

Targeted Disruption of HLA genes via CRISPR-Cas9 generates iPSCs with Enhanced Immune Compatibility

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Summary

Induced pluripotent stem cells (iPSCs) have strong potential in regenerative medicine applications, however, immune rejection caused by HLA-mismatching is a concern. B2M gene knockout and HLA-homozygous iPSC stocks can potentially address this issue, but the former approach may induce NK cell activity and fail to present antigens, and it is challenging to recruit rare donors for the latter method. Here, we show two genome-editing strategies for making immune compatible donor iPSCs. First, we generated HLA pseudo-homozygous iPSCs with allele-specific editing of HLA heterozygous iPSCs. Second, we generated HLA-C-retained iPSCs by disrupting both HLA-A and -B alleles to suppress the NK cell response while maintaining antigen presentation.

1 Importantly, HLA-C-retained iPSCs could evade T cells and NK cells *in vitro* and
2 *in vivo*. We estimated that 12 lines of HLA-C-retained iPSCs combined with
3 HLA-Class II knockout are immunologically compatible with over 90% of the
4 world-wide population, greatly facilitating iPSC-based regenerative medicine
5 applications.

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10 11 **INTRODUCTION**

12 An abundance of research has shown the potential of pluripotent stem cells
13 such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)
14 in the field of regenerative medicine (Mandai et al., 2017; Schwartz et al., 2012).
15 However, one major concern that must be overcome before their widespread
16 clinical use is potential HLA mismatching, which can cause immune rejection
17 (Williams et al., 2017). When considering HLA matching, HLA class I and HLA
18 class II molecules are paramount. HLA class I molecules (HLA-A, -B, -C) are
19 expressed on all nucleated cells and display antigens from pathogens or
20 transformed tumor cells to CD8⁺ cytotoxic T cells, while HLA class II molecules
21 (HLA-DP, -DQ, -DR) are almost exclusively expressed in antigen presenting
22 cells, such as macrophages and dendritic cells, to activate CD4⁺ helper T cells.
23 In addition, class II expression can also be induced in other cell types upon
24 stimulation by interferons

25 From an immunological perspective, autologous transplantation is ideal, but
26 it is time and cost consuming. For allogeneic transplantation, although iPSC
27 stocks isolated from HLA homozygous donors are being considered for covering
28 most HLA haplotypes, the recruitment of HLA homozygous donors that serve an
29 entire population is not trivial. It has been estimated that more than 150,000
30 donors must be screened to establish 140 HLA-A, -B, and -DR homozygous
31 iPSC lines that serve 90% of the Japanese population (Okita et al., 2011), and
32 that number is even higher for multiethnic societies (Taylor et al., 2012).

33 As an alternative to HLA homozygous iPSCs, researchers have been using

genome-editing technologies to knockout the β 2-microglobulin (B2M) gene. B2M protein forms a heterodimer with HLA class I proteins and is required for HLA class I presentation on the cell surface (Gornalusse et al., 2017; Lu et al., 2013; Mandal et al., 2014; Riobobos et al., 2013). Hence, suppression of the B2M gene would prevent an immune response from cytotoxic CD8⁺ T cells by depleting all HLA class I molecules (HLA-A, -B, -C, -E, -F, and -G). However, the lack of HLA would activate a response by natural killer (NK) cells and risk the proliferation of cells that are infected by pathogens or are oncogenic (Ichise et al., 2017; Marty et al., 2017; McGranahan et al., 2017). Overexpression of single-chain HLA-E fused with B2M could suppress NKG2A⁺ NK cells (Gornalusse et al., 2017), but not KIR2DL1-4⁺ NK cell populations, as these are suppressed through HLA-C or HLA-G binding. In addition, the single-chain HLA-E fused with B2M has no antigen presentation ability. Overexpression of immunosuppressive receptors CTLA4-Ig and PD-L1 in hESCs have been shown to prevent allogeneic immune-rejection (Rong et al., 2014), however, the transgene approach might suffer from transgene silencing before or after differentiation from the pluripotent state.

Owing to recent advances of genome-editing technologies, other groups have eliminated HLA-A genes by zinc finger nucleases in hematopoietic stem cells (Torikai et al., 2016; 2013) for increasing donor compatibility, yet these studies did not address matching HLA-B and HLA-C. Moreover, another group showed HLA-A, -B, and -C knockout in a HEK293T cell line (Hong et al., 2017) however, this strategy shares the same concerns as B2M knockout.

As advanced options for HLA-matching in allogeneic settings, we explored two strategies. First, we generated HLA class I pseudo-homozygous iPSCs from HLA heterozygous donors by allele specific genome editing to reduce the number of donors needed. Second, given the pivotal role of HLA-C for suppressing NK cells, we deleted HLA-A and HLA-B bi-allelically and retained a single haplotype of HLA-C to generate HLA-C-retained iPSCs (also preserving non-canonical HLA-E, -F and -G), which greatly expand donor compatibility. In both cases, the cells were capable of suppressing T and NK cell activity while retaining HLA expression and antigen presentation to a certain extent. Moreover, this strategy can be combined with HLA class II depletion by targeted knockout

of CIITA, if necessary.

RESULTS

Design of gRNAs for HLA disruption

HLA genes are highly polymorphic and the allelic HLA information of the target cells must be considered when designing genome-editing strategies. Furthermore, each HLA gene has sequence similarity with one another, which raises the risk of targeting non-specific HLA allele. To target a specific HLA allele, we generated a customized gRNA database for the *Streptococcus pyogenes*-derived CRISPR-Cas9 system to cover all publicly available HLA haplotype sequences from IPD-IMGT/HLA (Robinson et al., 2015). Among the 7,955 gRNAs with an “NGG” PAM sequence (Table S1), we identified 7,118 gRNAs that can target individual HLA genes (Figure 1A), with many of them located near exons 2 and 3 (Figure 1B). Only 2,388 gRNAs are able to target single alleles, and the other gRNAs target multiple alleles (Figure 1C).

Enrichment of HLA-edited cells by IFN- γ treatment and sorting

To identify successful knockout clones, we initially tried an HLA allele-specific antibody to sort the HLA-negative population. Although it is reported that HLA-ABC is expressed in primed-state iPSCs (Collier et al., 2017), flow cytometry analysis revealed that single HLA-A2 expression levels were low (Figure S1A). To boost HLA cell surface expression, we tested cytokine treatment (LPS, TNF- α or IFN- γ), and found that IFN- γ could efficiently increase HLA-expression levels (Figure 1D) enough to clearly distinguish HLA-A2 positive and negative iPSC populations after gRNA transfection (Figure 1E). HLA expression plateaued at 10 ng/mL IFN- γ (Figure S1B). The surface presentation of HLA began as early as eight hours post-stimulation (Figure S1C) and persisted for more than one week (Figure S1D). To ensure IFN- γ treatment did not affect the pluripotency core network, *NANOG* expression was checked by RT-qPCR immediately or five days after cytokine stimulation, but it was unchanged compared with unstimulated cells (Figure S1E).

Following gRNA transfection, IFN- γ treatment and HLA-A2 negative sorting

of iPSCs, we analyzed genomic DNA by Sanger sequencing and found 100% (16/16 clones) of sorted cells harbored indels, whereas only 47.6% (10/21 clones) of the non-sorted bulk population contained indels. Importantly, we also found no off-target mutations in the non-targeted HLA-A32 allele (19 clones), which have 3 bp mismatches in the gRNA sequence (Figure 1F).

Generation of HLA pseudo-homozygous (haploid) iPSCs

We next tried to generate class I HLA haploid iPSCs by allele-specific HLA disruption of heterozygous cells to generate “pseudo-homozygous” iPSCs, which should possess similar donor potential as HLA homozygous iPSCs. Because HLA matching in clinical transplantation is often done for HLA-A and HLA-B, but not for HLA-C, we first tried to knockout a single allele of HLA-A and of HLA-B simultaneously by genome editing (Figure 2A and Table S2). We co-transfected Cas9 protein, and A0101-ex3g2 and B0702-ex2g1 gRNAs into iPSCs. We then sorted the HLA-A1 and -B7 double-negative population (Figure 2B) and found up to 75% indel induction (9/12 clones) (Table S3). We selected two out-of-frame iPSC clones, #8 and #10, for further immunological assays (Figure 2C). As controls, we also generated B2M-KO cells from 604B1, 1383D1 and 585A1 iPSCs (Figures S1F-I).

CD8⁺ T cell activity on HLA-AB pseudo-homozygous cells

To assess the immunogenicity of iPSC-derived cells, we differentiated iPSCs from the 604B1-wild type (WT) clone, HLA-A1⁻B7⁻ clones (#8 and #10), 604B1-B2M-KO #1 and 1383D2 iPSCs to CD43⁺ blood cells. The differentiated blood cells were co-cultured with CD8⁺ T cells isolated from PBMCs of donor A (27 year-old Hispanic Female, LP_194) (Table S2) to perform CFSE assays (Figure 2D). CD8⁺ T cells were stimulated with cytokines to mimic the presence of CD4⁺ helper T cells. Only 604B1-WT and 1383D2 cells with HLA mismatch stimulated CD8⁺ T cell proliferation, whereas our edited cells and B2M-KO clones did not (Figure 2E).

