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# HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells

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Polymorphisms in the human leukocyte antigen (HLA) class I genes can cause the rejection of pluripotent stem cell (PSC)-derived products in allogeneic recipients. Disruption of the *Beta-2 Microglobulin (B2M)* gene eliminates surface expression of all class I molecules, but leaves the cells vulnerable to lysis by natural killer (NK) cells. Here we show that this ‘missing-self’ response can be prevented by forced expression of minimally polymorphic HLA-E molecules. We use adeno-associated virus (AAV)-mediated gene editing to knock in HLA-E genes at the *B2M* locus in human PSCs in a manner that confers inducible, regulated, surface expression of HLA-E single-chain dimers (fused to B2M) or trimers (fused to B2M and a peptide antigen), without surface expression of HLA-A, B or C. These HLA-engineered PSCs and their differentiated derivatives are not recognized as allogeneic by CD8<sup>+</sup> T cells, do not bind anti-HLA antibodies and are resistant to NK-mediated lysis. Our approach provides a potential source of universal donor cells for applications where the differentiated derivatives lack HLA class II expression.

Cellular products derived from human pluripotent stem cells (PSCs) have the potential to treat many human diseases, but their clinical use is limited by the host rejection of transplanted cells owing to expression of the highly polymorphic human leukocyte antigen (HLA) genes. Various solutions have been proposed for this allogeneic rejection problem, including HLA-typed PSC banks<sup>1</sup>, or the derivation of induced PSCs from each patient<sup>2</sup>, but these approaches require that multiple cell lines be derived<sup>3</sup>, differentiated into therapeutic products and approved for human administration. The development of a single PSC line that can avoid allogeneic rejection would greatly reduce the time and expense required to advance cellular therapies to the clinic.

HLA class I molecules are expressed on most cells and play a central role in allogeneic rejection through their presentation of peptide antigens to CD8<sup>+</sup> T cells<sup>4</sup>. *Beta-2 Microglobulin (B2M)* is a non-polymorphic gene that encodes a common protein subunit required for surface expression of all polymorphic HLA class I heavy chains<sup>5</sup>. Our group<sup>6</sup> and others<sup>7–9</sup> have created *B2M*<sup>-/-</sup> human PSCs in order to eliminate class I surface expression and prevent the stimulation of allogeneic CD8<sup>+</sup> T cells. However, a major limitation of this simple engineering strategy is that HLA class I-negative cells are lysed by natural killer (NK) cells through the missing-self response<sup>10,11</sup>. In mice, host NK cells eliminate transplanted *B2m*<sup>-/-</sup> donor cells<sup>10</sup>, and a similar phenomenon occurs *in vitro* with HLA class I-negative human leukemic lines<sup>12</sup>. This NK cell-dependent lysis is normally inhibited through interactions with class I molecules<sup>13</sup>, including the minimally polymorphic HLA-E protein<sup>14–16</sup>, which presents peptides derived from the signal sequences of other HLA class I molecules<sup>17</sup>, and is a ligand for the inhibitory CD94/NGK2A complex expressed on the majority of NK cells<sup>14,15,18</sup>. Like other HLA class I molecules, HLA-E

forms a heterodimer with a B2M subunit so it is not expressed on the surface of *B2M*<sup>-/-</sup> cells. We surmised that *B2M*<sup>-/-</sup> cells could be engineered to express HLA-E as a single-chain protein fused to B2M, and thereby create cells that express HLA-E as their only surface HLA class I molecule.

Here we use recombinant adeno-associated virus (rAAV)-mediated gene editing to create such PSCs. rAAV vectors deliver single-stranded linear DNA genomes that efficiently recombine with homologous chromosomal sequences in up to 1% of infected human cells without the use of potentially genotoxic nucleases<sup>19,20</sup>, including human PSCs<sup>21–23</sup>. We show that single-chain HLA-E molecules prevent the NK-mediated lysis of *B2M*<sup>-/-</sup> cells without stimulating allogeneic T cells, addressing a major problem in the creation of universal donor cells for regenerative medicine applications.

## RESULTS

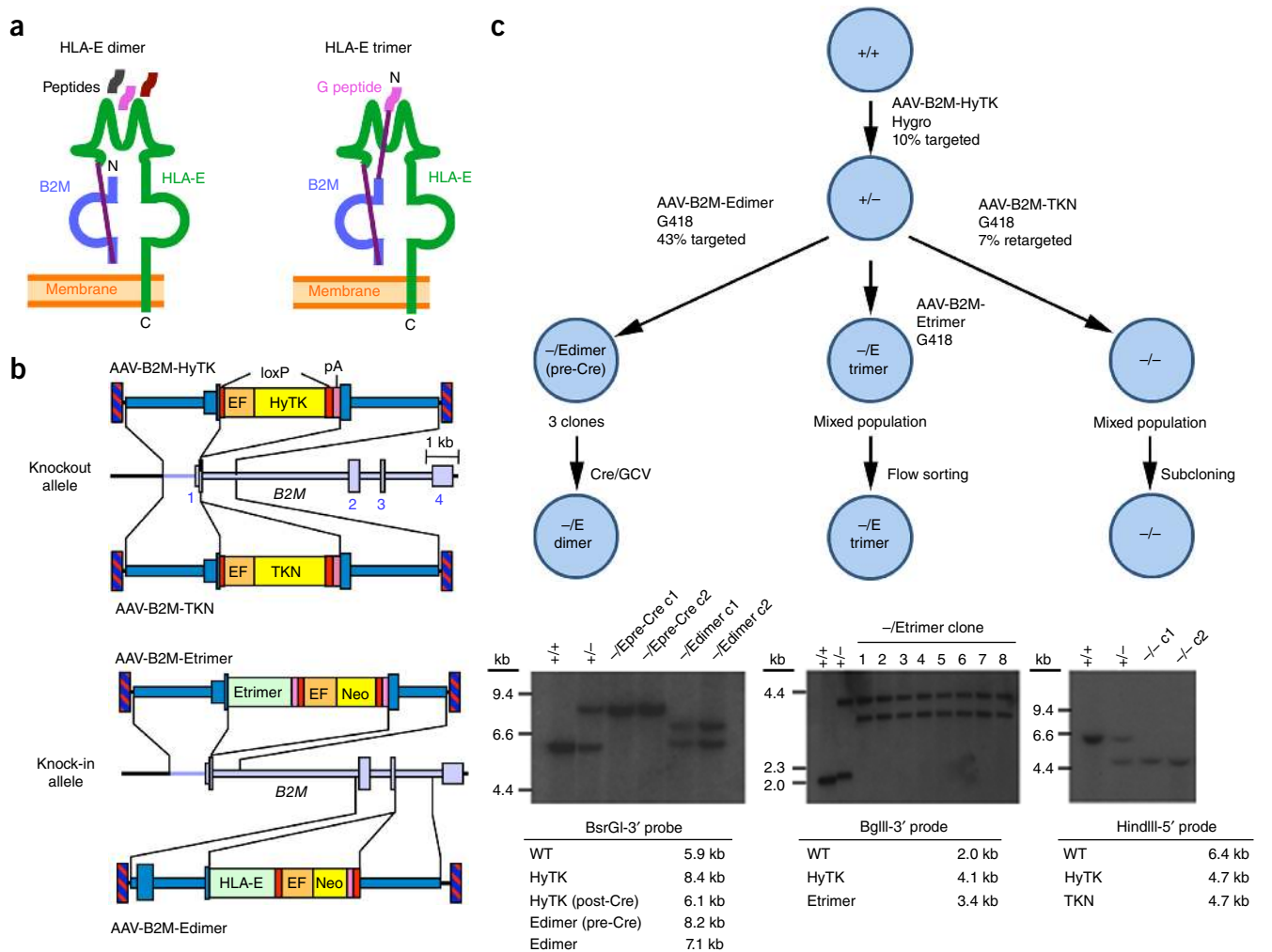
### Generation of HLA-E knock-in cells at the *B2M* locus

Two types of HLA-E single-chain fusion constructs were designed for our experiments (Fig. 1a). The HLA-E single-chain dimer consists of an HLA-E heavy chain covalently fused to B2M through a flexible (G<sub>4</sub>S)<sub>4</sub> linker, such that it can bind a normal repertoire of peptides for antigen presentation. The HLA-E single-chain trimer contains an additional (G<sub>4</sub>S)<sub>3</sub> linker fused to a peptide derived from the signal sequence of HLA-G (another HLA class I molecule), which is a non-polymorphic peptide normally presented by HLA-E that inhibits NK cell-dependent lysis through its binding to CD94/NGK2A<sup>15</sup>.

