Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients

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Autologous induced pluripotent stem cells (iPSCs) constitute an unlimited cell source for patient-specific cell-based organ repair strategies. However, their generation and subsequent differentiation into specific cells or tissues entail cell line-specific manufacturing challenges and form a lengthy process that precludes acute treatment modalities. These shortcomings could be overcome by using prefabricated allogeneic cell or tissue products, but the vigorous immune response against histo-incompatible cells has prevented the successful implementation of this approach. Here we show that both mouse and human iPSCs lose their immunogenicity when major histocompatibility complex (MHC) class I and II genes are inactivated and CD47 is over-expressed. These hypoimmunogenic iPSCs retain their pluripotent stem cell potential and differentiation capacity. Endothelial cells, smooth muscle cells, and cardiomyocytes derived from hypoimmunogenic mouse or human iPSCs reliably evade immune rejection in fully MHC-mismatched allogeneic recipients and survive long-term without the use of immunosuppression. These findings suggest that hypoimmunogenic cell grafts can be engineered for universal transplantation.

Treatment of heart disease with adult multipotent, bone marrow-derived stem cells has shown marginal efficacy in patients with acute myocardial infarction or chronic ischemic cardiomyopathy. This has been attributed to the limited plasticity of adult hematopoietic stem cells, which do not differentiate into cardiomyocytes and thus cannot replace contractile elements. Pluripotent stem cells are more promising cell sources for regenerative strategies as they can produce an unlimited amount of progeny cells that can be differentiated into functional tissue cells. Although reprogramming technology allows the generation of autologous iPSCs for patient-specific treatments, this is laborious, costly, associated with uncertain quality and efficacy of individual cell products and is only practical for chronic diseases. Thus, most regenerative approaches relying on autologous iPSC generation have been abandoned. Allogeneic cell therapies targeting large patient populations could be more economically feasible, but are subject to forceful immune rejection.

The use of allogeneic iPSC- or embryonic stem cell (ESC)-based products would require strong immunosuppression.

We envisioned engineering hypoimmunogenic pluripotent stem cells as a source for universally compatible cell or tissue grafts not requiring any immunosuppression. During pregnancy, the maternal immune system is tolerant of allogeneic paternal antigens although it would reject cells from the baby later in life. We examined syncytiotrophoblast cells, which form the interface between maternal blood and fetal tissue, and found low MHC class I and II expression (Supplementary Fig. 1) as well as strong expression of CD47, a ubiquitous membrane protein that can interact with several cell surface receptors to inhibit phagocytosis. We used this knowledge to design hypoimmunogenic mouse iPSCs (miPSCs).

C57BL/6 wild type (WT) miPSCs give rise to classical teratomas with ectodermal, mesodermal and endodermal features in SCID-beige mice (Supplementary Fig. 2). To achieve hypoimmunogenicity, these miPSCs underwent a three-step gene-editing process (Supplementary Fig. 3a). First, CRISPR guide RNAs targeting the coding sequence of the mouse β2-microglobulin (B2m) gene were ligated into vectors containing the Cas9 expression cassette and subsequently transfected into miPSCs. B2m is a ubiquitous membrane protein that can interact with several cell surface receptors to inhibit phagocytosis.

Fig. 3b–d). We confirmed that the miPSC lines we generated lacked MHC class I and II expression and negligible Cd47 expression (Supplementary Fig. 3a). First, CRISPR guide RNAs targeting the coding sequence of the mouse β2-microglobulin (B2m) gene were ligated into vectors containing the Cas9 expression cassette and subsequently transfected into miPSCs. B2m is a ubiquitous membrane protein that can interact with several cell surface receptors to inhibit phagocytosis. We used to transduce B2m−/−Ciita−/− miPSC clones followed by antibiotic selection and expansion of B2m−/−Ciita−/− Cd47 transgene (tg)-expressing miPSCs. WT miPSCs had interferon-γ (IFN-γ)-inducible MHC class I surface expression, low but detectable MHC class II expression and negligible Cd47 expression (Supplementary Fig. 3b–d). We confirmed that the miPSC lines we generated lacked MHC class I and II expression, and over-expressed Cd47 roughly 4.5-fold in flow cytometry. All three lines maintained their expression of pluripotency genes (Supplementary Fig. 3e–h).

Next, we transplanted WT miPSCs or engineered miPSCs into syngeneic C57BL/6 (H2b) and allogeneic (H2k) BALB/c recipients without immunosuppression. As expected, WT miPSCs showed...
100% teratoma growth in syngeneic recipients, but all cell grafts were rejected in allogeneic BALB/c mice (Fig. 1a,b). After 5 days, splenocytes from allogeneic BALB/c recipients showed a strong IFN-γ and a moderate IL-4 response relative to baseline responder cell activity (not shown); syngeneic mice showed no responsiveness (Fig. 1c). Only allogeneic BALB/c recipients mounted a strong IgM antibody response against the WT miPSCs relative to baseline MFI (not shown) (Fig. 1d). Engineered miPSCs developed comparable teratomas to WT miPSCs in syngeneic recipients, with enhanced survival in allogeneic recipients that depended on their level of hypoimmunogenicity and increased with every engineering step (Supplementary Fig. 4a–h). Our final B2m−/−Ciita−/− Cd47 tg miPSC line showed 100% teratoma formation and induced no IFN-γ or antibody responses (Fig. 1e–h).

We further evaluated the contribution of Cd47 overexpression by comparing B2m−/−Ciita−/− miPSCs to B2m−/−Ciita−/− Cd47 tg miPSCs in natural killer (NK) cell toxicity assays. Gene editing did not enhance the expression of stimulatory ligands for the mouse NK cell NKG2D or NKp46 receptors (Supplementary Fig. 4i), which are constitutively expressed in the NK cell-sensitive target cell line YAC-1. B2m−/−Ciita−/− miPSCs induced IFN-γ release that was significantly elevated when compared to unchallenged NK cells, but lower than IFN-γ release triggered by YAC-1 (Supplementary Fig. 4j). This suggested that Cd47 overexpression completely prevented any miPSC-induced NK cell IFN-γ release in vitro. To further assess innate miPSC clearance in vivo, a 1:1 mixture of CFSE-labeled WT miPSCs and either B2m−/−Ciita−/− miPSCs or B2m−/−Ciita−/− Cd47 tg miPSCs were injected into the innate immune cell-rich peritoneum of syngeneic C57BL/6 mice (Supplementary Fig. 5a). Using a syngeneic host for this assay precluded relevant T cell-mediated cytotoxicity. After 48 h, the peritoneal fluid was aspirated and CFSE-labeled cells were analyzed by flow cytometry. B2m−/−Ciita−/− Cd47 tg miPSCs, but not B2m−/−Ciita−/− miPSCs, were resistant to innate immune clearance and the 1:1 ratio with WT miPSCs could be maintained. We observed the same pattern of cell clearance when mice were pretreated with clodronate to eliminate macrophages (Supplementary Fig. 5b). Notably, a blocking antibody against mouse Cd47 completely abolished the protective effect of Cd47 in macrophage-depleted mice and B2m−/−Ciita−/− Cd47 tg miPSCs were rapidly eliminated (Supplementary Fig. 5c). Collectively, these data suggest an inhibitory effect of Cd47 on NK cells in vivo.

