641281 |
| PE anti-human HLA-A,B,C antibody [W6/32] | BioLegend | Cat#311406; RRID: AB_314875 |
| PE anti-human HLA-E antibody [3D12] | BioLegend | Cat#342604; RRID: AB_1659249 |
| Monoclonal Anti-HLA-G PE [MEM-G/9] | Quimigen | Cat#1P-292-C100; RRID: AB_10736086 |
| Alexa Fluor 647 anti-human HLA-DR, DP, DQ Antibody [Tu39] | BioLegend | Cat#36104; RRID: AB_2563169 |
| PE-Cy7 Mouse anti-human CD3 [SK7] | BD Biosciences | Cat#557851; RRID: AB_396896 |
| Alexa Fluor 647 Mouse Anti-Human CD16 [3G8] | BD Biosciences | Cat#557710; RRID: AB_396819 |
| PE-Cy5 Mouse Anti-Human CD56 [B159] | BD Biosciences | Cat#555517; RRID: AB_395907 |
| Human CD155/PVR Alexa Fluor 488-conjugated antibody [300907] | R&D Systems | Cat#FAB25301G; RRID: AB_2269068 |
| Human B7-H3 PE-conjugated antibody [185504] | R&D Systems | Cat#FAB1027P; RRID: AB_2073697 |
| Human B7-H6 PE-conjugated antibody [875001] | R&D Systems | Cat#FAB7144P; RRID: AB_2638610 |
| Human Nectin-2/CD112 APC-conjugated antibody [610603] | R&D Systems | Cat#FAB2229A; RRID: AB_19073290 |
| Human MICA Alexa Fluor 488-conjugated antibody [159227] | R&D Systems | Cat#FAB1300G; RRID: AB_10891134 |
| Human MICB PE-conjugated antibody [236511] | R&D Systems | Cat#FAB1599P; RRID: AB_10973208 |
| Human ULBP-1 APC-conjugated antibody [170818] | R&D Systems | Cat#FAB1380A; RRID: AB_2687471 |
| Human ULBP-2/5/6 PE-conjugated antibody [165903] | R&D Systems | Cat#FAB1298P; RRID: AB_2214693 |
| Human ULBP-3 Alexa Fluor® 488-conjugated antibody [166510] | R&D Systems | Cat#FAB1517G; RRID: AB_1079122 |
| Human ULBP-4/RAET1E Alexa Fluor 750-conjugated antibody [709116] | R&D Systems | Cat#FAB6285S; RRID: AB_10888662 |
| Human NKG2A/CD159a Alexa Fluor 488-conjugated antibody [131411] | R&D Systems | Cat#FAB1059G; RRID: AB_2132978 |
| Human NKG2C/CD159c APC-conjugated [134591] | R&D Systems | Cat#FAB138A; RRID: AB_416838 |
| Human NKG2D/CD314 [149810] | R&D Systems | Cat#MAB139; RRID: AB_2133263 |
| Human NKG2D/CD314 PE-conjugated [149810] | R&D Systems | Cat#FAB139P; RRID: AB_2133264 |
| Human Nkp30/NCR3 [210845] | R&D Systems | Cat#MAB1849; RRID: AB_2149446 |
| Human Nkp30/NCR3 PE-conjugated [210845] | R&D Systems | Cat#FAB1849P; RRID: AB_2149447 |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human NKp46/NCR1 APC-conjugated [195314] | R&D Systems | Cat#FAB1850A; RRID: AB_416861 |
| Human DNAM-1/CD226 Fluorescein-conjugated [102511] | R&D Systems | Cat#FAB666F; RRID: AB_2072626 |
| Human CD96 v2 Alexa Fluor 488-conjugated [628211] | R&D Systems | Cat#FAB6199G; RRID: AB_2608850 |
| Human LAMP1/CD107a Alexa Fluor 488-conjugated antibody [508921] | R&D Systems | Cat#C4800G; RRID: AB_2296836 |
| Anti-Interferon gamma antibody [4S.B3] | Abcam | Cat#ab234193; AB_2747846 |
| Anti-TNF alpha antibody [EPR20972] | Abcam | Cat#ab225576; RRID: AB_2893364 |
| Alexa Fluor 647 Mouse Anti-OCT3/4 [40/OCT-3] | BD Biosciences | Cat#560307; RRID: AB_1645319 |
| PE Mouse anti-CD184 [12G5] | BD Biosciences | Cat#557145; RRID: AB_2292652 |
| Alexa Fluor® 647 Mouse Anti-Nestin [25/NESTIN] | BD Biosciences | Cat#560393; RRID: AB_1645170 |
| Alexa Fluor 488 Mouse Anti-PDX-1 [658AS] | BD Biosciences | Cat#652274; RRID: AB_10611998 |
| PE Mouse Anti-NKX6.1 [R11-560] | BD Biosciences | Cat#563023; RRID: AB_2716792 |
| Alexa Fluor 647 Mouse Anti-Insulin [T56-706] | BD Biosciences | Cat#565689; RRID: AB_2739331 |
| Mouse IgG1 Isotype Control | R&D Systems | Cat#MAB002; RRID: AB_357344 |
| Mouse IgG2A Isotype Control | R&D Systems | Cat#MAB003; RRID: AB_357345 |
| Mouse IgG2B Isotype Control | R&D Systems | Cat#MAB004; RRID: AB_357346 |
| Goat Anti-Mouse Ig, Human ads-Alexa Fluor 488 | Southern Biotech | Cat#1010-30; RRID: AB_2794130 |
| Mouse F(ab)2 IgG (H+L) PE-conjugated Antibody | R&D Systems | Cat#F0102B; RRID: AB_622014 |
| Mouse F(ab)2 IgG (H+L) APC-conjugated Antibody | R&D Systems | Cat#F0101B; RRID: AB_622013 |
| Human/Mouse/Rat BAT3/BAG6 antibody | Biotechne | Cat#AF6438; RRID: AB_10717417 |
| Human/Mouse/Rat Vimentin antibody | Biotechne | Cat#AF2105; RRID: AB_355453 |
| Human/Mouse/Rat Vimentin antibody [280618] | R&D Systems | Cat#MAB2105; RRID: AB_2241653 |
| Human Properdin antibody [10-18] | Novus Biologicals | Cat#NB100-64749; RRID: AB_963443 |
| Human OCT4 antibody [GT486] | Novus Biologicals | Cat#NP2-15052; RRID: AB_2895225 |
| Human NANOG antibody | R&D Systems | Cat#AF1997; RRID: AB_355907 |
| Human/Mouse/Rat SOX2 antibody [245610] | R&D Systems | Cat#MAB2018; RRID: AB_358009 |
| Anti-SSEA4 antibody [MC813-70] | Abcam | Cat#ab16287; RRID: AB_778073 |
| Anti-PDX-1 antibody [EPR3358(2)] | Abcam | Cat#ab134150; RRID: AB_2631338 |
| Anti-Insulin antibody | Abcam | Cat#ab63820; RRID: AB_1925116 |