To isolate HLA-reactive T cells, we sorted the CD8⁺ T cells that reacted with 604B1-WT CD43⁺ blood cells (HLA-reactive T cells). We next differentiated 604B1-WT and edited iPSC clones to CD43⁺ blood cells or cardiomyocytes

(Figure S1J) and performed ^{51}Cr release assays using the HLA-reactive T cells (Figure 2D). Despite that the effector HLA-reactive T cells and target blood cells differentiated from iPSCs have mismatches not only in class I HLAs but also in class II HLAs (Table S2), differentiated cells from pseudo-homozygous iPSCs could escape from T-cell cytolytic activity due to the lack of mismatched class I HLA-A and HLA-B, similar to B2M-KO cells (Figure 2F).

Generation of HLA-ABC pseudo-homozygous (haploid) iPSCs

In addition to HLA-A and HLA-B matching, several groups have reported HLA-C is also important for immune rejection (Woolfrey et al., 2011). Thus, we next generated HLA-A/B/C pseudo-homozygous iPSCs by targeting HLA-A, -B and -C from 585A1 (Figure S2A). We co-transfected B0702-ex2g1 and C0702-ex3g3 gRNAs simultaneously, performed HLA-B7-negative sorting after INF- γ treatment (Figure S2B) and picked 28 colonies. We chose three clones, #2, #11 and #26, for further evaluation (Table S3).

We also transfected HLA-A0101-ex3g2 and -C0602-ex3g1 gRNAs into 604B1 iPSCs (Figure S2E), sorted the HLA-A1 negative population (Figure S2F), and chose A1 and Cw6 double-knockout clone #3 (Table S3). Then, we transfected B-ex2g1 gRNA and sorted the HLA-B7 positive population (Figure S2G). Two of the 12 clones, #3-11 and #3-12, had indels only in the B37 allele. (Table S3)

For both HLA-ABC pseudo-homozygous 585A1 and 604B1 iPSCs, we prepared two sets of PBMCs to perform CFSE and ^{51}Cr release assays (Figure S2C, S2D, S2H, S2I). In both cases, HLA-ABC pseudo-homozygous cells could evade T cell cytolytic activity.

HLA-C-retention strategy and generation of HLA-C7-retained cells

Although HLA pseudo-homozygous iPSCs could help to fill the shortage of HLA homozygous iPSCs, it would remain a challenge to establish enough donor iPSC lines to cover an entire population. To expand the recipient coverage, we considered iPSCs that retain homozygous or haploid HLA-C gene while deleting both alleles of HLA-A and -B. Subsequently, we wanted to test whether the lack of HLA-A and -B is effective for evading T cell immunity and the retention of

HLA-C (and untouched on HLA-E, -F, and -G) is sufficient for suppressing NK cell activity.

First, we tried to generate HLA-C7-retained cells, which is one of the most common HLA-C alleles in the world. To prepare HLA-C7-retained iPSCs from 585A1 (Figure 3A), we transfected Cas9 protein and two gRNAs, HLA-A-ex3g1 and HLA-B-ex2-g1, which respectively target the HLA-A and HLA-B alleles and sorted the HLA-A24 and -B7 negative population (Figure S3A, B). Out of 6 clones picked, we chose #3 as the HLA-A/-B negative colony and transfected it with HLA-C12-ex3g1, leading us to pick 4 clones. Finally, we chose clones #3-1 and #3-3 as HLA-A/-B/-C12 knockout (HLA-C7-retained) clones (Table S3). We also generated HLA-C12-retained clones and HLA-C6-retained clones from 585A1 and 604B1 iPSCs (Figure S3A-E and Table S3). Pluripotency of the HLA-C7 lines was confirmed by teratoma formation in NRG mice (Figure S4A). Potential off-target sites for the two gRNAs identified by CRISPOR software (Haeussler et al., 2016) were checked by Sanger sequencing in 585A1-HLA-AB-KO #3 and 604B1-HLA-AB-KO #4 clones, and no mutations were found (Figure S4B).

CD8⁺ T cell response to HLA-C7-retained cells

We prepared HLA-reactive T cells from two donors that have a monoallelic HLA-C7 but mismatched HLA-A, HLA-B, and HLA class II alleles to assess reactivity with HLA-C7-retained iPSCs (Figure 3A). We performed ⁵¹Cr release assays using HLA-reactive T cells (Figure S3F) and found that CD43⁺ blood cells differentiated from HLA-C7-retained iPSCs could evade CD8⁺ T cell-mediated cytolytic activity comparably with B2M knockout cells (Figure 3B, S3G).

To assess the graft survival of the HLA-edited iPSCs *in vivo*, we first labeled iPSCs by Luciferase and then differentiated them into CD43⁺ blood cells. The differentiated blood cells were transplanted into NRG mice (lacking endogenous T, B, and NK cells) intraperitoneally and challenged with HLA-reactive human CD8⁺ T cells (Figure 3C). We assessed the survival of the transplanted cells over time by IVIS. HLA-C7-retained cells survived more than one week after injection *in vivo*, in contrast to the non-edited cells (Figure 3D, E).

NK cell response to HLA-C7-retained cells

Next, we analyzed whether the positive expression of HLA-C (and -E, -F, -G) with the negative expression of -A and -B is sufficient to suppress NK cell activity. First, we assessed CD107a (LAMP-1) expression as a functional marker of active NK cells by co-incubating NK cells with iPSC-derived CD43⁺ blood cells. As expected, a subset of NK cells reacted to B2M-KO cells, but not to WT cells or HLA-C7-retained cells (Figure 4A, S4C). We then sorted HLA-null-reactive NK cells that were sensitized to the B2M-KO population and performed ⁵¹Cr release cytotoxicity assays (Figure 4B). NK cell-mediated cytotoxicity was significantly less for HLA-C7-retained clones than for either B2M-KO cells or HLA-E retaining HLA-ABC-KO cells, suggesting HLA-C is important for suppressing NK cell activity. These results were consistent with NK cells isolated from two other donors (Figure 4C, S4D).

As above, we assessed the graft survival of the HLA-edited iPSCs *in vivo* in the presence of NK cells that are reactive to HLA-null cells. We transplanted CD43⁺ blood cells differentiated from 585A1-B2M-KO and 585A1-C7-retained #3-1 iPSCs into immunodeficient NRG mice intraperitoneally and then injected HLA-null-reactive human NK cells (Figure 4D). Consistent with the *in vitro* results, the survival ratio of the B2M-KO cells quickly decreases after NK cell injection compared with mock (medium) injection (Figure 4E, S4E). On the other hand, HLA-C7-retained cells showed significantly better survival *in vivo* even when NK cells were injected (Figure 4E, S4E).

HLA-C7-retained cells challenged with the mixture of CD8⁺ T and NK cells

In peripheral blood, CD8⁺ T cells and NK cells co-exist at roughly 3:1 cell number ratio. We challenged our HLA-C-retained cells by the mixture of HLA-reactive T cells and HLA-null-reactive NK cells. As expected, WT and B2M-KO cells were lysed by T cells and NK cells, respectively. On the other hand, our HLA-C retained cells were not lysed by either, which suggest that our HLA-C-retained cells can evade cytotoxicity of both T and NK cells simultaneously (Figure 4F).

Depletion of class II HLAs in HLA-C-retained iPSCs

HLA class I is recognized by CD8⁺ killer T cells for cytotoxic activities, whereas HLA class II is recognized by CD4⁺ helper T cells for regulating other immune cells, including CD8⁺ T cells, through cytokine release. To further expand the immune-compatibility of our HLA-C-retained iPSCs, disruption of class II HLA might be beneficial, especially when generating antigen presenting cells from iPSCs. CIITA (MHC class II trans activator) gene is known to be essential for transcription of class II HLA genes, and is specifically expressed in class II HLA positive cells and tissues. Functional loss of the CIITA gene leads to MHC class II deficiency, due to the lack of antigen presentation abilities through class II HLAs. We performed CIITA knockout by transfecting Cas9 protein and sgRNA CIITA-ex3g5 in HLA-C-retained iPSCs (C7 #3-1) and picked 28 colonies (Figure 5A, B, and Table S3). Subsequently, we isolated the clone #3-1-3 as HLA-C7-retained (CIITA⁻) iPSCs (Figure 5C).

CD4⁺ helper T cell activity on HLA-C-retained (CIITA⁻) cells

To assess the immunogenicity of CIITA⁻ C7-retained cells, we differentiated 585A1-WT, 585A1-C7 #3-1 and 585A1-C7 (CIITA⁻) #3-1-3 iPSC clones to CD43⁺ blood cells. After IFN- γ treatment, we confirmed that a subset of CD43⁺ blood cells induced HLA class II expression, detected by flow cytometry. Since HLA class II binding is specific to CD4 but not CD8, CD4⁺ helper T cells were isolated from PBMCs (Donor G: 33 year-old, Asian, male) that only match HLA-C7 but not for other HLAs including class II HLA-DRB1 (Table S2) to perform CSFE assay. As expected, HLA class II mismatched 585A1-WT and C7-retained cells stimulated proliferation of CD4⁺ helper T cells, whereas CIITA deficient 585A1-C7-retained cells did not (Figure S5A). This result shows that depletion of HLA class II molecules using a CIITA knockout approach, in addition to HLA-A and -B depletion, effectively suppresses the response of CD4⁺ T cells.