rAAV gene editing vectors were designed to introduce HLA-E constructs at the *B2M* locus, such that an HLA-E single-chain dimer or trimer would be expressed under the control of the *B2M* promoter

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**Figure 1** AAV-mediated knock in of HLA-E. **(a)** Schematic representation of HLA-E single chain (SC) molecules. The HLA-E heavy chain is covalently linked to B2M (by a  $(G_4S)_4$  linker). In the trimer, the HLA-G signal peptide is covalently attached to the N terminus by a  $(G_4S)_3$  linker. **(b)** AAV knock-out and knock-in vectors used to edit *B2M*. Inverted terminal repeats (hatched boxes), *B2M* exons (large boxes), homology arms (dark blue), *loxP* signals (red), synthetic poly(A) signals (pink), *HyTK* or *TKN* antibiotic-resistance cassettes (yellow), human elongation factor-1 alpha (EF-1  $\alpha$ ) promoter (orange) and HLA-E heavy chain or trimer reading frames (green) are shown. **(c)** Stepwise targeting strategy to generate *B2M*<sup>-/Edimer</sup>, *B2M*<sup>-/Etrimer</sup> and *B2M*<sup>-/-</sup> ESC lines. Bottom panels show corresponding Southern blots demonstrating accurate gene editing with *B2M* genotypes of each sample shown, and digests with predicted fragment sizes below (allele maps in **Supplementary Fig. 1a**).

(**Fig. 1b**). In our strategy, the first allele of *B2M* is edited with a vector that inserts a *loxP*-flanked (floxed) *HyTK* fusion gene encoding hygromycin resistance and thymidine kinase at exon 1 to create a null allele (AAV-B2M-HyTK), and the second *B2M* allele is then edited to express HLA-E. Vector AAV-B2M-Edimer inserts an HLA-E heavy chain reading frame at exon 3 of *B2M* for HLA-E single-chain dimer expression, and AAV-B2M-Etrimer inserts a full HLA-E single-chain trimer gene at exon 1 (including a codon-optimized, wobbled *B2M* reading frame). Both the dimer and trimer vectors also contain floxed *Neo* expression cassettes for G418 selection. A second *B2M* knockout vector was also constructed that inserts a floxed *TKNeo* fusion gene at exon 1 in order to create *B2M*<sup>-/-</sup> control cells (AAV-B2M-TKN).

Human Elf-1 embryonic stem cells (ESCs) were serially infected with these rAAV vectors, and 7–43% of selected clones were accurately edited, based on screening by PCR with primers in the transgene and outside of the homology arms (**Fig. 1c** and **Table 1**). PCR-positive clones infected with AAV-B2M-Edimer were further screened by Southern blot analysis to determine which allele was edited, with

6 of 13 clones edited at the second *B2M* allele (**Table 1**). Three of these *B2M*<sup>-/Edimer (pre-Cre)</sup> clones were subsequently infected with a non-integrating foamy virus vector to transiently express Cre, as described<sup>6</sup>, and 42–46% of ganciclovir-resistant clones had lost both the *HyTK* and *Neo* cassettes to produce a *B2M*<sup>-/Edimer</sup> genotype. To create cells with a *B2M*<sup>-/Etrimer</sup> genotype, we used flow cytometry to sort a polyclonal population of G418-resistant cells infected with AAV-B2M-Etrimer for the absence HLA-A,B,C expression, which ensured that the second allele had been edited. Southern blot analyses confirmed that accurate editing occurred (**Fig. 1c** and **Supplementary Fig. 1a**) and reprobings showed that none of the edited clones had random integrants (data not shown). *B2M*<sup>-/Edimer</sup> clones c1 and c2 and *B2M*<sup>-/Etrimer</sup> clones c5 and c6 had normal karyotypes (data not shown) and were selected for subsequent studies.

#### Generation of *B2M*<sup>-/-</sup> control cells

We attempted to establish *B2M*<sup>-/-</sup> control cells by infecting *B2M*<sup>+/-</sup> cells with the second knockout vector AAV-B2M-TKN (**Fig. 1**), but we

**Table 1** *B2M* editing by different AAV vectors in H1 and Elf-1 ESCs

Vector	Comments	ESC line	Editing step	Percent edited CFU <sup>a</sup>	Total no. PCR <sup>+</sup> CFU analyzed <sup>b</sup>	Targeted at 1st allele	Targeted at 2nd allele	Retargeted at 1st allele	Transiently targeted at 2nd allele <sup>c</sup>
AAV-B2M-HyTK	Total knockout	H1	First	16.5	15	15	NA	NA	NA
AAV-B2M-HyTK	Total knockout	Elf-1	First	10.1	2	2	NA	NA	NA
AAV-B2M-Edimer	HLA-E <sup>+</sup>	Elf-1	First	33.3	8	8	NA	NA	NA
AAV-B2M-Edimer	HLA-E <sup>+</sup>	Elf-1	Second	42.7	13	NA	6	7	0
AAV-B2M-TKN	Total knockout	H1	Second	5.2	5	NA	0	3	2
AAV-B2M-TKN	Total knockout	Elf-1	Second	7.2	8	NA	0	8	0
AAV-B2M-TKwN	Total knockout, wobbled	H1	Second	6.5	2	NA	0	0	2
AAV-B2M-TKwN	Total knockout, wobbled	Elf-1	Second	9.3	8	NA	0	7	1
AAV-B2M-ETKNpA <sup>d</sup>	Leaky knockout	H1	First	30.0	9	9	NA	NA	NA
AAV-B2M-EHyTKpA <sup>d</sup>	Leaky knockout	H1	Second	10.0	6	NA	2	4	0

CFU, colony-forming unit. NA, not applicable.

<sup>a</sup>(Edited CFU/resistant CFU) × 100. <sup>b</sup>CFU identified by primers for targeted alleles. <sup>c</sup>PCR<sup>+</sup> colonies that were mixed and subsequently lost the portion of cells targeted at the 2nd allele.

<sup>d</sup>These AAV vectors were used to create our initial *B2M* knockout model in H1 cells.

consistently obtained G418-resistant cells that had been retargeted at the allele previously edited by the AAV-B2M-HyTK vector, in contrast to the approximately 50:50 allele ratio obtained with the AAV-B2M-Edimer vector (Table 1). In addition, the rare G418-resistant colonies that appeared to be targeted at the second allele by PCR were mixed populations when examined by flow cytometry, and upon further subcloning, we were unable to obtain pure populations that lacked *B2M* expression and maintained a normal karyotype (Supplementary Fig. 1b). Ultimately, we were able to isolate two pure *B2M*<sup>-/-</sup> clones by repeated subcloning and flow cytometry screening of two independently edited cell populations (Supplementary Fig. 1c), but in both cases the clones had abnormal karyotypes (Supplementary Fig. 1d).

These results suggested that euploid *B2M*<sup>-/-</sup> Elf-1 ESCs grow poorly, which was surprising given that we had previously generated euploid H1 ESCs with both *B2M* alleles edited by the nearly identical knockout vectors AAV-B2M-EHyTKpA and AAV-B2M-ETKNpA (Table 1)<sup>6</sup>. This was not a unique property of Elf-1 cells, since we also failed to generate *B2M*<sup>-/-</sup> H1 ESCs with the newer vectors (Fig. 1 and Table 1). Another possible explanation was that the identical *TK* sequences present in both vectors were preferentially promoting recombination at the previously edited allele, but when the *TK* reading frame was wobbled to reduce homology, we were still unable to isolate ESCs edited at both alleles (Table 1). These failures led us to re-examine the euploid *B2M*<sup>-/-</sup> H1 ESCs edited with our original vectors, and we found that low levels of class I proteins could be detected by flow cytometry after interferon (IFN)- $\gamma$  induction of HLA gene expression (Supplementary Fig. 2a), suggesting that these edited alleles were leaky. This was confirmed by RT-PCR analysis, which detected an mRNA from the edited alleles generated by read-through transcription beyond the pA signal into an open reading frame with an ATG initiation codon in a *loxP* site and a novel signal sequence in-frame with downstream *B2M* sequences (Supplementary Fig. 2b). This leakiness was not a feature of alleles edited by the newer vectors AAV-B2M-HyTK and AAV-B2M-TKN, which contained additional stop codons downstream from the pA signal to block *B2M* protein production (Supplementary Fig. 2C). Taken together, these results suggest that a complete lack of *B2M* expression slows the growth of normal, euploid ESCs. Importantly, this was not a feature of ESCs that uniquely expressed HLA-E single-chain dimers or trimers, which grew well and had normal karyotypes.