(Continued on next page)
**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Goat anti-Mouse IgG (H+L), Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat#A11001; RRID: AB_2534069 |
| Donkey anti-Sheep IgG (H+L), Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat#A11015; RRID: AB_141362 |
| Rabbit anti-Goat IgG (H+L), Superclonal Recombinant Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat#A27012; RRID: AB_2536077 |

**Biological samples**

| Biological samples | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| Healthy donor PBMC-derived CD8 naïve cells | San Raffaele Immunohematology and Transfusion Medicine Service | N/A |
| Healthy donor PBMC-derived NK cells | San Raffaele Immunohematology and Transfusion Medicine Service | N/A |

**Chemicals, peptides, and recombinant proteins**

| Chemicals, peptides, and recombinant proteins | SOURCE | IDENTIFIER |
|-----------------------------------------------|--------|------------|
| Calcium chloride | Sigma-Aldrich | Cat#C1016 |
| Sodium alginate | Sigma-Aldrich | Cat#W201502 |
| rhIL-2 | Peprotech | Cat#200-02 |
| rhIL-12 p80 | Peprotech | Cat# 200-12p80H |
| rhIL-15 | Peprotech | Cat#200-15 |
| Aki5 II | Selleckchem | Cat#S2750 |
| L-3,30,5-Triiodothyronine (T3) | Sigma-Aldrich | Cat#T2877 |
| Nicotinamide | Sigma-Aldrich | Cat#0636 |
| rhIFN-γ | Peprotech | Cat#300-02 |
| rhTNF-α | Peprotech | Cat#300-01A |
| Y27632 (ROCK inhibitor) | StemCell Technologies | Cat#72304 |
| IBMX 3-Isobutyl-1-methylxanthine | Gibco | Cat#PHZ1124 |
| Nocodazole | Sigma-Aldrich | Cat#M1404 |
| Cell Proliferation Dye eFluor670 | Thermo Fisher Scientific | Cat# 65-0840-85 |
| LIVE/DEAD Fixable Violet Dead Cell Stain | Thermo Fisher Scientific | Cat#L34955 |
| Propidium Iodide (PI) | Sigma-Aldrich | Cat#P4170 |

**Critical commercial assays**

| Critical commercial assays | SOURCE | IDENTIFIER |
|---------------------------|--------|------------|
| STEMdiff Trilineage Differentiation Kit | StemCell Technologies | Cat #05230 |
| TaqMan™ hPSC Scorecard™ Kit, Fast 96-well | Thermo Fisher Scientific | Cat#A15871 |
| Naive CD8^+ T Cell Isolation Kit | MACS, Miltenyi Biotec | Cat#130-093-244 |
| CD56^+ CD16^+ NK Cell Isolation Kit | MACS, Miltenyi Biotec | Cat#130-092-660 |
| BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit | BD Biosciences | Cat#554714 |
| GeneArt Precision gRNA Synthesis Kit | Thermo Fisher Scientific | Cat#A29377 |
| SuperScript IV First-Strand Synthesis System | Thermo Fisher Scientific | Cat#18091050 |
| TaqMan Universal PCR Master Mix | Applied Biosystems | Cat#4305719 |
| PowerUp Green Master Mix | Applied Biosystems | Cat#A25741 |

**Deposited data**

| Deposited data | SOURCE | IDENTIFIER |
|----------------|--------|------------|
| Single cell RNAseq datasets of hiPSC during differentiation into pancreatic beta cells | Pellegrini et al., 2020 | GEO: GSE149613 |

**Experimental models: Cell lines**

| Experimental models: Cell lines | SOURCE | IDENTIFIER |
|-------------------------------|--------|------------|
| Human: iPS cells | Cell and Gene Therapy Catapult, London, UK | CGTRCiB10 |
| Human: iPS cells | Institute of Experimental Neurology (INSPE) - IRCCS San Raffaele Scientific Institute | CB4 |
| Human: iPS cells | Institute of Experimental Neurology (INSPE) - IRCCS San Raffaele Scientific Institute | NeoFiWT |
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human: iPS cells     | Diabetes Research Institute (DRI) - IRCCS San Raffaele Scientific Institute | DRI1#11 |
| Human: iPS cells     | Diabetes Research Institute (DRI) - IRCCS San Raffaele Scientific Institute | DRI2#3 |
| Human: H1-hESC       | San Raffaele Telethon Institute for Gene Therapy (SR-TIGET) | N/A |
| Human: Jurkat [Clone E6-1] | ATCC | TIB-152 |
| Human: MOLT-4        | ATCC | CRL-1582 |
| Human: JY            | San Raffaele Telethon Institute for Gene Therapy (SR-TIGET) | N/A |

Experimental models: Organisms/strains

Mouse: NSG: NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ Charles River Laboratories JAX: 005557