Bulk CD4⁺, CD8⁺ and NK cell activities on class II HLA-null (CIITA⁻) HLA-C-retained cells

Additionally, we mixed our edited iPSC-derived blood cells with bulk PBMCs

(Donor E) for 7 days to mimic a more physiologically relevant situation and checked activities of CD3⁺ CD8⁺ T cell, CD3⁺ CD4⁺ T cell and CD3⁺ CD56⁺ NK cell compartments simultaneously by CFSE labeled proliferation assay (Fig 5D). Similar to above experiments, CD8⁺ killer T cells were only activated in the class I HLA mismatched situation with unedited 585A1 cells (5 loci mismatch, see Fig.3A), but not in the HLA-C retained cells (HLA-C7, HLA-C7 CIITA⁻). On the other hand, CD4⁺ helper T cells were activated in the class II mismatched situations (585A1 WT, HLA-C7), but not in the class II HLA-null situations (HLA-C7 CIITA⁻), similarly to what we observed with CD4⁺ T cells alone from Donor G (Fig. S5A). Notably, CD8⁺ T cells were not activated even if CD4⁺ cells were activated with class II HLA mismatched HLA-C7 cells, suggesting the importance of editing class I HLAs to prevent activation of cytotoxic CD8⁺ T cells. These results suggest class II HLA editing is beneficial for suppressing CD4⁺ T cells.

Toward generation of clinical grade HLA-C-retained iPSC stocks

For future cell therapy applications, the genome editing process must adhere to regulatory guidelines for generating clinical grade HLA-C-retained iPSCs. For instance, the subcloning process should be minimized, to avoid clonal bias and prolonged culture. In addition, HLA negative sorting is an efficient way to establish desired clones in basic research, however, clinical grade sorting is challenging due to the requirements of a validated instrument and clinical grade antibodies. To this end, we sought to use HLA-homozygous iPSCs as a platform for an HLA-C retention strategy, as bi-allelic knockout of HLA-A or HLA-B is feasible by single sgRNAs for each and allele-specific disruption of HLA-C is not required (Figure 5E). In addition, as a proof-of-concept, we chose the iPSC line from a healthy donor with blood type O, to minimize immunity against ABO antigens.

A clinical grade iPSC line (Ff-XT28s05) was generated from a healthy volunteer who has a homozygous HLA haplotype, which is the third most frequent Japanese HLA-haplotype (HLA-A*24:02, B*07:02, C*07:02 and DRB1*01:01). First, we edited both HLA-A and -B by transfecting Cas9 protein and two gRNAs (HLA-A-ex3g1 and HLA-B-ex2-g1) by electroporation. After

confirming the depletion of both HLA-A and -B by flow cytometry analysis (Figure 5F), we performed limiting dilution of the edited iPSCs for subcloning without HLA negative sorting. Out of 15 isolated clones, 9 clones had detectable indels on either the HLA-A or -B locus, and we successfully isolated a homozygous knockout clone (Ff-XT28s05-ABo #14) on HLA-A and -B genes (Table S3). Further, to knockout class II HLAs, we transfected the clone #14 with Cas9 protein and CIITA-ex3g5 sgRNA to obtain subclones without sorting. Out of 12 clones, 7 clones were homozygous for CIITA gene knockout, in addition to HLA-A and -B (Ff-XT28s05-ABo_To #14-3, #14-4) (Figure 5G and Table S3). Moreover, we generated B2M-KO clones (Ff-XT28s05-Mo #1-X") and B2M CIITA DKO clones (Ff-XT28s05-MTo #1-X) from Ff-XT28s05 iPSCs without sorting (Table S3).

Genomic integrity tests

To test the genomic integrity of HLA-edited iPSC lines, we first performed karyotyping analysis. Out of 15 iPSC clones analyzed, nearly all clones (14/15, 93.3%) showed a normal karyotype, except for one clone which showed an abnormal elongation on chr 15 (Figure 6A and S5B-C). We further characterized the abnormal clone by mFISH and mBAND analyses, and identified that the chr 2 segment distal to 2p16~22 was translocated to the terminal end of the long arm of chr 15. Karyotypically abnormal clones should be excluded for clinical applications.

To further evaluate the potential impact of genome editing on protein coding regions in the genome, we performed whole exome sequencing (WES) analysis of the same 15 iPSC clones mentioned above. The exome sequencing analysis identified several SNVs (single nucleotide variations) and indels, including on-target indels on the target HLA genes (Figure S6A). To assess whether the identified SNVs/indels were associated with the sgRNA sequences used, we searched for potential sgRNA off-target sites using Cas-OFFinder (Bae et al., 2014). While, recent off-target papers generally search for potential off-target sites by allowing up to 6 bp mismatches, we used up to 9 bp mismatches for covering as many potential sites as possible. As shown in Fig. S6B, we found 1.7-4.1 million potential binding sites in the human genome for each HLA

targeting sgRNA, which indicates one site per every 765-1,845 bp. Next, we calculated the relative distance of the identified SNVs/indels from the potential sgRNA binding sites. As expected, all of the on-target SNVs/indels on the HLA genes overlapped with the listed sgRNA binding sites and clustered as a sharp peak located 3 bp distal from the PAM sequence, where Cas9 induced a DNA break (Figure 6B, Green line). On the other hand, when we excluded on-target SNVs/indels (Figure 6B, Orange line), the sharp peak disappeared and remaining SNV/indels distributed relatively independent from potential sgRNA binding sites, suggesting that most of the SNVs/indels identified by WES were not associated with Cas9/sgRNA mediated cleavage, rather probably due to spontaneous mutagenesis resulting from culturing or subcloning (Figure S6C).

Estimation of the T cell compatibility of HLA-C-retention strategy

Finally, we estimated the required number of unique iPSC lines for covering most of the population. For HLA-A/B/C matching of the HLA pseudo-homozygous iPSC strategy, 73 cell lines are needed to cover >95% of the Japanese population. For other races (Mairers et al., 2007), we estimated 82 lines for European American, 282 lines for African American, 213 lines for Asian and 308 lines for Hispanics (Figure 6C and Table S4). On the other hand, for the HLA-C retention strategy, owing to the lower variation of HLA-C haplotype, only 7 lines for Japanese, 8 lines for European, 9 lines for African, 10 lines for Asian, and 11 lines for Hispanic are required to cover more than 95% in each population, albeit the rank order of frequency was significantly variable among the 5 ethnic groups (Figure 6C and Table S4). Furthermore, we estimated that only 12 common HLA-C-retained lines (HLA-C*01:02, *02:02, *03:03, *03:04, *04:01, *05:01, *06:02, *07:01, *07:02, *08:01, *12:02, and *16:01) are sufficient to cover more than 90% of all the five ethnicities (Table S5).

Discussion

Here, we propose two alternative donor cell sources, HLA pseudo-homozygous (or HLA haploid) iPSCs and HLA-C-retained iPSCs. We showed that IFN- γ -induced HLA expression and sorting of genome-edited iPSCs

greatly facilitated the precise editing of individual HLA class I genes. These cells could evade both T cell activity caused by HLA-mismatching and NK cell activity caused by a lack of HLA-class I expression. HLA pseudo-homozygous iPSCs can be generated from an ordinary HLA heterozygous healthy donor, but various HLA haplotypes (maximum 8 haplotypes for HLA-A, -B, and -C loci) can be generated by allele-specific genome editing. Based on these findings, we propose HLA pseudo-homozygous iPSCs could supplement the on-going global effort to prepare HLA-homozygous iPSC stocks for cell therapy applications (Turner et al., 2013).

The HLA pseudo-homozygous iPSC approach would retain surface expression of all HLA genes, offering an advantage over B2M knockout cells engineered to express a single-chain HLA-E/B2M fusion (Gornalusse et al., 2017), as antigen presentation in the event of pathogen infection and oncogenesis and NK cell suppression via KIR and CD94/NKG2A receptors would be retained. In the HLA-C-retention approach, where HLA-C is capable of antigen presentation (Schlott et al., 2018), antigen presentation might be slightly compromised compared with unedited cells as HLA-A and -B are absent, however, it is still more advantageous than the B2M-KO approach since a single antigen-MHC complex per target cell is sufficient to trigger T cell activity (Sykulev et al., 1996).

NK cell activity is known to be regulated by various inhibitory receptors and their ligands. Examples include suppression of the CD94/NKG2A receptor by HLA-E, KIR 2DL2/3 receptors by HLA-C(C1), KIR 2DL1 receptor by HLA-C(C2) and KIR3DL1 by HLA-Bw4 (i.e. HLA-B52, -B37, etc.). Therefore, HLA-C retention could be more effective at suppressing NK activity against transplanted donor cells compared with HLA-E overexpression in B2M-KO cells. Indeed, our results showed that HLA-C-retained cells (HLA-C⁺, E⁺) evaded NK cell activity more efficiently than B2M KO (HLA-C⁻, E⁻) or HLA-A/-B/-C KO cells (HLA-C⁻, E⁺). Nonetheless, to completely suppress NK cell activity in some individuals with HLA-Bw4 and/or heterozygous C1/C2 haplotypes, the retention of HLA-Bw4, HLA-C(C1) and -C(C2) should also be considered (Ichise et al., 2017). Furthermore, it is challenging to edit all other minor antigens, such as an ABO blood type or minor histocompatibility antigen. Given that some iPSC-derived

cell types might trigger immune rejection even in an autologous setting (Zhao et al., 2015), use of mild immunosuppressant should be considered for allogenic transplantation of the HLA-edited iPSC derivatives (Swijnenburg et al., 2008).