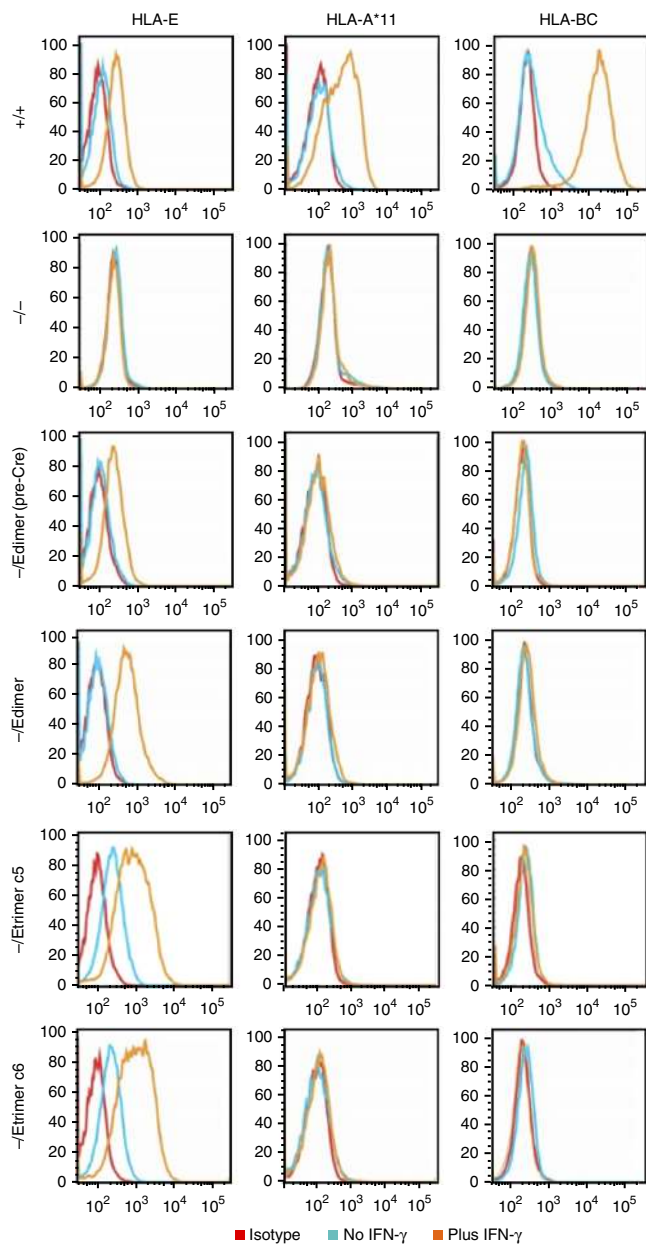
### HLA expression in edited cells

Our editing strategy produces HLA-E single-chain genes that are expressed from the *B2M* promoter, so they should be regulated appropriately in response to cytokines. Undifferentiated ESCs with the *B2M* genotypes shown in Figure 1 were cultured with and without IFN- $\gamma$ , and the levels of cell surface HLA class I molecules were determined by flow cytometry (Fig. 2). In the absence of IFN- $\gamma$ , *B2M*<sup>+/+</sup> ESCs expressed nearly undetectable amounts of both HLA-E and polymorphic class I molecules (HLA-A, B and C), all of which were upregulated significantly in the presence of IFN- $\gamma$ . In contrast, *B2M*<sup>-/-</sup> ESCs had no surface expression of HLA class I molecules, even in the presence of IFN- $\gamma$ , confirming that these cells have a complete knockout of *B2M* (although they acquired an abnormal tetraploid karyotype).

*B2M*<sup>-/-</sup>*Edimer*(*preCre*) cells expressed low levels of HLA-E upon IFN- $\gamma$  induction, which explains why these clones could be easily obtained by gene editing, even though they did not express HLA-A, B or C molecules. A similar pattern was observed with *B2M*<sup>-/-</sup>*Edimer* cells after Cre-mediated removal of the *Neo* cassette, but with higher HLA-E single-chain dimer expression. *B2M*<sup>-/-</sup>*Etrimer* cells were distinct in that they also expressed HLA-E without IFN- $\gamma$  induction, as observed for two independent clones, as well as higher levels upon IFN- $\gamma$  induction. It is possible that in the absence of IFN- $\gamma$ , the wild-type (WT) HLA-E and HLA-E dimer molecules were not effectively loaded with peptides, which reduces surface expression levels. Both *B2M*<sup>-/-</sup>*Edimer* and *B2M*<sup>-/-</sup>*Etrimer* cells expressed higher than normal levels of HLA-E, reflecting the fact that all the proteins expressed from one edited *B2M* allele were HLA-E single-chain molecules, in contrast to WT *B2M* proteins that are shared by all types of class I heavy chains. To assess whether expression of HLA-E as the only HLA class I surface molecule influenced differentiation, we derived retinal pigmented epithelium (RPE) cells from *B2M*<sup>-/-</sup>*Edimer* ESCs *in vitro*, and the results were similar to those obtained with *B2M*<sup>+/+</sup> ESCs. Both lines had efficient RPE differentiation, as shown by their cuboidal morphology, and staining for PMEL and MITF (Supplementary Fig. 3).

### HLA-E single-chain expression prevents NK-mediated lysis

Undifferentiated ESCs have limited immunogenicity and do not induce NK-mediated lysis<sup>24</sup>, so we differentiated ESCs into CD45<sup>+</sup> hematopoietic derivatives (Fig. 3a) before characterizing their immune reactivity. Flow cytometry showed there were >89% CD45<sup>+</sup> hematopoietic cells on day 38 in all edited cell lines except



**Figure 2** Inducible HLA-E expression without other class I molecules. Flow cytometry of HLA-E, HLA-A\*11 (present in WT E1f-1 cells) and HLA-BC expression in the presence (orange) or absence (light blue) of IFN $\gamma$  treatment. Isotype control tracings are shown in red. Single-cell suspensions of E1f-1 ESCs with each indicated B2M genotype were derived by trypsin digestion of IFN- $\gamma$ -treated ESCs. See **Supplementary Table 2** for full HLA genotypes.

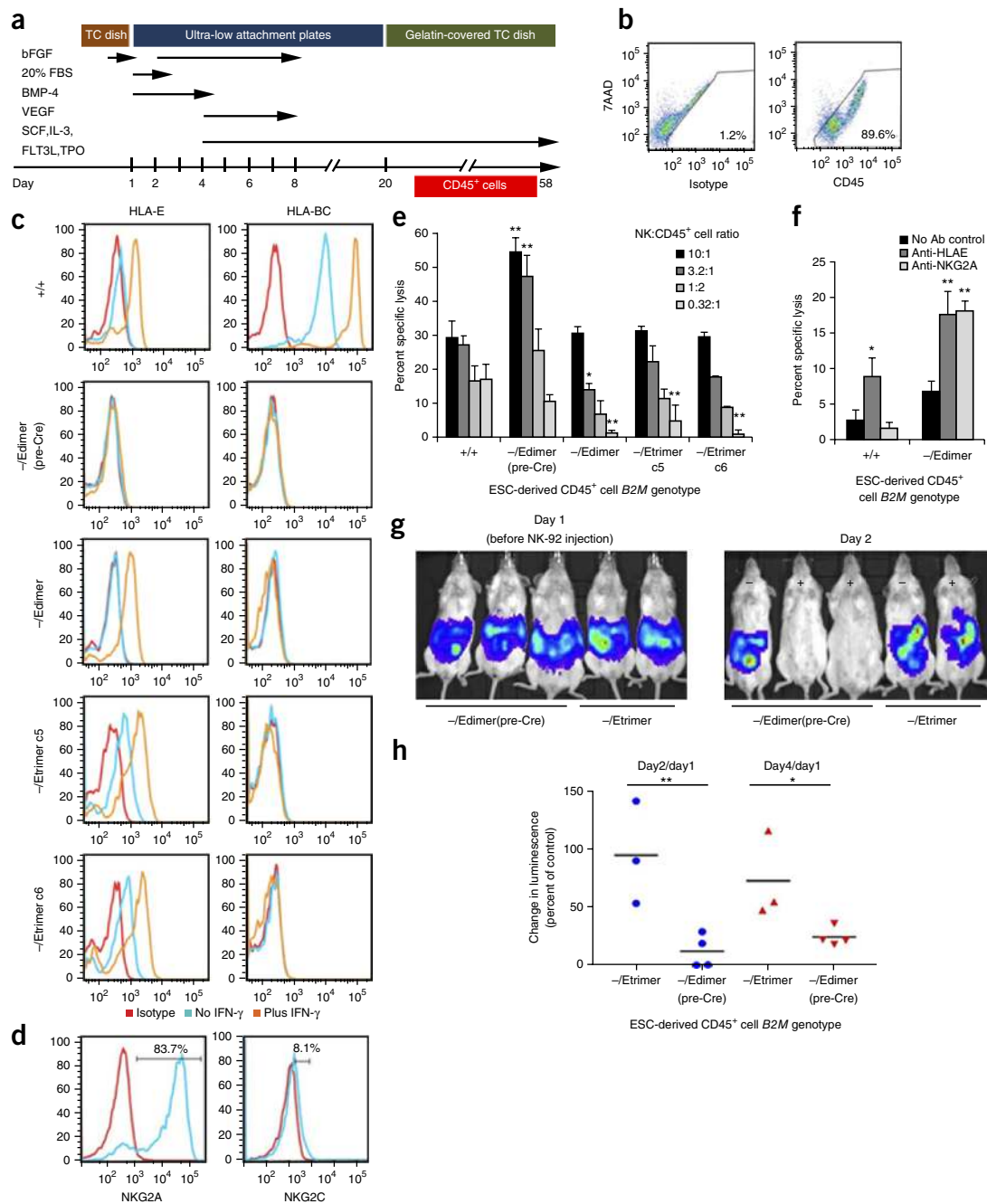
$B2M^{-/-}$  cells (**Fig. 3b** and **Supplementary Fig. 4a**), where the total yield of suspension culture cells (containing CD45 $^{+}$  hematopoietic cells) was  $\sim 10$  times lower (**Supplementary Fig. 4b**), presumably because of impaired differentiation in these karyotypically abnormal lines. HLA class I expression in ESC-derived CD45 $^{+}$  cells was similar to that observed in undifferentiated ESCs, with IFN- $\gamma$ -inducible HLA-E expression and no other surface class I molecules in  $B2M^{-/Edimer}$  and  $B2M^{-/Etrimer}$  cells (**Fig. 3c**). One notable difference was that the  $B2M^{-/Edimer(preCre)}$  cells had virtually no detectable HLA-E expression, so for some experiments they were used as