To test whether hypoimmunogenic B2m−/−Ciita−/− Cd47 tg miPSCs could give rise to hypoimmunogenic cardiac tissue, they were differentiated into endothelial cells (miECs), smooth muscle cells (miSMCs) and cardiomyocytes (miCMs) with WT miPSC derivatives serving as controls (Supplementary Fig. 6). All derivatives showed the morphologic appearance, cell marker immunofluorescence and gene expression characteristic of their mature target tissue cell lines, and cultures achieved >90% purity of VE-Cadherin+ miECs, Smα1+ miSMCs and troponin I+ miCMs. The expression of MHC class I and II molecules in WT derivatives markedly varied by cell type (Supplementary Fig. 7a–c) but, as expected, miECs had by far the highest expression of IFN-γ induced MHC class I and II, miSMCs had moderate MHC class I and II expression and miCMs had moderate MHC class I but very low MHC class II expression. All B2m−/−Ciita−/− Cd47 tg derivatives appropriately showed a complete lack of MHC class I and II and significantly elevated Cd47 compared to their WT counterparts. None of the B2m−/−Ciita−/− Cd47 tg derivatives showed upregulation of NKG2D or NKp46 ligands (Supplementary Fig. 7d,e).

We next assessed the in vivo immunogenicity of WT and hypoimmunogenic miPSC derivatives. miECs, miSMCs or miCMs derived from WT or B2m−/−Ciita−/− Cd47 tg were transplanted intramuscularly into syngeneic C57BL/6 or allogeneic BALB/c mice and adaptive immune responses were assessed after 5 days. All allogeneic recipients mounted a strong cellular IFN-γ response, as well as a strong IgM antibody response against all differentiated WT cell grafts (Supplementary Fig. 8a–c). In contrast, neither of the corresponding B2m−/−Ciita−/− Cd47 tg derivatives showed detectable increases in IFN-γ Elispot frequencies or IgM antibody production (Supplementary Fig. 8d–f). To assess the efficacy of Cd47 to mitigate the susceptibility to innate immune killing, we performed NK cell Elispot assays with antibody-coated magnetic bead-enriched BALB/c NK cells and B2m−/−Ciita−/− or B2m−/−Ciita−/− Cd47 tg derivatives (Supplementary Fig. 8g–i). While B2m−/−Ciita−/− derivatives triggered NK cell IFN-γ release, none of the B2m−/−Ciita−/− Cd47 tg derivatives produced IFN-γ spot frequencies significantly exceeding those of unchallenged NK cells. Accordingly, in vivo innate immune assays showed rapid clearance of all B2m−/−Ciita−/− derivatives, but confirmed that none of the B2m−/−Ciita−/− Cd47 tg derivatives showed susceptibility to innate elimination (Supplementary Fig. 8j–l). To confirm an inhibitory effect of Cd47 on NK cells, we next performed real-time in vitro killing assays with confluent miECs and highly purified NK cells. Both allogeneic (BALB/c) and syngeneic (C57BL/6) NK cells rapidly killed B2m−/−Ciita−/− miECs, but not WT and B2m−/−Ciita−/− Cd47 tg miECs (Supplementary Fig. 5d,e). However, antibody blocking of mouse Cd47 resulted in the rapid killing of B2m−/−Ciita−/− Cd47 tg miECs (Supplementary Fig. 5f). The effect of Cd47 is species-specific as human NK cells rapidly killed both B2m−/−Ciita−/− and B2m−/−Ciita−/− Cd47 tg miECs (Supplementary Fig. 5g).

We next examined survival of WT and hypoimmunogenic miPSC derivatives in vivo. WT and B2m−/−Ciita−/− Cd47 tg derivatives were transduced to express firefly luciferase, and Matrigel plugs containing differentiated cells were transplanted into syngeneic C57BL/6 or allogeneic BALB/c mice. All three WT derivatives showed long-term (50 days) survival in syngeneic C57BL/6recipient mice, but were rejected in allogeneic mice (Fig. 11–k). In contrast, all three B2m−/−Ciita−/− Cd47 tg derivatives showed 100% long-term survival in both syngeneic and allogeneic recipients (Fig. 11–n).

Matrigel plugs containing WT or B2m−/−Ciita−/− Cd47 tg miECs were transplanted into allogeneic BALB/c recipients (Supplementary Fig. 9a). ECs are the most immunogenic cardiac cell type due to their high expression of MHC class I and II, which allows them to function as antigen-presenting cells. We observed infiltrating immune cells containing mostly T and B lymphocytes, but also some NK cells and macrophages in WT miEC plugs. B2m−/−Ciita−/− Cd47 tg miEC-containing plugs had almost no immune cell infiltration (Supplementary Fig. 9b). In the WT plugs, cytokine arrays on day 10 revealed an inflammatory milieu that included upregulated T helper cell (T_h)-1 cytokines (IFN-γ and IL-2) and T_h-2 cytokines (IL-4, IL-5, IL-10 and IL-13). In contrast, in plugs containing B2m−/−Ciita−/− Cd47 tg miECs, the cytokine milieu was very similar to that of cell-free plugs containing only Matrigel, with no indication of immune activation (Supplementary Fig. 9c). Over time, transplanted B2m−/−Ciita−/− Cd47 tg miECs organized in circular structures and formed primitive vessels that contained erythrocytes (Supplementary Fig. 10a). Similarly transplanted B2m−/−Ciita−/− Cd47 tg miSMCs (Supplementary Fig. 10b) or miCMs (Supplementary Fig. 10c) did not form three-dimensional structures, and their in vivo maturation and integration potential in cardiac tissue remains to be studied.