Oligonucleotides

PVR (Hs00197846_m1) Thermo Fisher Scientific Cat#4331182
CD276 (Hs00987207_m1) Thermo Fisher Scientific Cat#4331182
NECTIN2 (Hs01071562_m1) Thermo Fisher Scientific Cat#4331182
MICA (Hs00741286_m1) Thermo Fisher Scientific Cat#4331182
MICB (Hs00792952_m1) Thermo Fisher Scientific Cat#4331182
ULBP1 (Hs00360941_m1) Thermo Fisher Scientific Cat#4331182
ULBP2 (Hs00607609_mH) Thermo Fisher Scientific Cat#4331182
ULBP3 (Hs00225909_m1) Thermo Fisher Scientific Cat#4331182
ULBP4 (Hs01026643_g1) Thermo Fisher Scientific Cat#4331182
ULBP5 (Hs01584111_mH) Thermo Fisher Scientific Cat#4331182

Other oligonucleotides: Table S1

Recombinant DNA

Plasmid : B2M-ETrimer-IRES-GFP This paper N/A
Lentiviral vector : PKG-FLuc-mCMV-NGFR This paper N/A

Software and algorithms

CHOPCHOP v.3 tool Labun et al., 2019 https://chopchop.cbu.uib.no/
FlowJo software v.10 Ashland https://www.flowjo.com/solutions/flowjo
Fiji/ImageJ v.1.52p Schindelin et al., 2012 https://imagej.net/Fiji/Downloads
PRISM v.8 GraphPad Software https://www.graphpad.com/scientific-software/prism/
R v.4.0.3 R Foundation for Statistical Computing https://www.R-project.org/

Other

High-capacity low-pulsatility peristaltic pump-based automated perfusion system v.2.0.0 BioRep https://doi.org/10.1177/0963689718798564

RESOURCE AVAILABILITY

Lead contact
Further information and requests resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Lorenzo Piemonti (piemonti.lorenzo@hsr.it).

Materials availability
This study did not generate new unique reagents.
**Data and code availability**

The RNA-Seq data have been deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (GEO: GSE149613) and are publicly available. All other raw data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell culture**

Human iPSC clone CGTRCiB10 was obtained from Cell and Gene Therapy Catapult (London, UK). Human iPSC CB4 and NeoFiWT were obtained from the Institute of Experimental Neurology (INSPe) at IRCCS San Raffaele Scientific Institute (Milan, Italy). Human iPSC DRI1 clone 11 (DRI1#11) and DRI2 clone 3 (DRI2#3) were generated by our laboratory following previous described Sendai virus-based protocol (Pellegrini et al., 2018). H1-hESC were supplied by Ditadi’s Lab at San Raffaele Telethon Institute for Gene Therapy (SR-Tiget) (Milan, Italy). Wild type iPSCs and CGTRCiB10-derived gene engineered stem cell lines were cultured in complete Essential 8 Flex Medium (Gibco, Thermo Fisher Scientific) added with 1% penicillin/streptomycin (P/S) (Lonza) on plates coated with recombinant human vitronectin (Thermo Fischer Scientific). Passages were performed when cell density reached 70–80% of confluence and detached using 0.5 mM pH 8.0 EDTA (Thermo Fisher Scientific) or 1X Trypsin/EDTA (Lonza). iPSC karyotyping, aCGH characterization and validation of pluripotency were described in supplementary methods. Jurkat (Clone E6-1, ATCC TIB-152), MOLT-4 (ATCC CRL-1582) and JY (obtained from the SR-TIGET, Telethon Institute for Gene Therapy, Milan, Italy) cell lines were cultured in RPMI-1640 (Lonza), added with 10% FBS (Lonza), 1 mM L-glutamine (Lonza) and 1% P/S. CD8+ T and NK cells were isolated from PBMCs derived from buffy coats purchased by the Immunohematology and Transfusion Medicine Service (ITMS) of Garbagnate, Milan, Italy, and managed (accepted, registered, and distributed) by the ITMS of San Raffaele hospital, Milan, Italy. Buffy coats were derived from healthy donors who donate blood and sign an informed consent in accordance with the D.M. November 2nd 2015 entitled "Provisions relating to the quality and safety requirements of blood components". In accordance with the ministerial provisions and the IOG 364 institutional procedure "Request and delivery of buffy coats for research purposes", it is not possible to retrieve any type of information (gender, age, HLA typing) of buffy coat donors.

**Human subject**

All PBMCs used in this study were derived from buffy coats purchased by the Immunohematology and Transfusion Medicine Service (ITMS) of Garbagnate, Milan, Italy, and managed (accepted, registered, and distributed) by the ITMS of San Raffaele hospital, Milan, Italy. Buffy coats were derived from healthy donors who donate blood and sign an informed consent in accordance with the D.M. November 2nd 2015 entitled "Provisions relating to the quality and safety requirements of blood components". In accordance with the ministerial provisions and the IOG 364 institutional procedure "Request and delivery of buffy coats for research purposes", it is not possible to retrieve any type of information (gender, age, HLA typing) of buffy coat donors.

**Animals**

Experiments involving mice were performed under protocols approved and monitored by Animal Care and Use Committee of San Raffaele Scientific Institute. The NOD-scid IL2Rgamma<sup>-/-</sup> (NSG) mice [sex: female; age: 6–8 weeks; weight: 20–24 g] used as recipients were obtained from Charles River Laboratories, Calco, Italy.