class-I-negative controls, since  $B2M^{-/-}$  CD45 $^{+}$  cells could not be produced in adequate numbers.

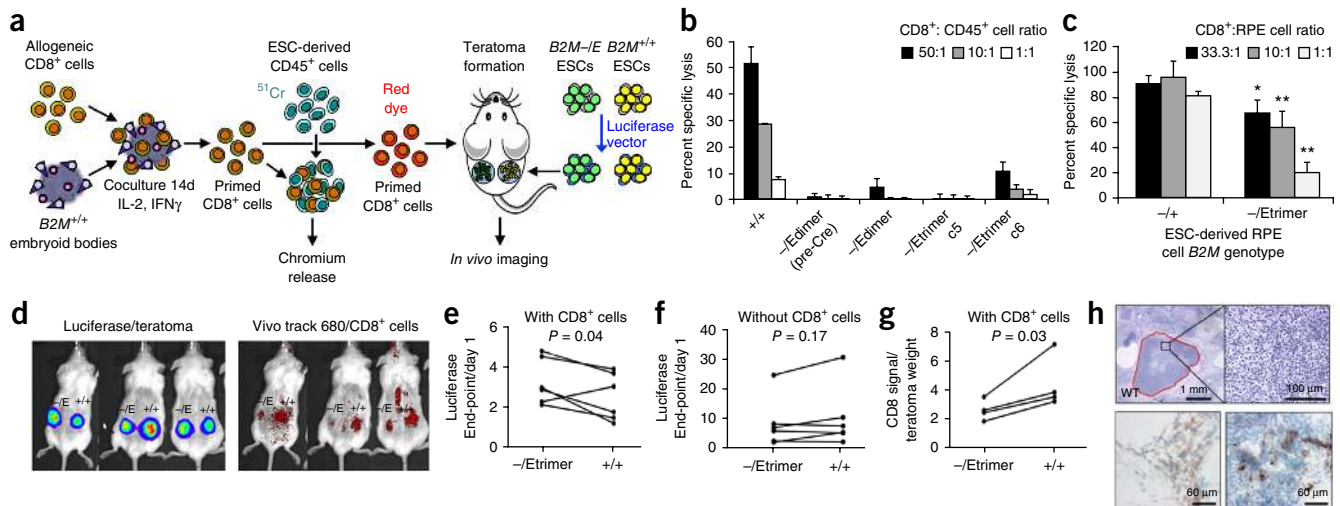
HLA-E binds to both the inhibiting and activating receptors NKG2A and NKG2C, respectively<sup>25</sup>, but NKG2A is expressed in most human NK cells<sup>18</sup>, so we expected that the overall effect of HLA-E should be inhibitory. Allogeneic peripheral blood CD56 $^{+}$  NK cells isolated from normal donors were incubated with <sup>51</sup>Cr-labeled ESC-derived CD45 $^{+}$  cells, and lysis was measured in chromium release assays. A typical NK donor with 83.7% NKG2A $^{+}$  cells and 8.1% NKG2C $^{+}$  cells (**Fig. 3d**) showed increased lysis of class-I-negative  $B2M^{-/Edimer(preCre)}$  CD45 $^{+}$  cells as compared to class I-expressing cells, confirming that the missing-self response observed in mice is also important for human hematopoietic cells (**Fig. 3e**). This increased lysis was completely reversed by expression of HLA-E single-chain molecules in  $B2M^{-/Edimer}$  and  $B2M^{-/Etrimer}$  CD45 $^{+}$  cells, and at lower NK/CD45 cell ratios, HLA-E single-chain expression reduced lysis to below that of  $B2M^{+/+}$  CD45 $^{+}$  cells, perhaps due to their higher than normal HLA-E levels, or a lack of interactions with activating Killer-cell Immunoglobulin-like Receptors (KIRs). Blocking antibodies against HLA-E or NKG2A increased the lysis of  $B2M^{-/Edimer}$  cells  $\sim 2.5$  fold, confirming that the HLA-E-NKG2A interaction was responsible (**Fig. 3f**). NK cells from a donor with unusually low NKG2A expression (49.86% NKG2A $^{+}$ , 3.66% NKG2C $^{+}$ ; **Supplementary Fig. 4c**) also preferentially lysed class-I-negative ESC-derived CD45 $^{+}$  cells, and this was partly reversed by HLA-E expression in  $B2M^{-/Edimer}$  cells (**Supplementary Fig. 4d**). Control experiments confirmed that both  $B2M^{-/-}$  and  $B2M^{-/Edimer(preCre)}$  CD45 $^{+}$  cells were lysed at equivalent levels by NK cells, and that HLA-E expression also prevented lysis by NK cells grown at lower interleukin (IL)-2 concentrations (**Supplementary Fig. 4e,f**). To provide an *in vivo* proof of concept, we monitored the survival of luciferase-expressing ESC-derived CD45 $^{+}$  cells and teratomas in immunodeficient mice and showed that HLA-E expression improved survival in mice that also received NK-92 cells, an NK cell line that expresses NKG2A<sup>26,27</sup> (**Fig. 3g,h** and **Supplementary Fig. 4g,h**). Taken together, these experiments demonstrate that HLA-E single-chain expression prevents NK-mediated lysis in otherwise HLA class-I-negative cells.

### HLA-E single-chain-expressing cells are not recognized as allogeneic

We used both *in vitro* and *in vivo* models to determine if allogeneic T cells recognize and respond to  $B2M^{-/Edimer}$  and  $B2M^{-/Etrimer}$  cells (**Fig. 4a**). Normal, human CD8 $^{+}$  T cells were first cocultured with embryoid bodies derived from  $B2M^{+/+}$  E1f-1 ESCs, to enrich for T cells that recognize E1f-1 class I molecules. Embryoid bodies contain several types of cells and express high levels of HLA class I molecules, but not HLA class II (e.g., HLA-DR; **Supplementary Fig. 5a**). The costimulatory molecules CD40 and CD54 were also present on embryoid body cells, but not CD86, CD80, CD83 or CD275 (**Supplementary Fig. 5b**). These embryoid-body-primed T cells were then incubated with <sup>51</sup>Cr-labeled ESC-derived CD45 $^{+}$  cells in chromium release assays to detect T-cell-mediated cytotoxicity. The CD45 $^{+}$  cells robustly expressed CD40, CD54 and CD86 costimulatory molecules (**Supplementary Fig. 5c**). While embryoid body-primed T cells efficiently lysed  $B2M^{+/+}$  CD45 $^{+}$  cells, they did not kill  $B2M^{-/Edimer(preCre)}$ ,  $B2M^{-/Edimer}$  or  $B2M^{-/Etrimer}$  CD45 $^{+}$  cells (**Fig. 4b**), and a similar protection from lysis was observed for ESC-derived RPE cells expressing HLA-E (**Fig. 4c**), demonstrating both that cytotoxic T-cell responses can be prevented by eliminating surface expression of polymorphic class I molecules, and that non-polymorphic HLA-E single-chain dimers and trimers do not stimulate lysis. These experiments also show