We next applied our engineering strategy to human iPSCs (hiPSCs) using a human episomal iPSC line derived from CD34+ cord blood that showed a normal human XX karyotype and features of pluripotency (Supplementary Fig. 11a–c–h). The gene-editing process included two steps (Fig. 2a). First, both the human B2M and human CITAH genes were simultaneously targeted for CRISPR/Cas9-mediated disruption. Second, these edited hiPSCs were transduced with a lentivirus carrying human CD47 complementary DNA with an EFS promoter and puromycin resistance. Antibiotic-resistant B2M−/−CiITA−/− Cd47 hiPSC colonies maintained their normal
Fig. 1 | Survival of miPSCs and miPSC derivatives. a, WT C57BL/6 miPSCs were injected into the thigh muscle of syngeneic C57BL/6 or allogeneic BALB/c mice. b, Teratoma formation was observed by measuring the thigh muscle (n = 10 per group). c, IFN-γ and IL-4 enzyme-linked immunospots (Elispots) with splenocytes recovered 5 days after the transplantation (box 25th to 75th percentile with median, whiskers min–max, six animals per group, two-tailed Student’s t-test). d, Mean fluorescence imaging (MFI) of IgM binding to WT miPSCs incubated with recipient serum after 5 days (box 25th to 75th percentile with median, whiskers min–max, six animals per group, two-tailed Student’s t-test). e, 82m<sup>−</sup>Ciita<sup>−/−</sup> Cd47 tg C57BL/6 miPSCs were transplanted into syngeneic C57BL/6 or allogeneic BALB/c recipients. f, Thigh volume C57BL/6 (n = 5) and BALB/c (n = 11) animals. The overall percentage of cell grafts that survived and formed teratomas in BALB/c was 100%. g, IFN-γ and IL-4 Elispots with splenocytes recovered 5 days after the transplantation and 82m<sup>−</sup>Ciita<sup>−/−</sup> Cd47 tg miPSCs stimulator cells (box 25th to 75th percentile with median, whiskers min–max, six animals per group, two-tailed Student’s t-test). h, MFI of IgM binding to 82m<sup>−</sup>Ciita<sup>−/−</sup> Cd47 tg miPSCs incubated with recipient serum after 5 days (box 25th to 75th percentile with median, whiskers min–max, six animals per group, two-tailed Student’s t-test). i-n, Grafts of Fluc<sup>+</sup> C57BL/6 miPSC derivatives in C57BL/6 or BALB/c recipients were longitudinally followed by bioluminescent imaging (BLI). One representative animal is depicted per group and the BLI values of all animals are plotted. All WT miPSC-derived miECs (i, eight animals in C57BL/6 and six animals in BALB/c), miSMCs (j, nine animals in C57BL/6 and eight animals in BALB/c) and miCMs (k, eight animals in C57BL/6 and seven animals in BALB/c) showed long-term survival in syngeneic C57BL/6 recipients but were rejected in allogeneic BALB/c recipients. In contrast, all 82m<sup>−</sup>Ciita<sup>−/−</sup> Cd47 tg miPSC-derived miECs (l, five animals in C57BL/6 and six animals in BALB/c), miSMCs (m, five animals in C57BL/6 and five animals in BALB/c) and miCMs (n, five animals in C57BL/6 and five animals in BALB/c) showed long-term survival in both syngeneic C57BL/6 and allogeneic BALB/c recipients.
human karyotype and pluripotency (Supplementary Fig. 11d–f,i–j) and successful depletion of HLA I and II surface expression, along with overexpression of CD47, was confirmed by flow cytometry (Fig. 2b). Both WT hiPSCs and B2M−/−CIITA−/− CD47 tg hiPSCs were differentiated into endothelial-like cells (hiECs) and cardiomyocyte-like cells (hiCMs) (Fig. 2c). All derivatives showed the morphologic features and protein expression of the differentiated target cells and lost their pluripotency genes (Supplementary Fig. 12a,b). Cultures showed >95% purity for VE-Cadherin+ hiECs and tropomin 1+ hiCMs. There were no alterations in the expression of stimulatory NK cell ligands with gene engineering (Supplementary Fig. 12c–i). WT hiECs and hiCMs upregulated IFN-γ induced HLA I expression roughly three- and two-fold, respectively, compared to WT hiPSCs and hiECs also showed roughly 11-fold elevated HLA II (Fig. 2d–e). B2M−/−CIITA−/− CD47 tg hiECs and hiCMs exhibited HLA I and II depletion and significant CD47 upregulation compared to their WT counterparts.

We next performed transplant studies in humanized CD34+ hematopoietic stem cell-engrafted NSG-SGM3 mice, which were allogeneic to the hiPSC, hiEC and hiCM grafts. Since no syngeneic controls are available in this humanized mouse model, background measurements were collected in naïve mice. After 5 days, recipients of WT hiPSCs (Fig. 2f) showed a high splenocyte IFN-γ spot frequency (Fig. 2g) and elevated IgM levels (Fig. 2h). Recipients of B2M−/−CIITA−/− CD47 tg hiPSCs did not mount any detectable cellular IFN-γ response or antibody response. NK cell activation was assessed using in vitro incubation with human enriched CD56+ NK cells. B2M−/−CIITA−/− hiPSCs resulted in an IFN-γ release reaching roughly one-third of the spot frequency of the highly NK cell susceptible K562 line, whereas B2M−/−CIITA−/− CD47 tg hiPSCs did not provoke any measurable response (Fig. 2i). The allogeneic transplantation of WT hiECs (Fig. 2j) and WT hiCMs (Fig. 2n) resulted in strong systemic IFN-γ reactions (Fig. 2k,a) and IgM antibody increases (Fig. 2l,p) of similar intensity as WT hiPSCs, whereas hypoimmunogenic hiECs and hiCMs did not induce any cellular or humoral immune response. Moreover, in vitro, hypoimmunogenic derivatives did not trigger NK cell activation (Fig. 2m,a) or NK cell killing (Supplementary Fig. 5b). As with the miPSCs, a blocking antibody specific for human CD47 completely abolished NK cell protection of B2M−/−CIITA−/− CD47 tg hiPSCs (Supplementary Fig. 5i).

We also assessed the survival of hiPSCs, as well as their derivatives in allogeneic humanized NSG-SGM3 mice. All cell lines were transduced to express Fluc to enable tracking by BLI. There was no significant difference in HLA-A mismatch between groups (Supplementary Fig. 12j). As expected, all WT hiPSC grafts in Matrigel plugs underwent rejection (Fig. 3a) and all B2M−/−CIITA−/− CD47 tg hiPSCs formed teratomas. Similarly, WT hiECs (Fig. 3b) and WT hiCMs (Fig. 3c) were rejected, although at slightly slower rate than in the corresponding miPSC derivative experiments. This difference may be based on the reduced number, diversity and function of human immune cells in mouse recipients, although the triple transgenic NSG-SGM3 mice specifically express human cytokines to minimize these limitations. All B2M−/−CIITA−/− CD47 tg hiEC and hiCM grafts showed long-term survival (50 days) and stable BLI signal levels over time. The hiECs gradually organized into structures resembling primitive vascular structures, which occasionally contained erythrocytes, and the hiCMs acquired a limited polarized architecture (Fig. 3d,e).

hiECs, the most immunogenic derivatives, were further tested in the humanized BLT mouse model. BLT humanized mice are bioengineered by implantation of human fetal liver and thymic tissue under the kidney capsule followed by intravenous transplantation with autologous CD34+ HSCs (Fig. 3f). This allows for T cell maturation in human thymic tissue and permits HLA restricted T cell responses. WT hiEC grafts in Matrigel plugs triggered a roughly 40% stronger IFN-γ response but 40% weaker IgM antibody response than in the previous NSG-SGM3 mice. No measurable immune activation was detected in recipients of B2M−/−CIITA−/− CD47 tg hiEC grafts (Fig. 3g,h). All WT hiEC grafts underwent rapid rejection while four out of five B2M−/−CIITA−/− CD47 tg hiEC grafts achieved survival (Fig. 3i). We assume a non-immune-related reason for the failure of the fifth graft since no immune activation could be detected in this specific recipient. We thus demonstrated that the combination of MHC class I and II depletion and CD47 overexpression renders both mouse and human stem cells, as well as their differentiated derivatives, hypoimmunogenic. In the models studied here, engineered differentiated derivatives achieved long-term survival in fully allogeneic hosts without any immunosuppression and retained basic cell-specific features after transplantation.