Additionally, we depleted HLA class II by knocking out the CIITA gene because HLA-class II mismatching can stimulate CD4⁺ helper T cells (Sugita et al., 2016). CD4⁺ helper T cells do not have direct cytolytic activity, however, CD4⁺ T cells activate a wide range of immune cells by cytokine release. For transplantation of iPSC-derived HLA class II-expressing cells, such as T cells and macrophages, disruption of HLA class II in HLA-C-retained iPSCs should be considered for better donor-host matching as an option.

Lastly, we performed rigorous genomic integrity tests to assess the safety of genome-edited iPSCs. Although we performed a complex genome editing strategy, including allele-specific targeting, multiplexed gene disruption, and sequential subcloning, the overall genomic integrity of edited cells were maintained. Moreover, we observed modest CRISPR-Cas9/sgRNA mediated off-target cleavage. Since HLA-edited cells acquire the ability to escape immune surveillance, risk of tumorigenicity must be rigorously assessed for future clinical applications by careful assessment of the genomic integrity, mutation in cancer related genes, and off-target mutagenesis. An iPSC clone with a cancer causing mutation (loss-of-function mutation in tumor suppressor gene or gain-of-function mutation in tumorigenic gene) should be excluded for future clinical applications, regardless of whether it is caused by genome editing off-target mutagenesis or not (Figure S6C).

Altogether, our HLA-C-retained stock strategy could greatly increase donor compatibility. We anticipate a core stock of 12 HLA-C-retained iPSC lines (Table S5) as a next generation donor cell source for regenerative medicine to cover most of world population.

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Author contributions

H.X. and A.H. designed the experiments; H.X., M.O., A.K., N.S. and T.T. performed the genome editing, sub-cloning, genotyping and off-target analysis; H.X. and B.W. performed the CD43⁺ blood cell differentiation; M.N. and Y.Y. performed the cardiomyocyte differentiation; H.X., B.W. and S.K. performed the immunological assay; H.X., B.W., A.K., K.F. and T.U. performed the *in vivo* experiments; H.X. and K.O. calculated the coverage; M.N., F. K. and A.H. performed deep sequencing analysis; H.X., B.W. and A.H. analyzed the results; H.X., P.G. and A.H. wrote the manuscript.

Declaration of interests

H.X., B.W., S.K. and A.H. are inventors on a patent for HLA editing filed by Kyoto University, Kyoto, Japan. The remaining authors declare no competing interests.

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FIGURE LEGENDS

Figure 1. IFN- γ treatment augments HLA surface expression in iPSCs for the detection of HLA knockout cells by HLA allele-specific gRNAs

(A) Venn diagram depicting the number of gRNAs that can target individual or combinations of HLA-ABC genes.

(B) Location of the designed gRNAs in each HLA-ABC gene locus. The peak distribution of the unique k-mer sequences (Li et al., 2015) is shown on the top in green to indicate the genome-wide uniqueness of the region. Orientations of the gRNAs are indicated below in red and blue.

(C) Number of perfectly matched HLA haplotypes for each gRNA and number of corresponding gRNA are plotted.

(D) Undifferentiated iPSCs were treated with three cytokines (100 ng/mL LPS, 100 ng/mL TNF- α or 50 ng/mL IFN- γ) for 2 days and assessed for HLA-ABC expression by flow cytometry.

(E) 1383D2 iPSCs were transfected with non-HLA-targeting DMD-gRNA#1, or HLA-targeting HLA-A0207-ex3g1 or HLA-A0207-ex3g4 gRNAs. With or without INF- γ treatment, HLA-A2 presentation was assessed by flow cytometry.

(F) 1383D2 iPSCs cells transfected with HLA-A0207-ex3g4 gRNA were sorted for HLA-A2 negative and genotyped for the gRNA target site by Sanger sequencing utilizing A*02:07 or A*32:01 allele-specific primers (n: number of clones analyzed). A*32:01 allele has 3 bp mismatches from HLA-A0207-ex3g4 gRNA.

See also Figure S1 and Tables S1 and S2.

Figure 2. Gene-edited HLA-AB pseudo-homozygous cells escape T cell activity

(A) Schematic depicting HLA types of WT and edited 604B1 iPSCs as well as those of a donor PBMCs (donor A: LP_194).

(B) Flow cytometry plots of 604B1 iPSCs transfected with A0101-ex3-g2 and B0702-ex2-g1 gRNAs simultaneously and stained with HLA-A1 and -B7 antibodies after INF- γ treatment.

(C) Indel pattern of two HLA-A1⁻B7⁻ 604B1 iPSC clones after HLA-A1 and B7 negative sorting.

(D) Schematic for immunogenicity of CD8⁺ T cells. Unedited or edited 604B1 iPSCs were differentiated into CD43⁺ blood cells, stimulated with IFN-γ and co-cultured with CD8⁺ T cells from a PBMC donor. HLA-reactive T cells were sorted and incubated with 604B1 iPSC-derived cardiomyocytes or CD43⁺ blood cells to assess *in vitro* T-cell cytotoxicity by ⁵¹Cr release assay.

(E) CFSE assay to detect proliferative CD8⁺ T cells reacting to HLAs on CD43⁺ blood cells differentiated from 604B1-WT, -#8, -#10, -B2M⁻ #1 or 1383D2 (allogenic control) iPSCs.

(F) CD43⁺ blood cells or cardiomyocytes differentiated from 604B1-WT, -#8, -#10, -B2M-KO or 1383D2-B2M-KO iPSCs were labeled with ⁵¹Cr and co-incubated with HLA-reactive T cells. Cytolysed and released ⁵¹Cr in the supernatant was quantified by a liquid scintillation counter. Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, One-way ANOVA followed by Tukey's test.

See also Figures S1 and S2, and Tables S2 and S3.

Figure 3. HLA-C-retained cells avoid T cell activity *in vitro* and *in vivo*

(A) Schematic of editing 585A1 iPSCs to first knockout HLA-A and -B biallelically and then HLA-C mono-allelically. PBMCs from two donors (donor D: LP_275 and donor E: LP_329) were used for the immunological assays.

(B) ⁵¹Cr release assay for CD43⁺ blood cells differentiated from 585A1-WT, C7-retained #3-1, C7-retained #3-3 and 585A1-B2M-KO iPSCs by using HLA reactive CD8⁺ T cells derived from two donors (donor D: LP_275 and donor E: LP_329, see also Figure S4F). Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, One-way ANOVA followed by Tukey's test.

(C) Schematic time course of *in vivo* experiments.

(D) Luciferase imaging of NRG mice transplanted with CD43⁺ blood cells differentiated from 585A1-WT or C7-retained #3-1 iPSCs. After one hour of the CD43⁺ blood cell injection, mice received an additional vehicle (mock) or

HLA-reactive human CD8⁺ T cell injection. Day 0 represents the time of the T cell injection, and 5 hour, 1, 3, 7 Day indicates 5hour, 1, 3, 7 day after the T cell injection.

(E) The change in luminescence at 5 hours, 1 day, 3 days and 7 days of WT or C7-retained #3-1 cells that were received with human CD8⁺ T cells. Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, Student's t test.

See also Figures S3 and S4, and Tables S2 and S3.

Figure 4. HLA-C-retained cells evade NK cell activity *in vitro* and *in vivo*

(A) CD43⁺ blood cells differentiated from 585A1-WT, C7-retained #3-1 and 585A1-B2M-KO cells were co-cultured with NK cells isolated from two donors (donor D: LP_275 and donor E: LP_329). NK cell activation was measured by CD107a expression (flow cytometry). K562 was used as a positive control for NK activation. Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, One-way ANOVA followed by Tukey's test.

(B) ⁵¹Cr release assay for CD43⁺ blood cells differentiated from 585A1-WT, C7-retained #3-1, C7-retained #3-3, HLA-AB-KO #3 (both HLA-C and HLA-E alleles intact), HLA-ABC-KO #5 (HLA-E intact) and 585A1-B2M-KO iPSCs by using HLA-null-reactive NK cells derived from two donors (donor D: LP_275 and donor E: LP_329). B2M-KO cells or ABC-KO cells showed significant cell lysis compared to C7-retained #3-1 and #3-3 lines. Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, One-way ANOVA followed by Tukey's test.

(C) CD107a expression was measured by flow cytometry after incubating CD43⁺ blood cells differentiated from 585A1-WT, C7-retained #3-1 and 585A1-B2M-KO with NK cells sorted from two donors (donor F: LP_118 and donor C: LP_266). K562 was used as positive control. Data are represented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, One-way ANOVA followed by Tukey's test.

(D) Schematic time course of *in vivo* experiments.

(E) The change in luminescence at 5 hours, 1 day, 3 days, 5 days and 7 days of B2M-KO or C7-retained #3-1 cells that were received with human NK cells.