**Figure 3** Protection of HLA-E-expressing cells from NK cell-mediated lysis. **(a)** Schematic representation of the hematopoietic differentiation protocol used for Elf-1 ESCs. Hematopoietic cells were harvested from day 24 to day 58. TPO, thrombopoietin. **(b)** Representative CD45 expression on day 38 suspension cells produced by *B2M*<sup>-/-Etrimer</sup> ESC clone c5 as measured by flow cytometry. Results for other *B2M*-edited lines are shown in **Supplementary Figure 4a**. 7AAD, 7-amino-actinomycin D. **(c)** Flow cytometry of HLA-E and HLA-BC expression in ESC-derived CD45<sup>+</sup> cells with the indicated *B2M* genotypes. **(d)** Expression of inhibiting and activating receptors NKG2A and NKG2C on NK cells derived from a healthy donor (donor 1). Isotype and specific antibody tracings shown in red and blue, respectively. Percents shown are calculated by subtracting corresponding isotype control frequencies. **(e)** Chromium release assay results obtained with ESC-derived CD45<sup>+</sup> cells of the indicated *B2M* genotype and normal NK cells (donor 1) at the indicated ratios (mean  $\pm$  s.d.,  $n = 3$ ). The  $P$  values (one-way ANOVA test) were  $< 0.002$  at all cell ratios. Asterisks indicate pair-wise comparisons with *B2M*<sup>+/+</sup> cells after applying the *post hoc* Tukey HSD test.  $*P < 0.05$  or  $**P < 0.01$ . **(f)** Chromium release assays performed as in **e** in the presence of neutralizing antibodies against either HLA-E or NKG2A at a NK/CD45<sup>+</sup> cell ratio of 10:1 (mean  $\pm$  s.d.,  $n = 3$ ).  $P$  values were calculated using the one-way ANOVA followed by Tukey HSD test; asterisks indicate statistical significant differences in regard to no antibody controls. **(g)** Luciferase imaging of five representative mice containing ESC-derived CD45<sup>+</sup> cells of *B2M*<sup>-/-Edimer(pre-Cre)</sup> (serves as class-I-negative control) or *B2M*<sup>-/-Etrimer</sup> genotypes, some of which also received NK-92 cells (- or + labels in the right panel). Results are shown for the same mice before NK-92 injection (day 1) and 1 d afterwards (day 2). **(h)** Quantification of luciferase-expressing CD45<sup>+</sup> cell levels in mice treated as in **g**. The change in luminescence between day 1 (pre NK-92 cell injection) and day 2 or day 4 (1 or 3 d after NK-92 cell injection) was measured for individual mice and divided by the average of control mice that did not receive NK-92 cells ( $n = 3$  for both *B2M*<sup>-/-Etrimer</sup> and *B2M*<sup>-/-Edimer(pre-Cre)</sup> control groups). Horizontal black bars indicate the mean for each group.  $P$  values were calculated using the unpaired Student's  $t$  test. For all panels,  $**P < 0.01$  and  $*P < 0.05$ .



**Figure 4** Allogeneic CD8<sup>+</sup> T cells do not recognize HLA-engineered cells. **(a)** Schema for priming allogeneic CD8<sup>+</sup> T cells against E1f-1 HLA antigens. **(b)** Chromium release assays measuring the cytotoxicity of allogeneic CD8<sup>+</sup> T cells toward ESC-derived hematopoietic cells with the indicated *B2M* genotypes (mean  $\pm$  s.d.,  $n = 3$ ;  $P$ -values determined by one-way ANOVA were  $<0.001$  and by HSD Tukey test  $P < 0.01$ , when comparing all the HLA-engineered lines to *B2M*<sup>+/+</sup> cells at all cell ratios). For representation purposes, negative specific lysis values were plotted as zero. **(c)** Analogous chromium release assays toward ESC-derived RPE cells with the indicated *B2M* genotypes (mean  $\pm$  s.d.,  $n = 3$  for each cell ratio,  $P$ -values determined by unpaired Student's  $t$ -test,  $*P < 0.05$  and  $**P < 0.01$ ). **(d)** Representative examples of teratoma and CD8<sup>+</sup> cell imaging in live animals. **(e)** Fold change of luciferase expression by *B2M*<sup>-/-Etrimer</sup> and *B2M*<sup>+/+</sup> teratomas from day 1 of CD8<sup>+</sup> cell infusion to endpoint (day 12,  $n = 4$  or day 16,  $n = 2$ ), with each line representing one mouse.  $P$ -value was determined by paired Student's  $t$ -test ( $n = 6$  mice). **(f)** Same as **e** but luciferase measurements of teratomas were made in control mice that did not receive CD8<sup>+</sup> T cells ( $n = 6$  mice per group). **(g)** Infiltration of teratomas with allogeneic CD8<sup>+</sup> T cells as measured by fluorescent signal divided by weight of teratomas harvested at the time of death ( $n = 4$  mice,  $P$ -value determined by paired Student's  $t$ -test). **(h)** Representative hematoxylin and eosin staining of a *B2M*<sup>+/+</sup> teratoma harvested from mice that received allogeneic CD8<sup>+</sup> T cells. Top images: necrotic area with abundant polymorphonuclear cells is indicated in red. The two bottom photos show immunohistochemistry staining of CD8<sup>+</sup> T cells infiltrating *B2M*<sup>+/+</sup> teratomas.

that indirect HLA class I antigen presentation as modeled by priming CD8<sup>+</sup> T cells with WT embryoid bodies does not stimulate responses against *B2M*<sup>-/-Edimer</sup> and *B2M*<sup>-/-Etrimer</sup> cells, which contain intracellular polymorphic heavy chains.

*In vivo* responses were examined by growing luciferase-expressing *B2M*<sup>+/+</sup> and *B2M*<sup>-/-Etrimer</sup> ESC-derived teratomas in immunodeficient mice, and transplanting primed allogeneic CD8<sup>+</sup> T cells labeled with VivoTrack 680 (examples in **Fig. 4d**). Serial luciferase measurements showed that *B2M*<sup>-/-Etrimer</sup> teratomas grew more than their *B2M*<sup>+/+</sup> counterparts in five of six mice after CD8<sup>+</sup> cell infusion (**Fig. 4e** and **Supplementary Fig. 6a**), but this did not occur in the absence of CD8<sup>+</sup> cells (**Fig. 4f**). Similar results were obtained in separate experiments with three different CD8<sup>+</sup> T cell donors (**Supplementary Fig. 6b**). CD8<sup>+</sup> cells also preferentially localized to *B2M*<sup>+/+</sup> teratomas as measured by *in vivo* tracking (**Fig. 4g**). Histological examination demonstrated multiple areas of necrosis in the *B2M*<sup>+/+</sup> teratomas as well as the presence of human CD8<sup>+</sup> cells at higher levels than found in *B2M*<sup>-/-Etrimer</sup> teratomas (**Fig. 4h** and **Supplementary Fig. 6c**).

We also examined possible B-cell responses against ESC-derived CD45<sup>+</sup> cells by performing a complement-dependent cytotoxicity (CDC) assay with human serum samples containing defined anti-HLA antibodies (**Table 2**). Cytotoxicity was detected when *B2M*<sup>+/+</sup> cells were exposed to 14 of 16 sera samples with antibodies against E1f-1 HLA-A or B alleles (A\*11, A\*24, B\*35 and B\*55), but not after exposure to 16 samples with antibodies against other HLA-A or B alleles, demonstrating the accuracy of the assay. No cytotoxicity was observed for *B2M*<sup>-/-Edimer(pre-Cre)</sup>, *B2M*<sup>-/-Edimer</sup> or *B2M*<sup>-/-Etrimer</sup> cells, except for one single sample reacting against *B2M*<sup>-/-Etrimer</sup> c6, which could reflect contaminating antibodies against non-HLA class I antigens, including possible class II alleles that may be expressed in some CD45<sup>+</sup> cells.