The initial concept of hypoimmunogenic pluripotent stem cells was based on an MHC class I knockdown and showed encouraging early results. However, according to the 'missing-self theory', MHC class I-deficient mouse and human PSCs become susceptible to NK

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Fig. 2 | Immune response against hiPSC derivatives. a. WT hiPSCs first underwent B2M and CIITA gene disruption and then CD47 transgene overexpression. b. Gene editing of hiPSCs was confirmed by flow cytometry (box 25th to 75th percentile with median, whiskers min–max, four independent experiments per graph, analysis of variance (ANOVA) with Bonferroni’s post-hoc test). c. WT and B2M−/−CIITA−/− CD47 tg hiPSCs were differentiated into both hiECs and hiCMs. d-e. The immune phenotype of WT and B2M−/−CIITA−/− CD47 tg hiECs (d) and hiCMs (e) is shown (box 25th to 75th percentile with median, whiskers min–max, four independent experiments per graph, two-tailed Student’s t-test). f-g. WT or B2M−/−CIITA−/− CD47 tg hiPSC grafts were injected into allogeneic humanized NSG-SGM3 mice. g. IFN-γ Elispots were performed after 5 days (mean ± s.d., n = 7 per group, two-tailed Student’s t-test), the background spot frequency in naïve mice is shown (mean ± s.d., four animals per group, two-tailed Student’s t-test). h. MFI of IgM binding to either hiPSC incubated with recipient serum after 5 days (mean ± s.d., five animals per group, two-tailed Student’s t-test), the background fluorescence in naïve mice is shown (mean ± s.d., three animals per group, Student’s t-test). i. IFN-γ Elispots with human NK cells were performed with B2M−/−CIITA−/− hiPSC or B2M−/−CIITA−/− CD47 tg hiPSCs (box 25th to 75th percentile with median, whiskers min–max, six independent experiments, ANOVA with Bonferroni’s post-hoc test). j. WT or B2M−/−CIITA−/− CD47 tg hiEC grafts were injected into allogeneic humanized NSG-SGM3 mice. k. IFN-γ Elispots were performed after 5 days (mean ± s.d., three animals per group, two-tailed Student’s t-test), the background spot frequency in naïve mice is shown (mean ± s.d., three animals per group, Student’s t-test). l. MFI of IgM binding to either hiEC incubated with recipient serum after 5 days (mean ± s.d., three animals per group, two-tailed Student’s t-test), the background fluorescence in naïve mice is shown (mean ± s.d., three animals per group, Student’s t-test). m. IFN-γ Elispots with human NK cells were performed with B2M−/−CIITA−/− hiECs or B2M−/−CIITA−/− CD47 tg hiECs (box 25th to 75th percentile with median, whiskers min–max, six independent experiments, ANOVA with Bonferroni’s post-hoc test). n. WT or B2M−/−CIITA−/− CD47 tg hiCM grafts were injected into allogeneic humanized NSG-SGM3 mice. o. IFN-γ Elispots were performed after 5 days (mean ± s.d., three animals per group, two-tailed Student’s t-test), the background spot frequency in naïve mice is shown (mean ± s.d., four animals per group, two-tailed Student’s t-test), the background fluorescence in naïve mice is shown (mean ± s.d., three animals per group, Student’s t-test). p. MFI of IgM binding to either hiCM incubated with recipient serum after 5 days (mean ± s.d., three animals per group, two-tailed Student’s t-test), the background fluorescence in naïve mice is shown (mean ± s.d., three animals per group, Student’s t-test). q. IFN-γ Elispots with human NK cells were performed with B2M−/−CIITA−/− hiCMs or B2M−/−CIITA−/− CD47 tg hiCMs (box 25th to 75th percentile with median, whiskers min–max, six independent experiments, ANOVA with Bonferroni’s post-hoc test).
Although isolated expression of HLA-E or HLA-G in human pluripotent stem cells has been used to mitigate NK cell cytotoxicity, we observed that CD47 is a very effective non-MHC ligand to silence all innate immune responses. However, cells eluding immune monitoring may pose the long-term risks of uncontrollable malignant transformation or impaired virus clearance, although for the latter alternative mechanisms have been shown. Inducible kill switches could enhance their overall safety.

The ability to generate substantial amounts of cardiac tissue from allogeneic iPSC-derived CMs has been well demonstrated in macaques. However, the amounts of toxic immunosuppressive drugs required to achieve allogeneic cell survival pose a major
Fig. 3 | Survival of hiPSCs and hiPSC derivatives in allogeneic hosts. Grafts of Fluc^+ WT or $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiPSCs, hiECs and hiCMs were transplanted into allogeneic humanized mice (NSG-SGM3 mice in a–e, BLT mice in f–i) and were longitudinally followed by BLI. One representative animal is depicted per group and the BLI values of all animals are plotted. a, BLI signals over time of WT and $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiPSC grafts ($n=5$ per group). b, WT and $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiECs were transplanted as in a ($n=5$ per group). c, WT and $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiCMs ($n=5$). d, $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiECs started to organize into a more complex structure, which included primitive vascular structures (representative pictures of three independent experiments). Scale bar, 50 μm. e, The $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiCMs began to organize into a more polarized framework and maintained their sarcomeric alpha-actinin cytoskeletal structure typical of cardiomyocytes (representative pictures of three independent experiments). Scale bar, 50 μm. f, WT or $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiECs were transplanted into allogeneic humanized BLT mice. g, IFN-γ Elispots were performed after 5 days (box 25th to 75th percentile with median, whiskers min–max, four animals per group, two-tailed Student’s t-test), the background spot frequency in naïve mice is shown. h, MFI of IgM binding to either hiEC incubated with recipient serum after 5 days (mean ± s.d., four animals per group, two-tailed Student’s t-test), the background fluorescence in naïve mice is shown (mean ± s.d., three animals per group, two-tailed Student’s t-test). i, Grafts of Fluc^+ WT or $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiECs were transplanted into allogeneic humanized BLT mice and were longitudinally followed by BLI. All WT hiEC grafts were rejected within roughly 14 days (four animals). Four of the five $B2M^{-/-}CIITA^{-/-}$ CD47 tg hiEC grafts permanently survived, the one failure is believed not to be immune-mediated (five animals).
hurdle for clinical use. Even with fully MHC class I- and II-matched allogeneic iPSC-derived CM grafts, macaque recipients required substantial and highly toxic immunosuppression to prevent cell rejection.20,29 Thus, the generation of universal hypoinnunogenic iPSCs that can be differentiated into the main components of cardiac tissue and achieve long-term survival in a fully allogeneic recipient without any immunosuppression may help to develop universal cell products to treat heart failure.