Relative survival ratio which received NK cells/ Mock was calculated. Data are

represented as mean $\pm$ SEM (n = 3-4).
(F) ^{51}Cr release assay for CD43⁺ blood cells differentiated from 585A1-WT, C7-retained #3-1 and 585A1-B2M-KO iPSCs by using both HLA-reactive T cells and HLA-null-reactive NK cells derived from donor (donor D: LP_275). WT cells and B2M-KO cells showed significant cell lysis compared to C7-retained #3-1 cells. Data are represented as mean $\pm$ SEM (n = 3). *P < 0.05, **P < 0.01, One-way ANOVA followed by Tukey's test.

See also Figure S4 and Table S2.

Figure 5. Depletion of HLA class II to suppress CD4⁺ helper T cells

(A) Schematic of editing 585A1-C7-retained #3-1 iPSCs to knockout CIITA.
(B) Number of genotyped iPSC clones with homozygous or heterozygous deletion of CIITA gene.
(C) CRISPR-sgRNA target site and indel patterns of the isolated CIITA^{-/-} iPSC clone #3-1-3.
(D) CFSE assay to detect proliferative CD4⁺, CD8⁺ T cells and NK cells reacting to HLAs on CD43⁺ blood cells differentiated from 585A1-WT, -C7 #3-1, -C7 (CIITA⁻) #3-1-3 and -C7 (CIITA⁻) #3-1-6 iPSC lines. "Mock" means no target cells and bulk PBMCs alone.
(E) Schematic of editing Ff-XT28s05 iPSCs to knockout HLA-A, -B and CIITA.
(F) Flow cytometry plots of Ff-XT28s05 iPSCs transfected with A-ex3-g1, B-ex2-g1 and CIITA-ex3g5 gRNAs simultaneously and stained with HLA-A24 and -B7 antibodies after INF- γ treatment.
(G) CRISPR-sgRNA target site and indel patterns of the isolated HLA-A^{-/-}, HLA-B^{-/-}, and CIITA^{-/-} iPSC clone #14-3 (Ff-XT28s05-ABo_To #14-3).

See also Figure S5 and Tables S2 and S3.

Figure 6. Genomic integrity of HLA-edited iPSCs and estimation of T cell compatibility

1 (A) Karyotyping analysis of HLA-edited iPSC clones identified only 1 clone out of
2 15 clones (6.6%) with abnormal translocation, and further assessed by mFISH
3 and mBAND analysis.

4 (B) Whole exome sequencing of 15 iPSC clones identified 421 SNVs and indels,
5 including on-target indels on the HLA genes. Relative position of the detected
6 SNV/Indels were plotted on putative CRISPR-Cas9/sgRNA binding sites (up to 9
7 bp mismatches). Green line indicates all the detected SNV/indels including the
8 on-target indels, which is clustered at the Cas9 cleavage site. Orange line
9 indicates the distribution of SNV/indels after exclusion of the on-target indels.
10 Purple line is a distribution of randomly generated virtual SNV/indels.

11 (C) The estimated number of required HLA-ABC pseudo-homozygous and
12 HLA-C-retained stocks to cover Japanese, European, African, Asian and
13 Hispanic populations are shown. To cover >95% of each population, 73-308
14 lines are needed for HLA-ABC (pseudo) homozygous cells, whereas only 7-11
15 lines for HLA-C-retained cells (See also Table S4 and S5).

16
17 See also Figure S6 and Tables S4 and S5.
18

1 MATERIALS AND METHODS

2 STAR★METHODS

3 Online

4 KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HLA class 1 A1, A11, A26 antibody [8.L.101]	Abcam	Cat# ab33883, RRID:AB_742131
BV421 mouse anti-Human HLA-A2 clone BB7.2 (RUO)	BD Biosciences	Cat# 740082, RRID:AB_2739845
Anti-HLA-A24 (human) mAb-Alexa Fluor 647	MBL International	Cat# K0208-A64, RRID:AB_1953030
Anti-HLA B7, Mouse-monoclonal (BB7.1)	Bio-Rad / AbD Serotec	Cat# MCA986, RRID:AB_322227
Pacific Blue™ anti-human HLA-A,B,C antibody	BioLegend	Cat# 311418, RRID:AB_493669
Alexa Fluor® 700 mouse anti-human CD3 clone UCHT1 (RUO)	BD Biosciences	Cat# 557943, RRID:AB_396952
Brilliant Violet 421 anti-human CD4 antibody (Clone: OKT4)	BioLegend	Cat# 317434, RRID:AB_2562134
CD8b Monoclonal Antibody (SID18BEE), PE-Cyanine7, eBioscience	Thermo Fisher Scientific	Cat# 25-5273-42, RRID:AB_11219680
APC/Cy7 anti-human CD56	BioLegend	Cat# 318332, RRID:AB_10896424
FITC anti-human CD107a (LAMP-1) antibody	BioLegend	Cat# 328606, RRID:AB_1186036
Pacific Blue™ anti-human CD34 antibody	BioLegend	Cat# 343512, RRID:AB_1877197
APC Mouse anti-human CD43 clone 1G10 (RUO)	BD Biosciences	Cat# 560198, RRID:AB_1645460

Alexa Flour 488 goat anti-mouse IgG(H+L)	Thermo Fisher Scientific	Cat# A-11001, RRID:AB_2534069
Alexa Flour 647goat anti-mouse IgG(H+L)	Thermo Fisher Scientific	Cat# A-21236, RRID:AB_2535805
Alexa Flour 488 goat anti-mouse IgM	Thermo Fisher Scientific	Cat# A-21042, RRID:AB_2535711
APC anti-human CD31 Antibody	BioLegend	Cat# 303116, RRID:AB_1877151
Alexa Fluor® 647 anti-human CD49a Antibody	BioLegend	Cat# 328310, RRID:AB_2129242
APC anti-mouse CD140b Antibody	BioLegend	Cat# 323608, RRID:AB_2162787
PE/Cy7 anti-human CD172a/b (SIRPα/β) Antibody	BioLegend	Cat# 323808, RRID:AB_1236409
APC Mouse Anti-Human CD90 clone 5E10 (RUO)	BD Biosciences	Cat# 559869, RRID:AB_398677
Bacterial and Virus Strains		
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Stem-Fit AK03N	Ajinomoto	AK03N
MTG (1-Thyoglycerol)	Sigma	M6145-25ml
L-Glutamine 200 mM	Invitrogen	25030-081
Transferrin 20 ml (600 mg)	Roche	652202
Ascorbic acid	Sigma	A4544-25G
Activin A recombinant human	R&D	338-AC-050
BMP-4 recombinant human	R&D	314-BP-010
Basic FGF recombinant human	R&D	233-FB-025
VEGF recombinant human	R&D	293-VE
IWP-3	Reprocell	04_0035

Matrigel (growth factor reduced)	BD	356230
Rock inhibitor Y-27632	Wako	253-00514
SB431542	Sigma	S4317
Dorsomorphin	Sigma	P5499
IMDM	Invitrogen	12440-053
PBS	Nakarai tesque	14249-24
FBS	Biosera	FB-1365
StemPro-34	Thermo Fisher Scientific	10639011
bFGF	Oriental Kobo	47107000
VEGF	R&D Systems	293-VE-050/CF
ITS	Thermo Fisher Scientific	41400-045
GlutaMax-I	Thermo Fisher Scientific	35050061
L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma	A8960-5G
MTG	Nakarai tesque	33709-62
SCF	R&D	255-SC
Flt3l	R&D	203-IL-050
TPO	Peprtech	300-18
Laminin 511 E8 (iMatrix-511 E8)	Nippi	892012
TrypLE Select	Thermo Fisher Scientific	12563011
LPS	Invivogen	tlrl-b5lps
TNF- α	R&D	210-TA-020
IFN- γ	Peprtech	300-02
Opti-MEM I	Thermo Fisher Scientific	31985-070
recombinant Cas9 protein	IDT	1074181
Propidium Iodide	Invitrogen	P3566
CFSE	Dojindo	C309
PHA-P	WAKO	161-15251
Human IL-2	Peprtech	200-02
Human IL-3	Peprtech	200-03
Human IL-6	Oriental Bio	NIB47066000

Human IL-7	Peprtech	200-07
Human IL-15	Peprtech	200-15
Critical Commercial Assays		
4D nucleofector	Lonza	V4XP-4032
MEGAscript kit	Thermo Fisher Scientific	AM1354
Deposited Data		
Experimental Models: Cell Lines		
1383D2 Human iPSCs, reprogrammed PBMC (LP_53, donor #40) isolated from 36 year-old male by episomal vectors.	(Nakagawa et al., 2014)	From Dr. Nakagawa
404C2 Human iPSCs, reprogrammed skin fibroblasts (HDF1388) isolated from 36 year-old female by episomal vectors.	(Okita et al., 2011)	From Dr. Okita RRID:CVCL_DP92
604B1 Human iPSCs, reprogrammed PBMC (healthy donor PB #1) isolated from 30s male by episomal vectors.	(Okita et al., 2013)	From Dr. Okita RRID:CVCL_DQ10
585A1 Human iPSCs, reprogrammed PBMC (healthy donor PB #3) isolated from 30s male by episomal vectors.	(Okita et al., 2013)	From Dr. Okita RRID:CVCL_DQ06
Ff-XT28s05 Human iPSCs, reprogrammed PBMC (healthy donor with Japanese 3 rd most frequent HLA-homogyzous haplotype) isolated from 20s male by episomal vectors.	This paper	From FiT (Facility for iPS Cell Therapy), CiRA, Kyoto University
Experimental Models: Organisms/Strains		