**METHOD DETAILS**

**iPSC karyotyping and aCGH, characterization, validation of pluripotency and trilineage differentiation test**

Large chromosome rearrangements and/or short chromosomal copy number changes at high-resolution scale were assayed by performing Q-banding karyotype analysis and array comparative genomic hybridization (aCGH) with over 600 probes and a median probe spacing of 41 kb (Agilent Technologies). Karyotyping and aCGH experiments and accompanying reports were fully managed by the Integrated System Engineering (ISENET Biobanking, Milan, Italy). iPSCs were evaluated on their morphological parameters and tested for pluripotency markers by flow cytometry (OCT4), qRT-PCR (OCT4, SOX2, NANOG) and immunofluorescence (OCT4, SOX2, NANOG, SSEA4), following the below protocols. Functional analysis to assess wild type iPSCs capability to correctly differentiate into all three germ layers was performed by using STEMdiff Trilineage Differentiation Kit (Stemcell Technologies), according to manufacturer’s instructions. Outcome of trilineage differentiation was evaluated by testing committed cells for pluripotency and endo-, meso- and ectodermal markers by flow cytometry (OCT4, CD56, CXCR4, NESTIN) and qRT-PCR using Fast 96-well panel of TaqMan hPSC Scorecard Assay (Life Technologies).

**iPSC differentiation into pancreatic β cells, characterization and maintaining**

Human wild type and genetic engineered iPSCs were differentiated into insulin-producing β cells following a previously described protocol (Pellegrini et al., 2018). Differentiation started with cells in adhesion, at 70–80% of confluence. Morphological changes
were followed by using digital phase contrast inverted microscope (Invitrogen EVOS XL; Thermo Fisher Scientific). During differentiation, cells were sampled at specific time points to evaluate stage-specific pancreatic markers by qRT-PCR and flow cytometry. Terminally differentiated cells were maintained in CMRL-1066 medium (Thermo Fisher Scientific), added with 10% FBS (Euroclone), 10 μM Alk5i II (Selleckchem), 1 μM L-3,30,5-Trifluorothymidine (T3) (Sigma-Aldrich), 10 mM nicotinamide (Sigma-Aldrich), 1 mM L-Glutamine (Lonza), 1% P/S (Lonza). In preparation for cytotoxicity test, cells were detached by gently pipetting and cultured in suspension at 60 rpm, adding to complete medium 10 ng/mL IFN-γ (Peprotech), 50 μg/mL DNase I (Roche) and 10 μM Y27632 (Stemcell Technologies).

**Alloreactive CD8+ T cell isolation**

6x10^5 PBMCs isolated from healthy donors were co-incubated with 50 Gy-irradiated MOLT-4. Co-incubation was performed for 4 weeks in X-VIVO (Lonza) supplemented with 5% FBS (Euroclone) at 100:1 PBMC/MOLT-4 ratio on flat-bottom 6-wells plates. After the first week, all cells in suspension were recovered and transferred in new plates to eliminate monocytes and other adherent cells. Additional 1x10^6 irradiated MOLT-4 were added to culture after 14 days. From the third week, clusters of naïve T lymphocytes primed against MOLT-4 antigens could be appreciated by inverted microscope. Finally, CD8+ T cells were isolated using magnetic separator and Naïve CD8+ T Cell Isolation Kit (MACS, Miltenyi Biotec), following producer’s protocol. Enriched CD3+ CD8+ T cells were resuspended in complete culture media or frozen in FBS with 10% DMSO (Sigma-Aldrich). Four days before cytotoxicity test, frozen CD8+ T cells were thawed and activated with 1000 U/ml of IL-2 (Peprotech).

**NK cell subsets isolation**

PBMCs isolated from healthy donors were processed by using magnetic separator and CD56+ CD16+ NK Cell Isolation Kit (MACS, Miltenyi Biotec). NK cell subsets isolation was performed in a two-step procedure: non-NK cells were labeled with a cocktail of biotin-conjugated antibodies and removed by negative selection. Pre-enriched fraction contained both CD56dim/CD16dim and CD56dim/CD16+ subsets. During the second step, pre-enriched fraction was labeled with CD16 MicroBeads following incubation for 5' at 4°C, then loaded into the separation MS columns. While CD56dim/CD16+ cells were retained into columns, unlabeled CD56dim/CD16dim was recovered from washing flow-through. Finally, CD56dim/CD16+ NK cells were isolated by flushing out magnetically labeled fraction. Identity and purity of NK subsets were evaluated by flow cytometry, using anti-human CD56 and CD155 conjugated antibodies and removed by negative selection. Pre-enriched fraction contained both CD56dim/CD16dim and CD56dim/CD16+ subsets. During the second step, pre-enriched fraction was labeled with CD16 MicroBeads following incubation for 5' at 4°C, then loaded into the separation MS columns. While CD56dim/CD16+ cells were retained into columns, unlabeled CD56dim/CD16dim was recovered from washing flow-through. Finally, CD56dim/CD16+ NK cells were isolated by flushing out magnetically labeled fraction. Identity and purity of NK subsets were evaluated by flow cytometry, using anti-human CD56 and CD16 antibodies. Both NKG2A- and NKG2C-positive NK cells were purified by cell sorting, by using FACSARia Fusion (BD Biosciences). NK cells used for in vitro cytotoxicity tests were maintained in culture for 2–4 days under stimulation with IL-2 (Peprotech) and IL-12 (Peprotech). NK cell used for in vivo experiments were expanded in vitro for a total of 12 days into NK MACS Medium (Miltenyi Biotec), added with 5% human AB Serum (Corning), 140 ng/mL IL-15 (Peprotech) and 500 U/ml IL-2 (Peprotech). Since the reactivity profile of each NK cell line can vary from donor to donor and merging them could result in confounding results (or induce cross-alloreactivity reactions), NK cells from different donors were never pooled to perform in vitro or in vivo cytotoxicity experiments.