Online content
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Author contributions
T.D. and S.S. designed the experiments, supervised the project, and wrote the manuscript. X.H. performed the adaptive and innate immunobiology experiments, molecular biology and imaging studies and cell culture work and analyzed the data. A.G. performed imaging studies and cell injections. C.D. and W.O.T. generated BLT mice and performed the BLT imaging experiments. A.W. and J.V.G. designed and supervised the project, and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Methods

Syncytiotrophoblast cells of mouse placenta. On isolated mouse syncytiotrophoblast cells, expression of MHC class I, MHC class II and Cd47 was performed using PCR. RNA was isolated with the RNasy Plus Mini kit (Qiagen) according to the manufacturer’s protocol. RT-PCR was performed to generate the cDNA (Applied Biosystems). The following primers were used: mouse MHC class I: 5'-AGGTGTCGTCAGCACTTACA-3'; reverse: 5'-GGTGACCTCAAACTTGTAGC-3', MHC class II forward: 5'-GATGCAGACCTGGG-3', reverse: 5'-TGATCTTCTGAGGGTTTT-3'; mouse Cd47 forward: 5'-GGCGCAAGACCCAGAAGAATGTT-3', reverse: 5'-CCATGGCATGCCGTTATCCATT-3'; PCR were performed on Mastercycler nexus (Eppendorf) and the amplification products were visualized by 2% agarose gel electrophoresis (Thermo Fisher).

Derivation of mouse iPSCs. Mouse tail tip fibroblasts of mice were dissociated and isolated with collagenase type IV (Life Technologies) and maintained with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% glutamine, 4.5g/l glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin (pen-strep) at 37°C, 20% O₂, and 5% CO₂ in a humidified incubator. 1 x 10⁶ mouse fibroblasts were then re-programmed using a mini-intronic plasmid carrying sequences of Oct4, Klf4, Sox2 and c-Myc as well as short hairpin RNA against p53 (10–12 μg plasmid carrying sequences of Oct4, Klf4, Sox2 and c-Myc as well as short hairpin RNA against p53). The transfection was done using lipofectamine 2000 (Invitrogen). The transfection efficiency was then determined by immunostaining the generated iPSC colonies for OCT4 and KLF4. The transfection was repeated 3 times and the iPSCs were expanded in the presence of LIF. The iPSCs were then induced to differentiate into three embryonic lineages: endodermal, mesodermal, and neural lineages using inducing medium (DMEM, 1% B2m, 10% serum, 1% competence factor). After the MEF feeder cells attached and were 100% confluent, miPSCs were grown on MEF in knockout DMEM 10829 with 15% complete B2m media. Cell cultures were regularly screened for mycoplasma infections using the kit's instructions (GeneArt CRISPR Nuclease Kit, Thermo Fisher). miPSCs were transfected with the AIO vectors containing Neomycin-resistance cassette and the target gene sequences. Antibiotic-selected pools were tested for Cd47 overexpression and B2m−/−Cd47 +/− miPSCs were expanded.

Generation of B2m−/− miPSCs. CRISPR technology was used for disruption of the B2m gene. For targeting the coding sequence of mouse B2m gene, the CRISPR sequence 5’-TCCGCTCCATCTCCTGGG(TGG)-3’ was annealed and ligated into the All-In-One (AIO) vectors containing the Cas9 expression cassette as per the kit’s instructions (GeneArt CRISPR Nuclease Kit, Thermo Fisher). miPSCs were transfected with the AIO vectors using Neon electroporation with 1,200 V pulses of 20 ms duration. The transfected iPSC cultures were dissociated to single cells using 0.05% trypsin (Gibco) and then sorted with FACSAria cell sorter (BD Biosciences) for removing doublets and debris by selective gating on forward and side light scatter properties. Single cells were expanded to full-size colonies and tested for CRISPR editing by screening for the presence of the altered sequence from the CRISPR cleavage site. Briefly, the target sequence was amplified via PCR using AmpliTag Gold Master Mix (Applied Biosystems) and the primers B2m gDNA forward: 5′-CTCGATACGACATAGTGTGGGGA-3′; reverse: 5′-GCAAAGGTTTTAATGCCACAGAC-3′. After cleanup of the obtained PCR product (PureLink Pro 96 PCR Purification Kit, Thermo Fisher), sequencing was performed. The Ion Personal Genome Machine (PGM) Sequencing was used for the identification of the homogeneity, through sequencing of the PCR amplified 250 bp fragment from the B2m gDNA of each transfection. Sanger sequencing was performed as above and prepared using the Ion PGM Hi-Q Template Kit (Thermo Fisher). Experiments were performed on the Ion PGM System with the Ion 318 Chip Kit v2.1 (Thermo Fisher).

Generation of B2m−/−Cd47 +/− miPSCs. CRISPR technology was used for the further disruption of the Cd47 gene. For targeting the coding sequence of mouse Cd47 gene, the CRISPR sequence 5′-GGTCCATCTGCGTACATTGGG(CGG)-3′ was annealed and ligated into the All-In-One (AIO) vectors containing the Cas9 expression cassette as per the kit’s instructions (GeneArt CRISPR Nuclease Kit, Thermo Fisher). miPSCs were transfected with the AIO vectors containing Cd47 mRNA under recombination. Antibiotic-selected pools were tested by RT–quantitative PCR amplification of Cd47 mRNA and flow cytometry detection of Cd47 on the surface of the cells. Single cells were expanded to full-size colonies and tested for CRISPR editing by screening for the presence of an altered sequence from the CRISPR cleavage site. Briefly, the target sequence was amplified via PCR using AmpliTag Gold Master Mix (Applied Biosystems) and the primers Cd47 gDNA forward: 5′-CCCCACAGAGGATGAGCATGTT-3′; reverse: 5′-GGATTCTTATGTCAGCGCCGGGT-3′. The PCR product was purified as described above and prepared using the Ion PGM Hi-Q Template Kit (Thermo Fisher). Experiments were performed on the Ion PGM System with the Ion 318 Chip Kit v2.1 (Thermo Fisher).

Pluripotency analysis by RT–PCR and immunofluorescence. miPSCs were plated in confluent dishes (MatTek) for immunofluorescence analysis 48h after plating using the miPSC Characterization Kit (Applied Stemcell). Briefly, cells were fixed, permeabilized, and stained overnight at 4°C with the primary antibodies for Sox2, SSEA-1 and Oct4. After several washes the cells were incubated with a secondary antibody and DNA staining solution. Alkaline phosphatase activity assay was performed (Applied Stemcell). Stained cells were imaged using a fluorescent microscope.

For RT–PCR, RNA was extracted using the RNeasy Plus Mini Kit (Qiagen). Genomic DNA contamination was removed using the gDNA spin column. cDNA was generated using Applied Biosystems High-Capacity cDNA Reverse Transcription kit. Gene-specific primers of the miPSC Characterization Kit (Applied Stemcell) were used to amplify target sequences. Actin was used as housekeeping gene, which encodes a cellular cytoskeleton protein. PCR reactions were performed on Mastercycler nexus (Eppendorf) and visualized on 2% agarose gels.