NOD.Cg-Rag1 ^{tm1Mom} Il2rg ^{tm1Wjl} /SzJ (NRG)	The Jackson Laboratory	IMSR Cat# JAX:007799, RRID:IMSR_JAX: 007799
Oligonucleotides		
Primers for gRNAs (see Table S6)	This paper	N/A
Primers for PCRs (see Table S6)	This paper	N/A
hNANOG-fwd: CTTCACTTCCCAGGTGCAA	(Ishida et al., 2018)	N/A
hNANOG-rev: AGGCTAGCCAACATGAGGAA	(Ishida et al., 2018)	N/A
hACTB-Fwd: CCAACCGCGAGAAGATGA	(Ishida et al., 2018)	N/A
hACTB-Rev: CCAGAGGCGTACAGGGATAG	(Ishida et al., 2018)	N/A
Recombinant DNA		
Software and Algorithms		
HLA-genotyper	https://pypi.python.org/pypi/hla-genotyper/0.4.2b1	Ver. 0.4.2
Bowtie program	http://bowtie-bio.sourceforge.net/index.shtml	Ver. 0.12.9
CRISPOR	http://crispor.tefor.net	Ver. 4.0-4.6
GGGenome	https://gggenome.dbcls.jp	n/a
bcl2fastq	Illumina	Ver. 2.17.1.14
BWA MEM		Ver. 0.7.15
Genomon 1 (Genomon-Fisher)	http://genomon.hgc.jp/exome_original/index.html	Ver. 1.0.1
Genomon 2 (Empirical Bayesian mutation Calling)	https://genomon.readthedocs.io/ja/latest/	Ver. 2.3.0
bedtools	https://bedtools.readthedocs.io/en/latest/	Ver. 2.27.0
Graphpad Prism software for Mac	https://www.graphpad.com	Ver 8

Other		
Japanese HLA database	HLA Laboratory	http://hla.or.jp
American HLA database	The National Marrow Donor Program®/ Be The Match® facilitates	https://bioinformatics.bethematchclinical.org
HLA allele sequence information	IPD-IMGT/HLA	https://www.ebi.ac.uk/ipd/imgt/hla/

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Akitsu Hotta (akitsu.hotta@cira.kyoto-u.ac.jp). Human iPS cell lines will be available upon ethical approval and MTA, to protect the rights of the donors to withdraw their consent in the future. Genome sequencing data is treated as personal information by the Act on the Protection of Personal Information in Japan, hence some restrictions will be applied for accessing the raw sequencing data.

Experimental Model and Subject Details

Human iPS cells

Human iPS cell lines 404C2 (Okita et al., 2011), 585A1, 604B1 (Okita et al., 2013), and 1383D2 (Nakagawa et al., 2014) were generated from healthy donors. Ff-XT28s05 (HLA homozygous iPS cell line with 3rd most frequent haplotype in Japan) iPS cell line was established in FiT (Facility for iPS cell Therapy) in CiRA, Kyoto University. All the iPS cell lines (404C2, 585A1, 604B1, 1383D2 and Ff-XT28s05) used in this study were generated under written consent with the approval by the Ethics Committee at Faculty of Medicine or CiRA, Kyoto University. Human iPS cell lines were cultured with StemFit AK03N media and iMatrix-511 as previously reported (Ishida et al., 2018). For cytokine

1 treatment, iPSCs were treated by 100 ng/mL LPS (Invitrogen, Cat. No. tlrl-b5lps),
2 100 ng/mL TNF- α (R&D Systems, Cat. No. 210-TA-020) or 10-100 ng/mL IFN- γ
3 (Peprotech, Cat. No. 300-02).

5 **NRG immunodeficient mouse**

6 NRG mice (NOD.Cg-Rag1tm1Mom Il2rgtm1Wjl/SzJ) were obtained from
7 Jackson Laboratory (Stock No: 007799). For cell transplantation experiments,
8 6-13 week-old mice with the same age and sex were used and randomly
9 assigned to experimental groups. All animal experiments were performed in
10 accordance with the guidelines of the Animal Experiment Committee and the
11 Recombinant DNA Experiment Committee at Kyoto University.

14 **Methods Details**

16 **HLA typing of donor iPSCs**

17 For 585A1 and 604B1 iPSCs, HLA alleles were determined by the
18 PCR-rSSO/Luminex method at HLA Laboratory (<http://hla.or.jp/>). For 604B1,
19 404C2 and 1383D2 iPSCs, because WES (whole exome sequencing) for 604B1
20 and WGS (whole genome sequencing) for 404C2 and 1383D2 data are
21 available in house, we performed NGS-based HLA haplotyping. Each BAM file
22 was made by mapping FASTQ sequence files to human reference genome
23 (hg19). We used HLA-genotyper
24 (<https://pypi.python.org/pypi/hla-genotyper/0.4.2b1>) to determine the HLA
25 haplotypes.

27 **HLA haplotype-specific gRNA design and database**

28 We obtained genomic DNA sequences of all HLA haplotypes (hla_gen.fasta)
29 from IPD-IMGT/HLA (<https://www.ebi.ac.uk/ipd/imgt/hla/>). As a candidate target
30 site for SpCas9 gRNA, we extracted all genomic sequences that match
31 “NNNNNNNNNNNNNNNNNNNNNNNGG”. Then, all gRNA candidate sequences
32 were mapped against all HLA haplotypes by using the Bowtie program
33 (<http://bowtie-bio.sourceforge.net/index.shtml>) to identify the targetability of HLA

genes and haplotypes. Next, we mapped all gRNA candidate sequences against human genome (hg19) to identify possible off-target sites (Table S1).

To target 1383D2, 604B1 and 585A1 iPSCs, we selected gRNA candidates from the exon 2-3 regions of the corresponding HLA alleles and confirmed the targetability of the picked gRNA sequences by using the WGS or WES data. We separately checked the potential off-target candidates of the selected gRNAs by using CRISPOR (<http://crispor.tefor.net>) or GGGenome (<https://gggenome.dbcls.jp>) tools. The designed gRNAs were prepared by *in vitro* transcription using a MEGAshortscript kit (Thermo Fisher Scientific, Cat. No. AM1354) according to the instruction manual. Table S6 shows the list of gRNA used in this study.

CRISPR-Cas9 genome editing experiments

Transfection was performed by using 4D-Nucleofector (Lonza). 0.3×10^6 iPSCs were transfected with a RNP (ribonucleoprotein) complex of 5 μ g recombinant Cas9 (IDT, Cat. No. 1074181 or ThermoFisher, Cat. No. A36498) and 1.25 μ g of IVT gRNA in 20 μ L Primary P4 buffer and the electroporation condition program CA-137. After electroporation, cells were transferred to 0.48 μ g/cm² iMatrix-511-coated 6-well plates and grown in AK03N medium containing ROCK inhibitor Y-27632.

Flow cytometry analysis

iPSCs were detached from plates by $0.5 \times$ TrypLE Select for 10 min at 37°C, dissociated and then passed through a 45- μ m cell strainer (BD, Cat. No. 352235) to obtain a single cell suspension. Staining and washing steps were carried out in PBS containing 2% FBS (Biosera, Cat. No, FB-1365). For staining, suspension of iPSCs was incubated with 50- μ L of diluted antibodies for 20-30 min on ice. After adding PI (Propidium Iodide, Invitrogen, Cat. No. P3566), samples were analyzed on a FACS Arianal (BD), and the data were processed using FlowJo software (Ver 9.9.6 or Ver 10.4.1). Anti-HLA-A1,11,26 (Abcam, Cat. No. ab33883, Clone 8.L.101), anti-HLA-A2 (BD Biosciences, Cat. No. 740082, Clone BB7.2), anti-HLA-A24 (MBL International, Cat. No. K0208-A64, Clone 17A10), anti-HLA-B7 (Bio-Rad / AbD Serotec Cat. No. MCA986, Clone BB7.1)

and anti-HLA-ABC (BioLegend Cat. No. 311418, Clone W6/32) antibodies were used for HLA staining.

Sub-cloning and genotyping

Genome-edited iPSCs were cultured for 24-76 hour with 25-50 ng/mL IFN- γ (Peprotech, Cat. No. 300-02) and stained by HLA antibodies as above. Then each single iPSC of the desired population was sorted by FACS ArianI (BD) into a well of iMatrix-511-coated 24-well plates and grown in AK03N medium containing ROCK inhibitor Y-27632. 10-14 days after single cell sorting when colony size reached greater than 1 mm² in diameter, then cells were passaged in a well of iMatrix-511-coated 6-well plate. After reaching confluency in the plate, half of the cells were frozen for cryogenic preservation and the other half were harvested for the isolation of genomic DNA. Genomic DNA was used for PCR and Sanger sequencing to analyze indel patterns, which were carried out with the PCR primers shown in Supplementary Table S6.

Cardiomyocyte differentiation.