**gRNA IVT, plasmid vector and genome editing**

Gene editing on iPSCs was achieved by applying CRISPR/Cas9 technology. All guide RNAs (gRNAs) were designed and chosen depending on the number of off-target sites using CHOPCHOP v.3 tool (Labun et al., 2019). We performed in vitro assembly of gRNAs for B2M (one sense gRNA on exon 1: 5'-AGTACGGGCGACACAGCCTA-3', B7-H3 (two sense gRNAs on exon 3: 5'-GCTGGTGCACAGCTTTGCTGA-3', 5'-CTGGTGACAGCTTTGCTGA-3'; one anti-sense gRNA mapping on exons 3 and 5: 5'-ACGGGACCCTG CAGCCTGTA-3') and CD155 (three sense gRNAs on exon 2: 5'-GGCCGTCTTCCACAAACGC-3', 5'-GAATTCGTGGCAGCCGCA GACT-3', 5'-GATGTTCCGGTTGCCGCGTAG-3'). Primer pairs were annealed to assemble by PCR the gRNA DNA template; then gRNAs were synthetized by in vitro transcription (IVT) and finally purified using the GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific), according to manufacturer's protocol. gRNAs concentration was measured by spectrophotometer (Epoch, Gen5 Software; BioTek). Targeting efficiencies reached up to 50–60%, 30% and 20% for B2M, B7-H3 and CD155 genes, respectively. Knock-in of HLA-E was performed using a plasmid vector as donor DNA, including right/left homology arms spanning exon 1 of B2M gene (chr15:44710847-44711568; chr15:44711569-44712268), with coding sequence for the HLA-G signal peptide and/or CD155 was set up delivering into wild type iPSCs 10 μg of TrueCut Cas9 protein v2 (Invitrogen, Thermo Fisher Scientific) and 5 μg of corresponding gRNAs as ribonucleoprotein (RNP) complex by using 4D-nucleofector (Lonza) with P3 electroporation solution and CB-150 program, according to manufacturer’s instructions. For knock-in, Cas9/gRNA RNP complex was electroporated with 1 μg of donor DNA into iPSCs treated for 16 hours with 1 μg/mL of Nocodazole (Sigma-Aldrich) to increase homology-directed repair (HDR) events from ~0.1% to >7%. After 7 days from electroporation, gene disruption and recombination efficiencies were evaluated by flow cytometry, then single clones of biallelic knock-out population of interest, as well as knocked-in HLA-E/GFP-expressing cells, were purified following cell sorting by using FACSAria Fusion (BD Biosciences).
RNA isolation, retro-transcription and qRT-PCR
Total RNA was extracted with mirVana Isolation Kit (Ambion) and quantified by using spectrophotometer (Epoch, Gen5 Software; BioTek). Up to 2 μg of total RNA was treated with 2 U/μl TURBO DNase (Ambion), then retrotranscribed by using the SuperScript IV RT (Thermo Fisher Scientific), according to manufacturer’s protocol. Both homemade primer pairs for SYBR Green-based protocol and commercial pre-designed gene-specific primer-probe sets from TaqMan Gene Expression Assays (Applied Biosystems) were used for the gene expression analysis. Quantitative RT-PCR were performed by using TaqMan Universal PCR Master Mix (Applied Biosystems) or PowerUp Green Master Mix (Applied Biosystems), according to manufacturer’s protocol. Expression levels were normalized applying 2−ΔΔCt method, using RPS18, RPS27 and GAPDH as housekeeping genes.

Immunofluorescence
For pluripotency test, cell lines were cultured in 4-Well Culture Slide (Falcon) until 70% confluence was reached, then fixed with 4% paraformaldehyde. Terminally differentiated β cell aggregates were embedded in 2–4% of low gelling temperature agarose (Sigma-Aldrich), fixed with 10% zinc formalin (Sigma-Aldrich), then included in paraffin. For intracellular staining cells were permeabilized with ice-cold Cytofix/Cytoperm Buffer III (BD), then incubation for 30’ at 4° with conjugated antibodies. Titration experiments were carried out following UWCCC Flow Cytometry Laboratory guidelines (Telford et al., 2009). For HLA and NK ligands overexpression induction experiments, staining was performed after overnight treatment with 10 ng/mL IFN-γ (Peprotech), 50 ng/mL TNF-α (Peprotech) or both. Results were analyzed by using FlowJo software v.10 (FlowJo).

Flow cytometry
Flow cytometry was performed with FACSCanto (BD) flow cytometer using FACS Diva Software. Cell surface staining was performed incubating cells for 20’ at RT in PBS supplemented with 2% FBS (Euroclone) and 0.1% sodium azide (Sigma-Aldrich). Cells were then analyzed immediately or after fixation with Cytofix/Cytoperm (BD), containing 4.2% formaldehyde. Intracellular staining required cell permeabilization with ice-cold Cytofix/Phosflow perm Buffer III (BD), then incubation for 30’ at 4° with conjugated antibodies. Titration experiments were carried out following UWCCC Flow Cytometry Laboratory guidelines (Telford et al., 2009). For HLA and NK ligands overexpression induction experiments, staining was performed after overnight treatment with 10 ng/mL IFN-γ (Peprotech), 50 ng/mL TNF-α (Peprotech) or both. Results were analyzed by using FlowJo software v.10 (FlowJo).

Dynamic perfusion of iPSC-derived β cells
Dynamic stimulation of terminally differentiated β cells was performed by using high-capacity low-pulsatility peristaltic pump-based automated perfusion system (BioRep Perfusion V2.0.0), as previously described (Pellegrini et al., 2018). Secretion was sampled every minute, collecting perfusate in round-bottom 96-wells plates. Released insulin was quantified by ELISA Kit (Mercodia), according to manufacturer’s instructions. ELISA plates were analyzed by ELISA Reader (MicroPlate Reader, Model 680, BioRad).