Pluripotency analysis by in vivo teratoma assay. Ten million miPSCs were injected intramuscularly into the hind limb of immunodeficient SCID-beige mice and teratoma development was observed within 14 days. Teratomas were recovered and fixed in 4% parafomaldehyde in PBS, dehydrated, embedded in paraffin, and cut into sections of 5 μm thickness. For histopathology, sections were rehydrated and stained with hematoxylin and eosin (Carl Roth). Images were taken with an inverted light microscope.

Generation of B2m−/−Cd47 +/− miPSCs. Cd47 transgene overexpression was generated using lentivirus-mediated delivery of a Cd47-expressing vector containing the antibiotic resistance cassette blasticidin. The Cd47 cDNA was synthesized and cloned into the lentiviral plasmid pLenti6/V5 (Thermo Fisher) with a blasticidin resistance. Sanger sequencing was performed to verify that no mutation had occurred. Lentiviruses generation was performed with a stock titer of 1x10⁶ TU per ml. The transduction was performed into 2x10⁶ B2m−/−Cd47 +/− miPSCs, grown on blasticidin-resistant MEF cells for 72 hours with a MOI ratio of 1:10 followed by antibiotic selection with 12.5 μg/ml blasticidin for 7 days. Antibiotic-selected pools were tested by RT–quantitative PCR amplification of Cd47 mRNA and flow cytometry detection of Cd47 on the surface of the cells. After the confirmation of Cd47, cells were expanded and validated by running pluripotency assays.

Transduction to express firefly luciferase. iPSCs were transduced to express Fluc. One hundred thousand miPSCs were plated in one gelatin-coated six-well plate and incubated overnight at 37°C at 5% CO₂. The next day, media was changed and iPSCs were exposed to Fluc lentiviral particles expressing luciferase II gene. A construct engineered EFla promoter (GenTarget) was added to 1.5 ml media. After 36 h, 1 ml of cell media was added. After further 24 h, complete media change was performed. After 2 days, luciferase expression was confirmed by adding D-Luciferin (Promega). Signals were quantified with IVIS 200 (Perkin Elmer) in maximum photons s⁻¹ cm⁻² sr⁻¹.

Karyotyping. Cell collection, slide preparation and G-banded karyotyping were performed using standard cytogenetics protocols optimized for human pluripotent...
cells. Cells were incubated with ethidium bromide and colcemid (Gibco) and then trypsinized to detach the cells from the plate. The cells were plated in hypotonic solution (0.075 M potassium chloride, 0.559 g KC1 in 100 ml water, Millipore), followed by fixation of each plate per group of five for each figure. Mice were purchased from Charles River Laboratories (Sulzfeld) and received humane care in compliance with the Guide for the Principles of Laboratory Animals. Animal experiments were approved by the Hamburger Amt für Gesundheit und Verbraucherschutz or the University of California San Francisco (UCSF) Institutional Animal Care and Use Committee and performed according to local and EU guidelines.

**Mice.** BALB/c (BALB/cAnN-SCID, H2), C57BL/6 (C57BL/6, H2) and SCID-beige (CBySmn.CB17-Prkdcscid/J) (all 6–12 weeks) were used as recipients for different assays. The number of mice per group of each figure is presented in parentheses. Mice were used: SMA forward 5′-CGGTTGCGCTGTGGATGATG-3′, reverse 5′-CATTCACACATTACCTCCGTGAT-3′; SM22 forward 5′-AAACCGCTTCACCGTATGG-3′, reverse 5′-GGATGAGCCACGCTTCTC-3′.

**Immunofluorescence (IF) staining.** The highly purified miECs in the flow-through were cultured in EGM-2 media and was changed every other day. Beating of cells started around days 11–14 and demonstrated their function. For enrichment, cells were plated on MACS according the manufacturer’s protocol using anti-CD15 mAb-coated magnetic microbeads (Miltenyi) for negative selection. The flow-through containing enriched miCMs were replated and used for different assays.

**Flow cytometry analysis.** The fluorescence intensity of HMC class I and II surface molecules on miPSCs, miECs, miSMCs and miCMs were plated on gelatin-coated six-well plates in medium containing 100 ng/ml of IFN-γ. After collection, cells were labeled with antibodies. For HMC class I: PerCP-eFlour710-labeled anti-MHC class I antibody (clone AF6-88.5.5.3, eBioscience) or PerCP-eFlour710-labeled mouse IgG2b isotype-matched control antibody (clone eB149/10H5, eBioscience). The anti-MHC class I antibody reacts with the H-2K2 MHC class I allotetrad. For HMC class II: PerCP-eFlour710-labeled anti-MHC class II antibody (clone M5/114.15.2, eBioscience) or PerCP-eFlour710-labeled mouse IgG2b isotype-matched control antibody (clone eB149/10H5, eBioscience). The MHC class II antibody reacts with the mouse MHC class II, both I-A and I-E subregion-encoded glycoproteins. Cyto-47: Alexa Fluor 647-labeled anti-mouse CD47 antibody (clone miap301, BD Biosciences) or Alexa Fluor 647-labeled mouse IgG2a isotype-matched control antibody (clone R35-95, BD Biosciences). The anti-CD47 antibody specifically binds to the extracellular domain of mouse CD47, also known as Integrin-Associated Protein. Cells were analyzed by flow cytometry (BD Bioscience) and results were expressed as fold change to isotype-matched control Ig staining.

**Donor-specific antibodies.** Sera from recipient mice were de-complemented by heating to 56 °C for 30 min. Equal amounts of sera and cell suspensions (5 × 10^6 ml) were incubated for 45 min at 4 °C. Cells were labeled with FITC-conjugated goat anti-mouse IgM (Sigma-Aldrich) and analyzed by flow cytometry (BD Bioscience). The anti-CD47 antibody specifically binds to the extracellular domain of mouse CD47, also known as Integrin-Associated Protein. Cells were analyzed by flow cytometry (BD Bioscience) and results were expressed as fold change to isotype-matched control Ig staining.

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**Elispot assays.** Uni-directional Elispot assays, recipient splenocytes were isolated from spleen 5 days after cell injection and used as responder cells. Donor cells were mitomycin-treated (50 μg/ml for 30 min) and used as stimulator cells. One hundred thousand stimulator cells were incubated with 1 × 10^6 recipient responder splenocytes for 24 h and IFN-γ and IL-4 spot frequencies were enumerated using an Elispot plate reader.
and stained with 5 μM CFSE. Cells in saline with IL-2 (1 ng/ml), Patelpro) were injected intraperitoneally into syngeneic C57BL/6 mice. After 48 h, cells were collected from the abdomen and stained with PerCP-εFlour701 labeled anti-MHC class I mAb for 45 min at 4 °C. The CFSE-positive and MHC class I negative population was analyzed by flow cytometry (BD Bioscience) and compared between the WT and the engineered miPSC group. The assay was performed with miPSCs, miECs, miSMCs and miCMs. Some animals were pretreated with in vivo Cd47-blocking antibody (BE0270, Fisher) and Sanger sequencing was performed for the prediction of indel frequency. After the confirmation of 2Bm and CIITA disruption, cells were further characterized through karyotype analysis and the TaqMan hiPSC Scorecard Panel (Thermo Fisher). The hiPSCs were found to be pluripotent and maintained a normal 46,XX karyotype during the genome editing process.