## DISCUSSION

In this report we show that HLA-E single-chain dimer and trimer expression in human cells lacking polymorphic HLA class I surface molecules can prevent the missing-self response. Most human NK cells express NKG2A<sup>18</sup>, an inhibitory receptor that binds HLA-E, and this interaction was enough to reduce NK-mediated lysis of CD45<sup>+</sup> cells that uniquely expressed HLA-E to a level below that of WT cells, and to protect cells from NK-92 cell-mediated lysis *in vivo*. This level of host NK cell population inhibition may also be adequate to protect transplanted cells in clinical trials, given that cumulative NK signaling can dictate the outcome<sup>28</sup>, and in mice NK cells can be tolerized to *B2m*<sup>-/-</sup> cells over time<sup>29</sup>. If necessary, the subset of host NK cells that are NKG2A-negative could be inhibited with additional gene editing steps, such as expressing single-chain HLA-G proteins<sup>13,30</sup> and/or other class I molecules targeting inhibitory KIR<sup>31</sup> or other NK receptors<sup>25</sup>, especially in recipients with fewer NKG2A<sup>+</sup> NK cells.

We were surprised that *B2M*<sup>-/-</sup> human ESCs completely lacking surface HLA class I proteins grew poorly and failed to differentiate robustly into CD45<sup>+</sup> cells, given the relative health of *B2m*<sup>-/-</sup> mice<sup>32,33</sup>. Other groups have described *B2M* knockouts in human cells<sup>7-9,34</sup>, with at least one report showing that the editing frequencies were lower than expected in normal T cells as compared to HEK293 cells<sup>34</sup>. These findings suggest that *B2M*-edited cells should be carefully screened for low level or inducible class I surface expression and/or compensatory mutations. The two independent *B2M*<sup>-/-</sup> lines that we eventually isolated by repeated subcloning had common cytogenetic abnormalities (tetraploidy, add20(q13), -15 and -22) that may have improved their survival. While the basis for the poor growth of *B2M*<sup>-/-</sup> human cells is not understood, it is not a feature of cells that express HLA-E as their sole class I surface

**Table 2 Human anti-HLA antibodies do not recognize HLA-E-expressing cells**

Serum anti-HLA type	<i>B2M</i> genotype of ESC-derived CD45 <sup>+</sup> cells				
	+/+	-/Edimer (pre-Cre)	-/Edimer	-/Etrimer c5	-/Etrimer c6
Positive control	++++	-	-	-	-
A1	-	-	-	-	-
A1, 9, 11, 24, 25, 26, 36, 66, 6802	++++	-	-	-	-
A1, 11, 26, 34, 36, 6601	+++	-	-	-	-
A1, 11, 36, 80	-	-	-	-	-
A2	-	-	-	-	-
A2, 68, 69	-	-	-	-	-
A3	-	-	-	-	-
A10, 28, 33	-	-	-	-	-
A11	++	-	-	-	-
A11, 66	+	-	-	-	-
A23	-	-	-	-	-
A23, 24	++++	-	-	-	-
A24	++++	-	-	-	-
A68, 69	-	-	-	-	-
B5, 18, 35, 49, 53	++++	-	-	-	-
B7	-	-	-	-	-
B7, 13, 41, 48, 60, 61	-	-	-	-	-
B7, 27, 42, 67	-	-	-	-	-
B7, 42	-	-	-	-	-
B7, 42, 55, 56, 67	++++	-	-	-	-
B7, 48	-	-	-	-	-
B12, 35, 50, 62, 70, 75, 76, 77	-	-	-	-	-
B13, 27, 47, 60, 61	-	-	-	-	-
B35	++++	-	-	-	++
B35, 53	++	-	-	-	-
B35, 49, 50, 53, 62, 70, 75, 77	++++	-	-	-	-
B35, 75	+++	-	-	-	-
B41, 45, 50	-	-	-	-	-
B54, 55, 56	++++	-	-	-	-
B55	+++	-	-	-	-
B60	-	-	-	-	-
B60	-/+	-	-	-	-

protein, which grew normally and produced hematopoietic cells and RPE cells as efficiently as WT cells.

*B2M*<sup>-/Edimer</sup> and *B2M*<sup>-/Etrimer</sup> ESCs and their differentiated derivatives were not lysed by CD8<sup>+</sup> T cells, and they were not recognized by human anti-HLA antibodies. Thus these cells were not only resistant to NK-mediated lysis, but they were not recognized as allogeneic, and their expression of single-chain HLA-E molecules was not immunogenic. These *in vitro* findings were confirmed by *in vivo* studies showing that allogeneic CD8<sup>+</sup> cells invaded *B2M*<sup>+/+</sup> teratomas more than *B2M*<sup>-/Etrimer</sup> teratomas, reducing their growth rate and producing necrosis. Our results support the use of HLA-E-expressing PSCs as a source of first-generation, universal donor cells, at least in applications where the transplanted cell type does not express HLA class II molecules. Additional engineering steps could be used to prevent class II expression, such as knocking out the genes responsible for bare lymphocyte syndrome<sup>35</sup>. While HLA-engineering should minimize the rejection of many types of PSC-derived cell products, especially relevant clinical applications include hematopoietic cell transplantation where HLA mismatches profoundly affect engraftment, and autoimmune or genetic diseases where the presentation of autoantigens and neoantigens would otherwise cause rejection.

Reduced HLA expression also raises specific safety concerns. Class I-deficient mice and humans are not tumor-prone<sup>36,37</sup>, but class I peptide presentation may contribute toward the elimination of malignant cells formed by other mechanisms, and it plays a role in clearing infections. The HLA-E dimer molecule could provide some protection in these settings, since HLA-E can present pathogen-specific peptides that are recognized by cytotoxic T cells<sup>38,39</sup>, and possibly tumor-specific peptides as well<sup>40</sup>. HLA-independent mechanisms

should also limit infections based on the partial immunity of class I-deficient humans<sup>37</sup> and mice<sup>41–43</sup>. In addition, many regenerative medicine applications will involve the treatment of recipients with normal immune systems, delivery to internal sites sequestered from pathogens and the transplantation of terminally differentiated and possibly irradiated PSC-derived cells that are resistant to transformation. Ultimately, the safety of these cell products may be enhanced by the inclusion of suicide genes such as the TK gene used in our editing steps, and the extensive pre-clinical testing that the use of a single universal donor cell line allows, which may not be practical when characterizing cellular products derived from multiple autologous induced PSCs or HLA-typed PSC banks.

## METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the [online version of the paper](#).

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper*

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#### AUTHOR CONTRIBUTIONS

G.G.G. and D.W.R. wrote the manuscript. G.G.G. and D.W.R. designed the experiments. G.G.G. and R.K.H. prepared AAV vectors stocks and performed gene editing. G.G.G. performed flow cytometry, hematopoietic differentiation, chromium release assays, CDC assays and data analysis. S.E.F. conducted *in vivo* experiments, PCR analysis and Southern blots. D.P. and R.K.H. performed flow sorting experiments. G.G.G., S.E.F. and G.M. did molecular cloning. G.M. performed RNA analysis. L.R. designed both the HLA-E dimer and trimer open reading frames, conducted preliminary studies with HLA-E vectors and optimized chromium release assay protocols. V.S.L., D.O.C. and G.G.G. performed RPE differentiation experiments and immunocytochemistry. L.-A.H. and C.T. did flow cytometry to detect costimulatory receptors. A.G.C. and L.R. each designed and built one of the AAV vectors. All authors discussed the results and commented on the manuscript.

#### COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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- Taylor, C.J., Peacock, S., Chaudhry, A.N., Bradley, J.A. & Bolton, E.M. Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient HLA types. *Cell Stem Cell* **11**, 147–152 (2012).
- Nishikawa, S., Goldstein, R.A. & Nierras, C.R. The promise of human induced pluripotent stem cells for research and therapy. *Nat. Rev. Mol. Cell Biol.* **9**, 725–729 (2008).
- Taylor, C.J. *et al.* Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. *Lancet* **366**, 2019–2025 (2005).
- Braciale, T.J. Antigen processing for presentation by MHC class I molecules. *Curr. Opin. Immunol.* **4**, 59–62 (1992).
- Arce-Gomez, B., Jones, E.A., Barnstable, C.J., Solomon, E. & Bodmer, W.F. The genetic control of HLA-A and B antigens in somatic cell hybrids: requirement for beta2 microglobulin. *Tissue Antigens* **11**, 96–112 (1978).
- Riolobos, L. *et al.* HLA engineering of human pluripotent stem cells. *Mol. Ther.* **21**, 1232–1241 (2013).
- Lu, P. *et al.* Generating hypoinmunogenic human embryonic stem cells by the disruption of beta 2-microglobulin. *Stem Cell Rev.* **9**, 806–813 (2013).
- Wang, D., Quan, Y., Yan, Q., Morales, J.E. & Wetsel, R.A. Targeted disruption of the  $\beta$ 2-microglobulin gene minimizes the immunogenicity of human embryonic stem cells. *Stem Cells Transl. Med.* **4**, 1234–1245 (2015).
- Feng, Q. *et al.* Scalable generation of universal platelets from human induced pluripotent stem cells. *Stem Cell Reports* **3**, 817–831 (2014).
- Bix, M. *et al.* Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature* **349**, 329–331 (1991).
- Liao, N.S., Bix, M., Zijlstra, M., Jaenisch, R. & Raulet, D. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* **253**, 199–202 (1991).
- Zarcone, D., Tilden, A.B., Friedman, H.M. & Grossi, C.E. Human leukemia-derived cell lines and clones as models for mechanistic analysis of natural killer cell-mediated cytotoxicity. *Cancer Res.* **47**, 2674–2682 (1987).
- Pazmany, L. *et al.* Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. *Science* **274**, 792–795 (1996).
- Braud, V.M. *et al.* HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391**, 795–799 (1998).
- Lee, N. *et al.* HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl. Acad. Sci. USA* **95**, 5199–5204 (1998).
- Lilienfeld, B.G., Crew, M.D., Forte, P., Baumann, B.C. & Seebach, J.D. Transgenic expression of HLA-E single chain trimer protects porcine endothelial cells against human natural killer cell-mediated cytotoxicity. *Xenotransplantation* **14**, 126–134 (2007).
- Miller, J.D. *et al.* Analysis of HLA-E peptide-binding specificity and contact residues in bound peptide required for recognition by CD94/NKG2. *J. Immunol.* **171**, 1369–1375 (2003).
- Horowitz, A. *et al.* Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci. Transl. Med.* **5**, 208ra145 (2013).
- Russell, D.W. & Hirata, R.K. Human gene targeting by viral vectors. *Nat. Genet.* **18**, 325–330 (1998).
- Khan, I.F., Hirata, R.K. & Russell, D.W. AAV-mediated gene targeting methods for human cells. *Nat. Protoc.* **6**, 482–501 (2011).
- Chamberlain, J.R. *et al.* Gene targeting in stem cells from individuals with osteogenesis imperfecta. *Science* **303**, 1198–1201 (2004).
- Li, L.B. *et al.* Trisomy correction in Down syndrome induced pluripotent stem cells. *Cell Stem Cell* **11**, 615–619 (2012).
- Khan, I.F. *et al.* Engineering of human pluripotent stem cells by AAV-mediated gene targeting. *Mol. Ther.* **18**, 1192–1199 (2010).
- Ichiryu, N. & Fairchild, P.J. Immune privilege of stem cells. *Methods Mol. Biol.* **1029**, 1–16 (2013).
- Pegram, H.J., Andrews, D.M., Smyth, M.J., Darcy, P.K. & Kershaw, M.H. Activating and inhibitory receptors of natural killer cells. *Immunol. Cell Biol.* **89**, 216–224 (2011).
- Gong, J.H., Maki, G. & Klingemann, H.G. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia* **8**, 652–658 (1994).
- Brooks, A.G., Posch, P.E., Scorzelli, C.J., Borrego, F. & Coligan, J.E. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J. Exp. Med.* **185**, 795–800 (1997).
- Brodin, P., Kärre, K. & Höglund, P. NK cell education: not an on-off switch but a tunable rheostat. *Trends Immunol.* **30**, 143–149 (2009).
- Huang, Y. *et al.* NK cells play a critical role in the regulation of class I-deficient hemopoietic stem cell engraftment: evidence for NK tolerance correlates with receptor editing. *J. Immunol.* **175**, 3753–3761 (2005).
- Rouas-Freiss, N., Gonçalves, R.M., Menier, C., Dausset, J. & Carosella, E.D. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytotoxicity. *Proc. Natl. Acad. Sci. USA* **94**, 11520–11525 (1997).
- Zappacosta, F., Borrego, F., Brooks, A.G., Parker, K.C. & Coligan, J.E. Peptides isolated from HLA-Cw\*0304 confer different degrees of protection from natural killer cell-mediated lysis. *Proc. Natl. Acad. Sci. USA* **94**, 6313–6318 (1997).
- Koller, B.H., Marrack, P., Kappler, J.W. & Smithies, O. Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8<sup>+</sup> T cells. *Science* **248**, 1227–1230 (1990).
- Zijlstra, M. *et al.* Beta 2-microglobulin deficient mice lack CD4-8<sup>+</sup> cytolytic T cells. *Nature* **344**, 742–746 (1990).
- Mandal, P.K. *et al.* Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. *Cell Stem Cell* **15**, 643–652 (2014).
- DeSandro, A., Nagarajan, U.M. & Boss, J.M. The bare lymphocyte syndrome: molecular clues to the transcriptional regulation of major histocompatibility complex class II genes. *Am. J. Hum. Genet.* **65**, 279–286 (1999).
- Street, S.E.A. *et al.* Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. *J. Exp. Med.* **199**, 879–884 (2004).
- Gadola, S.D., Moins-Teisserenc, H.T., Trowsdale, J., Gross, W.L. & Cerundolo, V. TAP deficiency syndrome. *Clin. Exp. Immunol.* **121**, 173–178 (2000).
- Pietra, G. *et al.* HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8<sup>+</sup> cytolytic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **100**, 10896–10901 (2003).
- Hansen, S.G. *et al.* Broadly targeted CD8<sup>+</sup> T cell responses restricted by major histocompatibility complex E. *Science* **351**, 714–720 (2016).
- Pietra, G., Romagnani, C., Manzini, C., Moretta, L. & Mingari, M.C. The emerging role of HLA-E-restricted CD8<sup>+</sup> T lymphocytes in the adaptive immune response to pathogens and tumors. *J. Biomed. Biotechnol.* **2010**, 907092 (2010).
- Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. & Doherty, P.C. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8<sup>+</sup> T cells. *J. Exp. Med.* **174**, 875–880 (1991).
- Hou, S., Doherty, P.C., Zijlstra, M., Jaenisch, R. & Katz, J.M. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8<sup>+</sup> T cells. *J. Immunol.* **149**, 1319–1325 (1992).
- Spriggs, M.K. *et al.* Beta 2-microglobulin-, CD8<sup>+</sup> T-cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses. *Proc. Natl. Acad. Sci. USA* **89**, 6070–6074 (1992).

## ONLINE METHODS

**Cell culture.** Human Elf-1 ESCs<sup>44</sup> were obtained from the University of Washington. H1 and H9 ESCs<sup>45</sup> were obtained from the WiCell Research Institute. Human Elf-1, H1 and H9 ESCs were cultured as described<sup>23,44</sup> and passaged every 5–6 d. All these ESC lines tested negative for mycoplasma contamination. For RPE differentiation, cells were adapted and maintained in 6-well plates coated with Matrigel (BD Biosciences, San Diego, CA, USA) in mTeSR1 media (Stem Cell Technologies, Vancouver, BC, Canada). Karyotyping of ESC lines was performed by the Fred Hutchinson Cancer Research Center (FHCRC) cytogenetics facility. Embryoid bodies were generated by dispase dissociation of ESC cultures, plating  $5 \times 10^6$  ESCs/per well of an ultra-low attachment 6-well plate containing X-VIVO 15 medium (Lonza) supplemented with 1 mM sodium pyruvate, 1 mM each non-essential amino acid, 50  $\mu$ M 2-mercaptoethanol and 2 mM L-glutamate. Medium was changed every 3 d and cultures were maintained for 15–20 d. Hematopoietic differentiation of ESCs was performed as described<sup>46</sup>, with an additional step of plating on 0.1% gelatin-coated dishes on day 20. Suspension culture cells were collected from day 24 onward for flow cytometry and functional studies. RPE differentiation was performed as described<sup>47</sup>. Normal human CD56<sup>+</sup> NK cells and CD8<sup>+</sup> T cells were isolated by positive enrichment (Miltenyi CliniMACS system) of blood cells collected by apheresis from healthy human donors (FHCRC Hematopoietic Cell Processing and Repository Core Facility). NK-92 cells (CRL-2407) were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described in the product sheet. G418 sulfate (Geneticin) was purchased from Thermo Fischer.