2×10^4 iPSCs were seeded into an ultra-low attachment 96-well plate (Corning, Cat. No. 7007) and embryoid bodies were formed with 2 ng/mL BMP4 (R&D, Cat. No. 314-BP-010) and 0.50% Matrigel (BD, Cat. No. 356230) in bFGF-free StemFit AK03N medium containing 10 μ M ROCK inhibitor Y-27632 (Wako, Cat. No. 251-00514), 1% L-Glutamine (Invitrogen, Cat. No. 25030-081), 150 μ g/mL Transferrin (Roche, Cat. No. 652202), 50 μ g/mL Ascorbic Acid (Sigma, Cat. No. A4544-25G), and MTG (Sigma, Cat. No. M6145-25ml) in a hypoxic (5% oxygen balanced with N₂) 5% CO₂ incubator for 1 day. Then, cardiac mesoderm was induced by adding the same amount of media containing 18 ng/mL BMP4 (final concentration 10ng/ml), 12 ng/mL Activin A (R&D systems, Cat. No. 338-AC-050) (final concentration 6 ng/ml) and 10 ng/mL bFGF (R&D systems, Cat. No. 233-FB-025) (final concentration 5ng/ml) from day 1 to day 3. From day 3, the cells were transferred into a 6-well plate (BD, Cat. No. 353046) with media containing 10 ng/mL VEGF (R&D systems, Cat. No. 293-VE), 1 μ M IWP-3 (Reprocell, Cat. No. 04_0035), 5.4 μ M SB431542 (Sigma, Cat. No. S4317) and 0.6 μ M Dorsomorphin (Sigma, Cat. No. P5499) to induce

cardiac progenitors, until day 7. From day 7, cardiomyocyte was induced by 5 ng/mL VEGF, without IWP-3, SB43154 and Dorsomorphin. From day 12, cardiomyocytes were incubated in a normoxia 5% CO₂ incubator. Day 21-28 cardiomyocytes were checked by flow cytometry by using CD31 (BioLegend, Cat. No. 303116), CD49a (BioLegend, Cat. No. 328310), CD140b (BioLegend, Cat. No. 323608), CD172a/b (BioLegend, Cat. No. 323808) and CD90 (BD Biosciences, Cat. No. 559869) antibodies, and used for the assays.

CD43⁺ blood cell differentiation.

0.3 × 10⁶ iPSCs were seeded into an ultra-low attachment 6-well plate (Corning, Cat. No. 3471) and cultured with StemFit AK03N medium (Ajinomoto) and 10 μM ROCK inhibitor Y-27632 (Wako, Cat. No. 251-00514) in a 5% O₂, 5% CO₂ incubator for 2 days. From day 2, the medium was changed to StemPro-34 (Thermo Fisher Scientific, Cat. No. 10639011) with 50 ng/mL bFGF (Oriental Kobo, Cat. No. 47107000), 50 ng/mL VEGF (R&D Systems, Cat. No. 293-VE-050/CF), 100× ITS (Thermo Fisher Scientific, Cat. No. 41400-045), 100× GlutaMax-I (Thermo Fisher Scientific, Cat. No. 35050061), 50 μg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma, Cat. No. A8960-5G), 100 nM MTG (Nakarai tesque, Cat. No. 33709-62) and 40 ng/mL BMP4 (R&D Systems, Cat. No. 314-BP-050) for 3 days. From day 5, BMP4 was removed, and 50 ng/mL SCF (R&D systems, Cat. No. 255-SC) was added for 2 days. From day 7, 30 ng/mL Flt3l (R&D systems, Cat. No. 203-IL-050) and 10 ng/mL TPO (Peprotech, Cat. No. 300-18) were added, and the cells were cultured in normal 5% CO₂ incubator. Day 21-28 floating CD43⁺ blood cells were checked by using CD34 (BioLegend, Cat. No. 343512) and CD43 (BD Biosciences, Cat. No. 560198) antibodies, and used for the assays.

CFSE-labeled CD8⁺ T cell proliferation assay

HLA typed PBMCs were obtained from CTL or Wako (see also Table S2). Upon thawing, PBMCs were cultured in RPMI-1640 supplemented with glutamine and 10% heat-inactivated human FBS (Biosera, Cat. No. FB-1365) for 3-5 hour and then sorted by FACS for CD8⁺ T cells by using anti-CD3 (BD Biosciences, Cat. No. 557943, Clone UCHT1) and anti-CD8b (Thermo Fisher

Scientific, Cat. No. 25-5273-42, Clone SIDI8BEE) antibodies. The next day the effector CD8⁺ T cells were labeled by 1 μ M CFSE (Dojindo, Cat. No. C309) for 8 minutes and then washed 2 times with PBS. Subsequently, iPSC-derived CD43⁺ blood cells were pretreated with 50 ng/mL IFN- γ for 48 hour and irradiated (with 40 Gy) to stop proliferation. Then, the target CD43⁺ cells were mixed with effector CD8⁺ T cells at a 1:1 ratio in a 96-well U-bottom plate for 7-9 days in RPMI-1640 supplemented with glutamine, 10% heat-inactivated human FBS, 25 ng/mL IFN- γ and 20 U/mL IL-2. Finally, the percentage of proliferative CD8⁺ T cells that became negative for CFSE labeling was measured by flow cytometry.

Expansion and activation of HLA-reactive CD8⁺ T cells

CD8⁺ T cells that reacted to HLA-expressing cells were sorted and co-cultured in 2 μ g/mL PHA, 10 U/mL IL-7 and 10 U/mL IL-15 with irradiated PBMC feeders in a 96-well U-bottom plate. After day 4, PHA was removed, and 100 $\times$ ITS (Thermo Fisher Scientific, Cat. No. 41400-045) and 50 μ g/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma, Cat. No. A8960-5G) were added. Activated CD8⁺ T cells were used between 10-17 days after sufficient expansion.

CD107a assay

PBMCs were purchased from CTL. Upon thawing, PBMCs were cultured in RPMI-1640 supplemented with glutamine and 10% heat-inactivated human FBS (Biosera, Cat. No. FB-1365) for 3-5 hour and then sorted by FACS for effector NK cells by using anti-CD3 (BD Biosciences, Cat. No. 557943, Clone UCHT1) and anti-CD56 (BioLegend, Cat. No. 318332, Clone HCD56) antibodies. The next day, 10⁴ NK cells were co-cultured with day 21-28 CD43⁺ blood cells, which were pretreated with 50 ng/mL IFN- γ for 48 hr at a 1:1 ratio in a 96-well U-bottom plate for 5 hour in 100 μ L RPMI-1640 supplemented with glutamine, 10% heat-inactivated human FBS and 2 μ L of anti-CD107a antibody (BioLegend, Cat. No. 328606, Clone H4A3). Finally, the percentage of CD107a positive NK cells was measured by flow cytometry.

Expansion and activation of HLA-null-reactive NK cells

NK cells that reacted to B2M-KO cells were sorted and co-cultured in 2 $\mu\text{g/mL}$ PHA, 10 U/mL IL-7 and 10 U/mL IL-15 with irradiated self-feeder PBMCs in a 96-well U-bottom plate. After day 4, PHA was removed, and 100 $\times$ ITS (Thermo Fisher Scientific, Cat. No. 41400-045) and 50 $\mu\text{g/mL}$ L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma, Cat. No. A8960-5G) were added. Activated NK cells were used between days 10-17 after sufficient expansion.

⁵¹Cr release assay by HLA-reactive CD8⁺ T cells

⁵¹Cr release assay was performed as previously reported (Ichise et al., 2017). Day 21-28 CD43⁺ blood cells or cardiomyocytes were first pretreated with 50 ng/mL IFN- γ for 48 hour and then ⁵¹Cr labeled for use as target cells. HLA-reactive CD8⁺ T cells or HLA-null-reactive NK cells were incubated at different ratios with 5,000 target cells for 5 hour at 37 °C.

***In vivo* immunoreactivity with HLA-reactive CD8⁺ cells**

In vivo immunoreactivity was assessed similarly to a previous report (Gornalusse et al., 2017). Stable luciferase-expressing iPSCs were generated by transfecting iPSCs with pPV-EF1 α -Luc2-iP-A and *piggyBac* transposase plasmids (pHL-EF1 α -hcPBase-A), followed by 1 μM puromycin selection for more than one week. Next, the luciferase-expressing iPSCs were differentiated into CD43⁺ blood cells, pretreated with 50 ng/mL IFN- γ for 48 hr and then transplanted into 6 week-old male NRG mice (NOD.Cg-Rag1tm1Mom Il2rgtm1Wjl/SzJ Stock No: 007799, Jackson Laboratory) by intraperitoneal injection (2.5×10^5 cells). Basal luciferase levels of the transplanted cells were measured by an IVIS imaging system (SPI, IVIS Spectrum). One hour post transplantation, 2.5×10^6 HLA-reactive CD8⁺ T cells were injected into one group of mice and re-imaged 5 hour, 1 day, 3 days and 7 days later. Luminescence was detected after the intraperitoneal injection of 150 mg/kg d-luciferin substrate (Promega, Cat. No. E1605) in D-PBS, and the average radiance was recorded using the IVIS imaging system.

***In vivo* immunoreactivity with HLA-null-reactive NK cells.**

1 5×10^5 luciferase-expressing iPSC-derived CD43⁺ blood cells were
2 pretreated with 50 ng/mL IFN- γ for 48 hr and intraperitoneally injected into 12
3 week-old female NRG mice, and IVIS-based imaging of pre-NK cell luciferase
4 levels were measured as a control. One hour after the injection, 1×10^6
5 HLA-null-reactive NK cells were injected into one group of the mice, and both
6 groups were imaged by IVIS. 25ng/ml IFN- γ was injected every 2 day
7 intraperitoneally.