In vitro cytotoxicity assay with CD8+ T and NK cells
Effector (CD8+ T or NK) cells were labeled following incubation for 10’ at 37°C with 5 μM Cell Proliferation Dye eFluor670 (Thermo Fisher Scientific). Labeling was stopped by adding 5 volumes of cold complete medium (X-VIVO with 5% FBS) and incubating cells in ice for 5’. Finally, after 3 washings with complete media, effector cells were recollected by centrifugation (5’ x 400 g) and resuspended in new fresh medium with 200 U/ml IL-2 (Peprotech) and 10 ng/ml IFN-γ (Peprotech). iPSC lines were detached by incubation with 1X Trypsin/EDTA for 5’ at 37°C, to reduce number and dimension of cell aggregates. Target (iPS cell lines, Jurkat, Molt4 or JY) cells were washed with PBS and counted. Target cells were stained with LIVE/DEAD Fixable Violet Dead Cell Stain (Thermo Fisher Scientific), following manufacturer’s protocol, to discriminate cells that die before the cytotoxicity test and exclude them from the analysis. LIVE/DEAD-stained target cells were resuspended in X-VIVO (Lonza) with 5% FBS (Euroclone), 200 U/ml IL-2 (Peprotech) and 10 ng/ml IFN-γ (Peprotech), then plated in round-bottom 96-well plate at a seeding density of 4x10^4 cells/well. Effector cells were added to target cells with different ratios (0.5:1, 1:1 and 1:5), mixing by pipetting to ensure that target and effector cells were homogeneously distributed. For each target cell type at least 3 wells were left without effectors, to measure spontaneous/basal cell death levels. Media volume was adjusted up to 50 μL and 96-wells plate was centrifugated for 1’ at 1000 rpm. Co-incubation proceeded for 4 hours at 37°C and 5% CO2. At the end of incubation time, cells were stained with 20 μg of propidium iodide (PI) (Sigma-Aldrich) and quickly analyzed by flow cytometry to identify dead cells. For cytotoxicity tests performed on terminally differentiated cells, about 20–30 suspension cultured iPSC-derived pancreatic β cell aggregates of 70–100 μm diameter were co-incubated with 5x10^5 NK cells for 4 hours. An aliquot of effector/target cell mix was used to quantify NK response and activation by cytofluorimetric analysis, performing surface staining for degranulation marker CD107a and intracellular staining for IFN-γ and TNF-α. For the blocking antibodies experiments, effector NK cells were pre-incubated for 20’ at 4° in PBS added with 2% BSA (Sigma-Aldrich) and mock unrelated antibody or anti-human NK receptor-specific antibodies. NK cells from different donors were never pooled together to perform cytotoxicity assays.

In vivo reactivity with donor-derived primary NK cells
Wild type, B2M−/−, B2M−/−/HLA-E−/+ and B2M−/−/B7-H3−/−/CD155−/− iPSC lines were stably transduced with a luciferase-expressing Red-Fluc-NGFR lentivirus vector (supplied by Lombardo’s Lab at the SR-Tiget, IRCCS San Raffaele Scientific Institute, Milan, Italy) and the NGFR-positive cells were used for in vivo experiments. Luciferase-expressing cells were differentiated to
pancreatic progenitors (PPs) following differentiation protocol as described above, then 2x10^6 of PPs were embedded in 4% alginate-matrix by dripping cell/alginate mixture into a gelling solution containing 100 mM of CaCl_2. NSG mice were divided into 2 groups: NK cell-injected group (N = 7) and control group (N = 7). NK cell-injected group’s mice were infused intravenously with 10^6 pre-activated expanded primary NK cells 24 hours before and 24 hours after PPs transplantation. Each NSG mice of the NK cell-injected group received NK cells derived from a single different donor. NK cells from different donors were never pooled or infused together within the same animal. PPs were transplanted sub-dermally into NSG mice and each recipient received all the four PP lines. In vivo monitoring of luciferase-expressing cells was done by the Lumina II IVIS imaging system (Caliper Life Science), at day of transplant and after 24 and 72 hours. Data analysis was done using the Living Image software v. 4.2 (Caliper Life Sciences). After seven days from last image acquisition, mice were sacrificed, and graft collected for H&E and IF staining. The histological procedures were conducted in the Animal Histopathology facility at San Raffaele Scientific Institute.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data are represented as means ± standard deviation. Statistical analysis was carried out by using PRISM v.8 (GraphPad). Numerosity indicated in figure legends refers to independent experiments carried out according to figure-related description. In particular, quantification of NK activating ligands on iPSC cell lines was performed by measuring mRNA and protein levels on batches of same cell lines at different passages and stage of confluence. Biological replicates for experiments on differentiated cells are intended performed on different independent in vitro differentiation, starting from undifferentiated iPSC at different passages. Each cytotoxicity experiment was replicated by using effector cells derived from different donors. As reported in Method Details section, NK cells from different donors were not pooled together, neither for in vitro nor for in vivo experiments. One-way analysis of variance (ANOVA) was performed for comparison of data belonging to more than two groups, followed by either Tukey’s honestly significant difference post hoc test for multiple comparison, or Dunnett’s test to compare means from several experimental groups against a single control group. For two groups comparison, assuming normal distribution of data, a two or one-tailed Student’s t-test was used. The specific test used to compute the significance is appropriately indicated in the text or in the figure legends. A p-value < 0.05 was considered significant (* for p < 0.05; ** for p < 0.01; *** for p < 0.001, **** for p < 0.0001).
Supplemental information

Engineering of immune checkpoints B7-H3 and CD155 enhances immune compatibility of MHC-I⁻/- iPSCs for β cell replacement