In the second step, the CD47 cDNA was synthesized and the DNA was cloned into a lentiviral plasmid with an EFS promoter and puromycin resistance cassette. Cells were transduced with lentiviral stocks and 8 μg/ml of Polybrene (Thermo Fisher). Media was changed daily after transduction. Three days after transduction, cells were expanded and selected with 0.5 μg/ml of puromycin. After 14 days of antibiotic selection, antibiotic-resistant colonies emerged and were further expanded to generate stable pools. The expression of CD47 transcripts was confirmed by quantitative PCR. Pluripotency assay (TaqMan hiPSC Scorecard Panel, Thermo Fisher) and karyotyping was performed again to verify the pluripotent status of the cells.

**Teratoma assays to study iPSC survival in vivo**. Six to eight week-old immunodeficient SCID-beige mice were used for transplantation of WT hiPSC or 2Bm−/−CIITA−/− CD47 tg hiPSCs. Here 1 × 10⁶ cells were resuspended in 100 μl saline solution and injected into the right thigh muscle of the mice. Teratomas were recovered, fixed, in 4% paraformaldehyde, dehydrated, embedded in paraffin and cut into sections of 5 μm thickness. For histopathology, sections were rehydrated and stained with hematoxylin and eosin. Images were taken with an inverted light microscope. For immunofluorescence, slides underwent heat-induced antigen retrieval in a steamer with Dako antigen-retrieval solution (Dako), followed by antigen blocking with Image-IT FX signal enhancer solution (Invitrogen). Tissue sections were incubated with a primary antibody against brachyury (Ab20680, Abcam), followed by a goat and a mouse secondary antibodies. Staining was visualized with Alexa Fluor 555 (Invitrogen). Subsequently, sections were incubated with primary antibodies against cytokeratin 8 (EP1628Y, Abcam) and GFAP (G5, Cell Signaling) conjugated with AF488 or AF647, respectively. DAPI was used to counterstain cell nuclei and images were acquired with a Leica SPS laser confocal microscope (Leica).

**Pluripotency analysis by RT-PCR and immunofluorescence**. hiPSCs were plated in conical dishes (MatTek) for immunofluorescence analysis 48h after plating using the hiPSC Characterization Kit (Applied Stem Cell). Briefly, cells were fixed, permeabilized and stained overnight at 4 °C with the primary antibodies for OCT4, SOX2, SSEA4, TRA-1-60 and TRA-1-81 (Applied Stem Cell). After several washes, the cells were incubated with a secondary antibody and DNA staining solution. Alkaline phosphatase activity assay was performed (Applied Stem Cell). Stained cells were imaged using a fluorescent microscope.

For RT-PCR, RNA was extracted using the RNeasy Plus Mini Kit (Qiagen). Genomic DNA contamination was removed using the gDNA spin column. cDNA was generated using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. Gene-specific primers of the hiPSC Characterization Kit (Applied Stem Cell) were used to amplify target sequences Actin was used as housekeeping gene. PCR reactions were performed on a Mastercycler nexus (Eppendorf) and visualized on 2% agarose gels.

**Humanized mice**. Humanized NSG-SGM3 mice (18–30 weeks) were purchased from Jackson Laboratories. Human CD34+ hematopoietic stem cell-engrafted NSG-SGM3 mice develop multi-lineage human immune cells, and demonstrate a functional human immune system displaying T cell-dependent immune responses with no donor cell immune reactivity towards the host. Animals were randomly assigned to experimental groups. The percentage of CD3+ cells among the human CD45+ cell population was assessed every two weeks and CD3+ cells were then expanded into full-size colonies, after which the colonies were tested for CRISPR editing by sequencing. CRISPR-mediated cleavage was assessed using the GeneArt Genomic Cleavage Detection Kit (Thermo Fisher) for testing of the initial edited pools. For screening of the isolated clones, genomic DNA was isolated from 1 × 10⁶ hiPSCs and the 2Bm and CIITA genomic DNA regions were PCR amplified using AmpliTaq Gold 301 Master Mix and the primer sets forward: 5′-TGGGGCCAAATCATGTAGACTC-3′ and reverse: 5′-TCAGGGGGTTGAATACCGTGTTG-3′ for 2Bm as well as forward: 5′-CTTAAAAACCATGCTTGACCTG-3′ and reverse: 5′-TGGCTTCACTTCCCTCCTCTCT-3′ for CIITA. For TIDE analysis, the obtained PCR product was cleaned using NEB核苷酸转运体 Purification Kit, Thermo Fisher) and Sanger sequencing was performed for the prediction of indel frequency. After the confirmation of 2Bm and CIITA disruption, cells were further characterized through karyotype analysis and the TaqMan hiPSC Scorecard Panel (Thermo Fisher). The hiPSCs were found to be pluripotent and maintained a normal 46,XX karyotype during the genome editing process.

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were expressed as fold change to isotype-matched control Ig staining. IgG1 isotype-matched control antibody (clone MOPC-21, BD Biosciences). Results were expressed as fold change to isotype-matched control Ig staining.

Human iPSC differentiation into hiECs. hiPSCs were plated on diluted Matrigel (356231, Corning) in six-well plates and maintained in Essential 8 Flex media (Thermo Fisher). The differentiation was started at 60% confluence and media was changed to RPMI-1640 containing 2% B-27 minus insulin (both Gibco) and 5μM CHIR-99021 (Selleckchem). On day 2, the media was changed to reduced media: RPMI-1640 containing 2% B-27 minus insulin (Gibco) and 2μM CHIR-99021 (Selleckchem). From day 4 to 7, cells were exposed to RPMI-1640 EC media, RPMI-1640 containing 2% B-27 minus insulin plus 50 ng/ml human vascular endothelial growth factor (VEGF, R&D Systems), 10 ng/ml human fibroblast growth factor basic (FGFb; R&D Systems), 10μM V-27632 (Sigma-Aldrich), and 1μM SB 431542 (Sigma-Aldrich). Endothelial cell clusters were visible from day 7 and 7 cells were maintained in EGM-2 SingleQuots media (Lonza) plus 10% FCS hi (Gibco), 25 ng/ml VEGF, 2 ng/ml FGFb, 1μM V-27632 (Sigma-Aldrich) and 1μM SB 431542 (Sigma-Aldrich). The differentiation process was completed after 14 days and undifferentiated cells detached during the differentiation process. For purification, cells were treated with 20μM PluriSin-1 (StemCell Technologies) for 48h. The highly purified ECs were cultured in EGM-2 SingleQuots media (Lonza) plus supplements and 10% FCS hi (Gibco). TrypLE Express was used for passing the cells 1:3 every 3–4 days.