8 **CFSE-labeled CD4⁺ T cell proliferation assay**

10 HLA typed PBMCs were obtained from healthy donor (also see Table S2).
11 Upon thawing, PBMCs were cultured in RPMI-1640 supplemented with
12 glutamine and 10% heat-inactivated human FBS (Biosera, Cat. No. FB-1365) for
13 3-5 hour and then sorted by FACS for CD4⁺ T cells by using anti-CD3 (BD
14 Biosciences, Cat. No. 557943, Clone UCHT1) and anti-CD4 (BioLegend, Cat.
15 No. 317434, Clone OKT4) antibodies. The next day the effector CD4⁺ T cells
16 were labeled by 1 μ M CFSE (Dojindo, Cat. No. C309) for 8 minutes and then
17 washed 2 times with PBS. Subsequently, iPSC-derived CD43⁺ blood cells were
18 pretreated with 50 ng/mL IFN- γ for 48 hour and irradiated (with 40 Gy) to stop
19 proliferation. Then, the target CD43⁺ cells were mixed with effector CD4⁺ T cells
20 at a 1:1 ratio in a 96-well F-bottom plate for 7 days in RPMI-1640 supplemented
21 with glutamine, 10% heat-inactivated human FBS, 25 ng/mL IFN- γ and 20 U/mL
22 IL-2. Finally, the percentage of proliferative CD7⁺ T cells that became negative
23 for CFSE labeling was measured by flow cytometry.

25 **CFSE-labeled bulk PBMC proliferation assay**

26 HLA typed PBMCs were cultured in RPMI-1640 supplemented with
27 glutamine and 10% heat-inactivated human FBS (Biosera, Cat. No. FB-1365) for
28 overnight. The next day the PBMCs were labeled by 2 μ M CFSE (Dojindo, Cat.
29 No. C309) for 8 minutes and then washed 2 times with PBS. Subsequently,
30 iPSC-derived CD43⁺ blood cells were pretreated with 50 ng/mL IFN- γ for 48 hour
31 and irradiated (with 40 Gy) to stop proliferation. Then, the target iPSC-derived
32 CD43⁺ cells were mixed with PBMCs at a 1:10 ratio in a 96-well flat-bottom plate
33 for 7 days in RPMI-1640 supplemented with glutamine, 10% heat-inactivated

human FBS and 25 ng/mL IFN- γ . Finally, the percentage of proliferative CD3⁺ CD4⁺, CD3⁺ CD8⁺ T cells and CD3⁻ CD56⁺ NK cells that became negative for CFSE labeling was measured by flow cytometry.

Teratoma formation assay

2-5 $\times 10^5$ iPSCs were mixed with matrigel at a 1:1 ratio and transplanted into the renal capsule of NRG mice. Mice were sacrificed 8 to 10 weeks later and analyzed by the hematoxylin and eosin staining of paraffin-embedded sections.

qRT-PCR

1383D2 and 604B1 were harvested in three conditions: (1) IFN- γ un-stimulated condition, (2) immediately after IFN- γ stimulation, and (3) 5 days after IFN- γ stimulation. mRNA was extracted from each condition. Subsequently, cDNA was amplified from the mRNA by using Rever Tra Ace qPCR RT Master Mix (Toyobo). Quantitative PCR was performed by using human Nanog specific primers. Human ACTB was used as a control. IFN- γ stimulation was done for 48 hour in a final concentration of 50 ng/mL.

Statistical analysis

For two group comparison Student's *t*-test was used with the T.TEST function in Excel software with two tailed, non-paired, unequal variance analysis options. For comparison of three groups or more, one-way ANOVA followed by the Tukey-Kramer post hoc test was used to calculate a corrected *p* value with GraphPad Prism 8 software. *p* < 0.05 was considered statistically significant.

Karyotyping analysis

All the karyotyping analyses were performed by chromocenter Inc. (Yanago, Japan). Preparation of metaphase chromosomes of hiPSCs was performed according to standard methods. The slides were stained with Hoechst 33258 for Q-banding karyotyping. Metaphase images were captured using an AxioImagerZ2 fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with the Ikaros software program (MetaSystems, Altlussheim, Germany). Chromosome abnormalities were described using An International System for

Human Cytogenetic Nomenclature (ISCN 2016). Human mFISH probes and mBAND probes for the chromosome 2 and chromosome 15 were purchased from MetaSystems. Procedures for the denaturation of metaphase chromosomes and mFISH/mBAND probes, hybridization, post-hybridization washes and fluorescent staining were performed in accordance with the manufacturer's instructions. Metaphase images were captured using an AxioImagerZ2 fluorescence microscope equipped with the ISIS mFISH/mBAND software program (MetaSystems).

Whole exome sequencing

Genomic DNA samples were extracted from iPS cells and used for exome capture with SeqCap EZ v3 (Roche) following the manufacturer's instructions with minor modification. Briefly, 100 ng of genomic DNA was randomly fragmented by the Covaris LE220 system. Adaptor-ligated genomic DNA libraries were prepared with the KAPA Hyper Prep Kit (KAPA Biosystems) according to the manufacturer's instructions. The libraries were amplified by the LM-PCR and purified with AMPure XP beads (Agencourt). Human COT-1 DNA and Illumina adaptor-specific blocking oligonucleotides were used during hybridization to block repetitive genomic sequences and adaptor sequences, respectively. The exome-enriched libraries were amplified by LM-PCR, purified with the AMPure XP beads and quantified by a KAPA library quantification Kit (KAPA Biosystems). Fifteen pM of libraries were used in the cluster generation on HiSeq PE Flow Cell v4 (Illumina) using an Illumina cBot. Paired-end sequencing for 2 × 126 cycles was performed using HiSeq SBS Kit v4 and HiSeq2500 (Illumina).

Deep sequencing data analysis

To generate FASTQ files from the raw sequencing data, bcl2fastq v2.17.1.14 (Illumina) was used. The sequenced FASTQ files were mapped to the reference human genome (hg19) by BWA MEM (Ver. 0.7.15) with or without -T 0 option, and duplicate reads were removed by NovoSort (Ver. 1.03.09). All samples were within 93-118 million sequenced reads, 89.6-91.4% mapping rate, and 93.1-118.8 average depth at CDS region, calculated by bedtools-2.17.0. Then,

each BAM file was analyzed by Genomon 1 (Genomon-Fisher, Ver. 1.0.1) (Yoshida et al., 2011) or Genomon 2 (Empirical Bayesian mutation Calling, Ver. 2.3.0) (Shiraishi et al., 2013) pipelines to call genome-edited iPSC clone specific SNV and indels. Subsequently, identified SNV/indels were pooled and filtered based on the following criteria: depth ≥ 15 , sample to control ratio ≥ 5 , control allelic frequency ≥ 0.01 and sample allelic frequency ≥ 0.05 . To further extract potentially pathogenic SNV/indel mutations, we extracted nonsynonymous and splicing mutations, and then searched for dbSNP, HGMD (Stenson et al., 2017), COSMIC (Ver. 85) (Forbes et al., 2017), and Cancer Gene Census (Sondka et al., 2018) plus the Shibata gene list (<http://www.pmda.go.jp/files/000152599.pdf>). Potential sgRNA binding sites were searched with Cas-OFFinder (Bae et al., 2014) with up to 9 bp mismatch allowance, and the closest distance between the potential sgRNA binding sites and the detected SNV/indels by WES were calculated with “bedtools closest” function and the same number of random SNV/indels were generated with “bedtools random” function. (Quinlan and Hall, 2010). The relative distribution of SNV/indels position was visualized by ngs.plot (Shen et al., 2014).

Estimation of T cell-based compatibility with HLA haplotypes

HLA haplotype information on HLA-A/B/C in Japanese population was obtained from HLA laboratory [http://hla.or.jp/med/frequency_search/en/haplo/], and that of other ethics (European American, African American, Asian, and Hispanics) were obtained from a previous report (Maiers et al., 2007). To estimate T cell-based compatibility with HLA-A/B/C or HLA-C matching, HLA haplotypes were sorted by the HLA allele frequency in each ethic and cumulative HLA-A/B/C or HLA-C coverage on a given rank was calculated by piling up all the allele frequencies that match with the given HLA haplotype (4-digit) on either paternal or maternal allele.

Supplemental Table 1. List of SpCas9 gRNAs that target specific HLA haplotypes (related to Figure 1).

Supplemental Table 2. HLA haplotype information of all iPSCs and PBMCs used in this study (related to Figures 1, 2, 3, 4 and 5).

Supplemental Table 3. Genomic indel patterns of the genome edited clones (related to Figures 2, 3 and 5).

Supplemental Table 4. Cumulative coverage percentage by HLA-A, -C, -B and HLA-C-retained in Japanese and four ethnicities in the U.S. population (related to Figure 6).

Supplemental Table 5. Cumulative coverage percentage by 12 core HLA-C haplotypes in Japanese and four ethnicities in the U.S. population (related to Figure 6).

Supplemental Table 6. Oligonucleotide lists for gRNA and PCR primers (related to STAR Methods).