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Figure S1. Human iPSCs express pluripotency markers and preserve their capability to differentiate in vitro into three germ layers. Related to Figure 1 and 2 (A) Pluripotency marker expression by flow cytometry (OCT4) and qRT-PCR (OCT4 and NANOG) into wild type iPSCs. GAPDH was used as normalizer gene. Error bars represent standard deviation. N = 3 independent experiments. Each experiment used the same cell line (CGTRCiB10) at different passages and state of confluence. (B) Immunofluorescence staining on wild type iPSCs for pluripotency markers OCT4, NANOG, SOX2 and SSEA4 (all in green). Cell nuclei are stained with Hoechst (blue). Scale bar 100 μm (20x magnification). (C) Phase contrast microscope view showing the morphology of iPSCs, and three germ layers obtained by using Trilineage Differentiation Kit. (D) Pluripotent, endoderm, mesoderm and ectoderm marker expression patterns assayed by flow cytometry. Gates were set by referring to scatter plot of related unstained or isotype controls. (E) Conventional staining of the metaphase I for karyotype analysis of iPSCs at passages 41 and 88, after treatment for 16 hours with 0.2 μg/ml Colchicine. (F) Relative mRNA expression of trilineage specific genes into iPSCs and iPSC-derived three germ layers. ACTB, EP300 and SMAD1 were used as normalizer genes. Data are plotted on the logarithmic scale as fold change over unrelated undifferentiated control. At the bottom-right of panel, algorithm scores for the samples showing up- or down-regulation of the endoderm, mesoderm or ectoderm markers relative to the reference set of nine undifferentiated pluripotent stem cell lines.
Figure S2. Expression pattern of intracellular NK activating ligands and B7 family genes on undifferentiated iPSCs and during in vitro differentiation into pancreatic β cells. Related to Figure 2 (A) Immunofluorescence staining for intracellular NK ligands vimentin (VIM), properdin (CFP) or BAG6/BAT3 (green). Cell nuclei were counterstained with Hoechst (blue). Scale bar 40 um (40X magnification) (B) Representative histograms of iPSCs after extracellular or intracellular staining with anti-CD112 mAb (red), using isotype Ab as staining control (light blue). (C) Violin plots of the normalized log-expression values calculated as Unique Molecular Identifier (UMI) of B7 family genes in the undifferentiated iPSC dataset. (D) Relative expression by qRT-PCR (mean ± SD; n = 3 independent experiments using batches of the same cell line at different passages) of B7-H1, -H2, -H3, -H4 and -H6 genes in undifferentiated iPSCs. GAPDH was used as normalizer gene. (E) Immunofluorescence staining on terminally differentiated cells (iBeta) for intracellular NK ligands VIM, CFP or BAG6/BAT3, followed by incubation with Alexa Fluor 488-conjugated secondary antibody (green). Cell nuclei were counterstained with Hoechst (blue). Scale bar 40 um (40X magnification). (F) Relative mRNA expression (mean ± SD; n = 3 independent in vitro differentiations) of NK ligand VIM, CFP or BAG6/BAT3 during pancreatic differentiation as assayed by qRT-PCR. GAPDH was used as normalizer gene. Data are reported as fold change over undifferentiated cells. (G) Average corrected total cell fluorescence (CTCF) of intracellular NK ligand VIM, CFP or BAG6/BAT3 positive cells in iPSCs and during pancreatic differentiation. Error bars represent standard deviation. N = 5 independent experiments. (H) Violin plots of the normalized log-expression values calculated as Unique Molecular Identifier (UMI) of B7 family genes in the iBeta dataset. (I) Relative expression by qRT-PCR (mean ± SD; n = 3 independent in vitro differentiations) of B7-H1, -H2, -H3, -H4 and -H6 genes in pancreatic terminally differentiated iPSCs. GAPDH was used as normalizer gene. Each experiment involving only undifferentiated iPSCs utilized the same cell line (CGTRCiB10) at different passages and state of confluence. Each experiment involving differentiated cells was carried out performing different independent in vitro differentiations of the same iPSC clone (CGTRCiB10) at different passages. iPSC = undifferentiated iPSC; PF = posterior foregut; PE = pancreatic endoderm; EN = endocrine cells; iBeta = iPSC-β cells. Statistical significances were obtained with two-tailed t-test and are referred to comparison with the iPSC stage. * p < 0.05; ** p < 0.01; *** p < 0.001.
Figure S3. Exposure of iPSC to IFN-γ and/or TNF-α caused slight changes in the expression of NK activating ligands. Related to Figure 2. Representative histograms of staining with mAbs (red) for B7-H3, DNAM-1 ligands CD155 and CD112, NKG2D ligands MICA, MICB and RAET1E, or with related isotype control (light blue). Staining was performed on untreated iPSCs or iPSCs treated overnight with 10 ng/ml IFN-γ, 50 ng/ml TNF-α or both (10 ng/ml IFN-γ and 50 ng/ml TNF-α).
A

B2M locus

Cas9 cleavage site

HLA-E

GFP

iPSC wild type

iPSC B2M−

iPSC B2M−/HLA-E−/−

B

C

OCT4

NANOG

SOX2

SSEA4

B2M−

B2M−/HLA-E−/−

D

Separation Index

IPSC wild type

IPSC B2M−

IPSC B2M−/HLA-E−/−
Figure S4. Gene engineered B2M−/− and B2M−/−/HLA-E+/+ iPSCs remain undifferentiated and maintain unaltered the expression pattern of NK activating ligands. Related to Figure 3, 4, 5, 6 and 7 (A) Schematic representation of the B2M locus, indicating cutting site (yellow triangle) of the CRISPR/Cas9 complex. HLA-E-containing construct knocked into B2M locus is also showed. GFP was used as reporter gene. HR/HL = right/left homology arms; pep = peptide; pA = polyadenylation signal. (B) FACS plots for pan class I MHC molecules, B2M and HLA-E proteins on wild type, B2M−/− and B2M−/−/HLA-E+/+ iPSCs. Knocked-in cells with HLA-E-IRES-GFP construct were enriched by cell sorting and assayed for HLA-E and GFP expression. Gates were set by referring to scatter plot of related unstained or isotype controls. (C) Immunofluorescence staining on B2M−/− and B2M−/−/HLA-E+/+ iPSC lines for pluripotency markers OCT4, NANOG, SOX2 and SSEA4 (green). Cell nuclei are stained with Hoechst (blue). Scale bar 100 μm (20x magnification). (D) Comparison by qRT-PCR of expression profile of the NK activating ligands between wild type and gene engineered B2M−/− and B2M−/−/HLA-E+/+ iPSCs. GAPDH was used as normalizer gene. Error bars represent standard deviation. N = 3. Each experiment was performed by using the same cell line at different passages and state of confluence. One-way ANOVA followed by Tukey’s post hoc test was performed.
Figure S5. Gene edited B2M<sup>+/−</sup>/B7-H3<sup>−/−</sup>, B2M<sup>+/−</sup>/CD155<sup>−/−</sup> and B2M<sup>+/−</sup>/B7-H3<sup>−/−</sup>/CD155<sup>−/−</sup> iPSCs preserve pluripotency, while specific knock-out of B7-H3 does not affects expression of other B7 family genes. 