Immunofluorescence staining was performed as described above to confirm their phenotype. Primary antibodies were used against CD31 (ab28364, Abcam) and VE-Cadherin (sc-6458, Santa Cruz Biotechnology), followed by the corresponding secondary antibody conjugated with AF488 or AF555 (Invitrogen). Cell nuclei were stained with DAPI. Imaging was performed using a Leica SP5 laser confocal microscope (Leica).

Human iPSC differentiation into hiCMs. hiPSCs were plated on diluted Matrigel (356231, Corning) in six-well plates and maintained in Essential 8 Flex media (Thermo Fisher). The differentiation was started at 60% confluence and media was changed to RPMI-1640 containing 2% B-27 minus insulin (both Gibco) and 5μM CHIR-99021 (Selleckchem). On day 2, the media was changed to reduced media: RPMI-1640 containing 2% B-27 minus insulin (Gibco) and 2μM CHIR-99021 (Selleckchem). After 2 days, media was changed to RPMI-1640 containing 2% B-27 minus insulin without CHIR. On day 3, 5μl IWR1 was added to the media for two further days. At day 5, the media was changed back to RPMI-1640 containing 2% B-27 minus insulin and left for 48h. At day 7, media was changed to B2M-1640 containing B27 plus insulin (Gibco) and replaced every 3 days thereafter with the same media. Spontaneous beating of cardiomyocytes was first visible around day 10. Purification of cardiomyocytes was performed as described above to confirm their phenotype. Primary antibodies were used against α-sarcomeric actinin (EA-53, Abcam) and troponin I (ab47003, Abcam), followed by the corresponding secondary antibody conjugated with AF488 or AF555 (Invitrogen). Cell nuclei were stained with DAPI. Imaging was performed using a Leica SP5 laser confocal microscope (Leica).

For the assessment of purity of hiPSC derivatives, antibodies against TRA-1-60 (Thermo Fisher), VE-Cadherin (Santa Cruz) and Troponin I (Santa Cruz) were used. The hiECs and hiCMs were generated with a purity of >95%.

Human NK cell Elispot assays. Human NK cells were co-cultured with B2M-‘‘CITA’’ or B2M-‘‘CIITA’’ CD47 hiPSCs and their IFN-γ release was measured. Human NK cells were purchased from StemCell Technologies and were >99% CD3- and CD56+. Flow cytometry revealed >95% NK cells and <5% other cells including myeloid cells. Donor cells were mitomycin-treated and used as stimulator cells. K562 cells (Sigma-Aldrich) served as positive control. Stimulator cells were incubated with NK cells (1:1) in RPMI-1640 containing 1% pen-strep and 1 ng/ml human IL-2 (Peprotech) for 24h and IFN-γ spot frequencies were enumerated using an Elispot plate reader.

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Matrigel plugs: cell morphology. One million B2M-‘‘CITA’’ or B2M-‘‘CIITA’’ CD47 hiECs or hiCMs in 1:1 pro survival scaffold, consisting of 50% (vol/vol) Matrigel (Corning), 100 μM ZVAD (Millipore), 50 μM Bcl-2 (Bcl-2 (Bcl-2 (Bcl-2 (Bcl-2)). Cell Index values were reach 0.7, NK cells were added with an effector cell / target ratio of 1:1. NK cells were incubated with NK cells (1:1) in RPMI-1640 containing 1% pen-strep and 1 ng/ml human IL-2 (Peprotech) for 24h and IFN-γ and IL-5 spot frequencies were enumerated using an Elispot plate reader.

DANGER. Sera from recipient mice were de-complemented by heating to 56°C for 30 min. Equal amounts of sera and cell suspensions (5×10^6 per ml) were incubated for 45 min at 4°C. Cells were labeled with FITC-conjugated goat anti-human IgM (BD Bioscience) and analyzed by flow cytometry (BD Bioscience).

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For BLI, D-luciferin firefly potassium salt (375 ng/μl) (Biosynth) dissolved in sterile PBS (pH 7.4) (Gibco, Invitrogen) was injected intraperitoneally (250 μl per mouse) into anesthetized mice. Animals were injected using the mini HT (Spectral Instruments Imaging) ROI bioluminescence was quantified in units of maximum photons per second per centimeter square per steradian (p s^-1 cm^2^-1). The maximum signal from an ROI was measured using Living Image software (MediaCybernetics). Humanized mouse were injected with 5×10^5 or 1×10^6 cells in pro survival scaffold as described above. Mice were monitored on day 0, day 1 and every 4 days until cells were rejected or up to 50 days.

In vitro NK cell killing. Mouse NK cells were isolated from fresh BALB/c or C57BL/6 spleens 18 h after pol L-1 injection (100μg intraperitoneally). After red cell lysis, NK cells were purified with MagneSort Mouse NK cell Enrichment Kit (Invitrogen), followed by CD49b MACS-sorting (Milteny). This cell population was highly selected for NK cells with a purity of >95%. Human NK cells from PBMCs were purchased from StemCell Technologies containing >99% NK cells.

NK cell killing assays were performed on the XCelligence SP platform (ACEA Biosciences). 96-well E-plates (ACEA Biosciences) were coated with collagen (Sigma-Aldrich) and 4×10^5 WT, B2M-‘‘CITA’’ or B2M-‘‘CIITA’’ CD47 hiECs or WT, B2M-‘‘CITA’’ or B2M-‘‘CIITA’’ CD47 hiECs were plated in 100 μl cell-specific media containing 1 ng/ml mouse or human IL-2 (Peprotech). After the Cell Index value reached 0.7, NK cells were added with an effector cell / target cell (E/T) ratio of 0.5/1, 0.8/1 or 1/1. As a negative control, cell treated with 2% Triton X100 was used. Some wells were pretreated with mouse CD47 or human CD47-blocking antibody (BioXcell) with 10μg/ml media for 2h. Data were standardized and analyzed with the RTCA software (ACEA).

NK cell stimulatory ligands. For the detection of NKGD2, NKp80, NKp46, NKp44 and NKp30 on hiPSCs, hiECs and hiCMs, cells were plated on gelatin-coated six-well plates. K562 cells were plated in six-well plates as suspension cells. After harvesting, cells were blocked with human FcR blocking reagent (Milteny) according to manufacturer’s protocol. Cells were labeled with anti-human NKGD2, NKp80, NKp46, NKp44 or NKp30 Fc chimera proteins or the recombinant control antibody IgG1 Fc protein (all R&D Systems) for 45 min at 4°C, followed by the secondary antibody IgG1 conjugated with FITC (Invitrogen). Data analysis was
carried out by Flow Cytometry (BD Bioscience) and results were expressed as fold change to isotype control.

**Statistics.** All data are expressed as mean ± s.d. or in box blot graphs showing the median and the minimum to maximum range. Intergroup differences were appropriately assessed by either an unpaired Student’s *t*-test or a one-way ANOVA with Bonferroni’s post-hoc test. Further information on experimental design and reagents is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
All data supporting the findings of this study are available in the paper and its Supplementary Information files.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.