Related to Figure 4, 5, 6 and 7 (A) Schematic representations of the PVR and B7-H3 loci, indicating cutting site (yellow triangle) of CRISPR/Cas9 complex. (B) FACS plots for B2M and CD155 or B7-H3 proteins on purified iPSC clones knocked-out for CD155 and/or B7-H3. (C) Immunofluorescence staining on B2M<sup>+/−</sup>/B7-H3<sup>−/−</sup>, B2M<sup>+/−</sup>/CD155<sup>−/−</sup> and B2M<sup>+/−</sup>/B7-H3<sup>−/−</sup>/CD155<sup>−/−</sup> iPSC lines for pluripotency markers OCT4, NANOG, SOX2 and SSEA4 (all in green). Cell nuclei are stained with Hoechst (blue). Scale bar 100 μm (20x magnification). (D) Pluripotency marker expression by flow cytometry (OCT4) and qRT-PCR (OCT4 and NANOG) into B2M<sup>+/−</sup>/B7-H3<sup>−/−</sup>, B2M<sup>+/−</sup>/CD155<sup>−/−</sup> and B2M<sup>+/−</sup>/B7-H3<sup>−/−</sup>/CD155<sup>−/−</sup> iPSCs. GAPDH was used as normalizer gene. Error bars represent standard deviation. N = 3 independent experiments. (F) Relative expression by qRT-PCR of B7-H1, -H2, -H3, -H4 and -H6 genes in both undifferentiated (iPSCs) and terminally differentiated (iBeta) B2M<sup>+/−</sup>/B7-H3<sup>−/−</sup> and B2M<sup>+/−</sup>/B7-H3<sup>−/−</sup>/CD155<sup>−/−</sup> cell lines. GAPDH was used as normalizer gene. Error bars represent standard deviation. N = 3 independent experiments. Each experiment used different independent in vitro differentiations of the edited cell lines at different passages. (G) Immune response of allogeneic CD8<sup>+</sup> T cells against unedited and edited iPSCs measured by cytotoxicity assay at 1:5 target:effector ratio. N = 3 independent experiments. Each experiment was performed by using CD8<sup>+</sup> T cells against the same cell line at different passages. One-way ANOVA followed by Dunnett’s post hoc test was performed using B2M<sup>+/−</sup> as control.
Figure S6. Activating ligand knock-out exerts protective effects against cytotoxic activity of NKG2C+ subpopulations. Related to Figure 5. (A) Representative gating strategy illustrating the total NK cells sub-gated in the NKG2A+ and NKG2C+ subsets. On the right are reported the relative contour plots of sorted NKG2A+/NKG2C- and NKG2A-/NKG2C+ NK cells used for cytotoxicity experiments. (B) Cytotoxicity on iPSC lines after co-incubation NKG2A+ and NKG2C+ NK cell subsets at the indicated target:effector ratio for 4 hours. Percentage of PI-positive target cells was measured by flow cytometry and data were normalized on basal cell death percentage occurring in target cells after incubation w/o effectors. Error bars indicate standard deviation. N = 6. All experiments were conducted using six different donors (one for each experiment). Each cytotoxicity experiment was carried out by using NKG2A+ and NKG2C+ cells derived from the same NK donor. One-way ANOVA followed by a Tukey’s test was performed. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.
Figure S7. Characterization of luciferase-expressing iPSC lines differentiated into PPs and of *in vitro* expanded donor-derived primary NK cells. Related to Figure 7 (A) Cartoon of 14-days differentiation to pancreatic progenitors (PPs). Markers of pluripotency (OCT4) and pancreatic progenitors (PDX-1, NKX6.1) are reported. (B) Relative light units (RLU) of luciferase-expressing cell lines, before (iPSCs) and after (PPs) differentiation. (C) FACS plot for PP makers PDX-1 and NKX6.1 on Wild type and edited cell lines after 14-days differentiation. Gates were set by referring to scatter plot of related unstained controls. (D) Representative phase contrast microscope view showing allogeneic donor-derived primary NK cells after isolation (day 0) and during expansion protocol with IL-2/IL-15. (E) Growth curve of NK cells during expansion protocol. Error bars represent standard deviation. N = 12 experiments carried out using twelve distinct donors. (F) Representative FACS plot of staining for CD56 and CD16 of NK cells after 12 days in expansion medium. Gates highlight the NK subsets CD56bright/CD16-, CD56bright/CD16+, CD56dim/CD16dim and CD56dim/CD16+. (G) Representative phase contrast microscope view showing NK cells at the end of 12-days *in vitro* expansion protocol (left) and after activation with IL-2/IL-12 (right). (H) Comparison between freshly isolated and expanded NK cells of their killing activity against K562 cells at different target:effector ratios. N = 6 experiments carried out using 6 different donor-derived NK cells. (I) Representative FACS plot of CD107a (degranulation maker), CD56 and CD16 expression of *in vitro* expanded NK cells before (left) and after (right) activation with IL-2/IL-12. Gates highlight the following NK subsets: CD56bright/CD16-, CD56bright/CD16+, CD56dim/CD16dim and CD56dim/CD16+.