**Viral vectors and genome editing.** All the AAV vectors used were serotype 3B and prepared as described<sup>20</sup>. AAV-B2M-EHyTKpA and AAV-B2M-ETKNpA have been described<sup>6</sup>. AAV-B2M-HyTK is similar to AAV-B2M-EHyTKpA and AAV-B2M-TKN is similar to AAV-B2M-ETKNpA, except they contain additional sequences with stop codons upstream of the 3' homology arm. AAV-B2M-TKwN is the same as AAV-B2M-TKN but with the TK reading frame wobbled to reduce homology. AAV-B2M-Edimer homology arms span B2M exon 3 (chr15:44,715,280–44,717,402 GRCh38/hg38 human assembly) with a (G<sub>4</sub>S)<sub>4</sub> flexible linker coding sequence, HLA-E 01:03 heavy-chain coding sequence and floxed EF1a promoter-Neo-pA cassette inserted at the terminal codon of B2M (chr15:44,716,339). AAV-B2M-Etrimer homology arms span B2M exon 1 (chr15:44,711,007–44,712,332), with coding sequences for the wobbled B2M signal peptide, HLA-G signal peptide VMAPRTLFL, (G<sub>4</sub>S)<sub>3</sub> flexible linker, wobbled B2M mature protein, (G<sub>4</sub>S)<sub>4</sub> flexible linker and HLA-E 01:03 heavy chain followed by a floxed EF1a promoter-Neo-pA cassette. These AAV vectors were used to infect ESCs, and drug-resistant colonies were picked and expanded as described<sup>6,20,23</sup>. The non-integrating foamy virus vector NIFV-EokCreW was used to express Cre and remove floxed transgenes as described<sup>6</sup>. Vector sequences are available upon request.

**DNA isolation and analysis.** Genomic DNA was isolated by using the Puregene DNA purification system (Gentra Systems, Minneapolis, MN). Southern blot analysis, plasmid preparation and restriction digestion were performed according to standard protocols<sup>48</sup>. Radiolabeled probes were synthesized by random priming using Rediprime II (GE Healthcare, Piscataway, NJ). The genomic coordinates for the 5' and 3' B2M probes were chr15:44,709,549–44,710,044 and chr15:44,711,550–44,712,205, respectively. For PCR analysis, cells were lysed in colony-PCR lysis buffer that consisted of 105 mM KCl, 14 mM Tris-HCl, pH 8.3, 2.5 mM MgCl<sub>2</sub>, 0.3 mg/ml gelatin, 0.45% (vol/vol) NP-40, 0.45% (vol/vol) Tween 20 and Proteinase K (100  $\mu$ g/ml), incubated at 55 °C for 1 h and heated at 95 °C for 15 min to inactivate Proteinase K<sup>49</sup>. The lysate was clarified by centrifugation on a Mini Strip Spin Microcentrifuge (Thomas Scientific) for 5 min and the supernatant was used as a PCR template. The primers and melting temperatures used for screening edited clones by PCR are described in **Supplementary Table 1**. PCR was performed under the following conditions: 1 $\times$  Green GoTaq Flexi buffer, 0.75 units GoTaq polymerase (Promega, Madison, WI), 5  $\mu$ l cell lysate (~150ng genomic DNA), 0.5  $\mu$ M each primer, 0.25 mM each dNTP and 2 mM MgCl<sub>2</sub> in a 25  $\mu$ l volume for 40 cycles in a PTC-200 Thermo Cycler (Biorad, MJ Research, Hercules, CA).

HLA-E genotypes were determined by sequence-specific PCR (PCR-SSP)<sup>50</sup> with two different 5' primers and a common 3' primer to distinguish the

non-synonymous difference of HLA-E\*01:01 and HLA-E\*01:03 at amino acid position 107. HLA-A,B,C typing was performed by the Immunogenetics/HLA Laboratory at the Bloodworks Northwest facility.

**Flow cytometry.** ESCs were harvested with trypsin for 3 min at 37 °C and single-cell suspensions were obtained by passing the suspension through a 70- $\mu$ m cell strainer. The subsequent staining and washes were performed in phosphate-buffered saline containing 1% bovine serum albumin. A 98- $\mu$ l suspension of ESCs was incubated with 2  $\mu$ l FcR blocking reagent (Miltenyi Biotec, Auburn, CA) for 10 min on ice and then stained with antibodies for 30–60 min on ice. 7-amino-actinomycin D (BD Biosciences, Bedford, MA) was added to identify dead cells. Single suspension cells derived from embryoid bodies were analyzed the same way. Samples were analyzed on a LSRII flow cytometer (BD Biosciences) and the data were plotted using FlowJo software (TreeStar, Ashland, OR). The antibodies used were: anti-HLA-E (clone 3D12HLA-E), anti-HLA-BC (clone B1.23.2), anti-HLA-ABC (clone W6/32), isotype controls mouse IgG1  $\kappa$  and mouse IgG2b, $\kappa$  (eBioscience); anti-HLA Class I A11 (4i93, Abcam); IgG( $\gamma$ ) goat anti-mouse (Invitrogen); anti-HLA-DR (clone AC122, MACS Miltenyi Biotec); anti-B2M (clone B2M-01) and isotype control mouse IgG2a, $\kappa$  (Santa Cruz Biotechnology); anti-NKG2A (clone Z199, Beckman Coulter); anti-NKG2C (clone 134591) and isotype control mouse IgG1 (R&D); anti-CD45 (clone HI30), anti-CD40 (clone 5C3), anti-CD54 (clone HA58), anti-CD80 (clone L307.4), anti-CD86 (clone 2331), anti-CD83 (clone HB15e), anti-CD275 (clone 2D3) and their corresponding isotype controls (BD Biosciences).

When sorting cells infected with AAV-B2M-Etrimer, ESCs were treated for 72 h with 25 ng/ml IFN- $\gamma$ , harvested by digestion with trypsin (3 min at 37 °C) and passed through a 70- $\mu$ m cell strainer. Single cells ( $4 \times 10^6$  in 0.1 ml) were preincubated with FcR blocking reagent and stained with 6  $\mu$ g of the pan-HLA-class I antibody (W6/32, eBioscience). HLA class-I-negative cells were sorted on a BD Aria III cytometer. 9,300 class-I-negative cells were collected, plated onto 10-cm dishes and individual colonies were picked for PCR and Southern blot analysis. 4 of 12 colonies analyzed were edited.

**Immunocytochemistry.** After 14 d of RPE differentiation, cells were plated on 8-chamber glass slides coated with Matrigel and kept for an additional 28 d in XVIVO-10 media (Lonza, Basel, Switzerland). The cells were washed with PBS, followed by permeabilization and treatment with blocking buffer (0.5% BSA, 0.05% saponin in PBS) for 45 min. Primary antibodies were prepared in blocking buffer and incubated with the cells for 1 h at room temperature. After washing with PBS, the cells were incubated with goat anti-mouse Alexa Fluor-488 secondary antibodies (Thermo Fisher Scientific, Carlsbad, CA, USA) for 45 min at room temperature. The glass slide was mounted with Fluoro-Gel II with DAPI (EMS, Hatfield, PA, USA) and images were taken on an Olympus BX51 upright microscope. The primary antibodies used in this study were: mouse anti-PMEL (Abcam, Cambridge, MA, USA; 1:100) and mouse anti-MITF (Millipore, Billerica, MA, USA; 1:100).

**Chromium release assays with NK cells.** Hematopoietic progenitors were evaluated for their susceptibility to NK-cell-mediated lysis by 4 h <sup>51</sup>Cr release assay (adapted from<sup>51</sup>). 48 h before the assay, NK cells were thawed and cultured in NK medium consisting of RPMI 1640-HEPES with 10% heat-inactivated human AB serum (Valley Biomedical), 400 U/ml IL-2 (100 U/ml IL-2 in the experiment shown in **Supplementary Fig. 4f**), 2 mM L-glutamate, 1 mM sodium pyruvate, 50  $\mu$ M 2-mercaptoethanol, 50 U/ml penicillin and 50  $\mu$ g/ml streptomycin. Hematopoietic cells were treated with 25 ng/ml IFN- $\gamma$  for 72 h, washed and  $2-5 \times 10^5$  cells were resuspended in 200  $\mu$ l hematopoietic medium (StemPro34 SFM by Life Technologies, supplemented with 1 mM each non-essential amino acid, 2 mM L-glutamate, 55  $\mu$ M 2-mercaptoethanol and 50  $\mu$ g/ml ascorbic acid) and labeled with 50  $\mu$ Ci of <sup>51</sup>Cr (PerkinElmer) for 2 h at 37 °C, followed by three washes with NK medium lacking IL-2 or IFN- $\gamma$ . Ten<sup>4,51</sup>Cr-labeled cells were plated per well of a 96-U-bottom plate (Corning Costar), NK cells were added at different ratios to target cells, and incubated for 4 h at 37 °C. The plate was spun at 1,000 r.p.m. for 5 min before and after the coculture. Controls included labeled cells without NK cells (spontaneous release) and labeled cells lysed with 1% Triton X-100 (total lysis). 100  $\mu$ l of each reaction supernatant was mixed with 1 ml liquid scintillation cocktail (Ultima

Gold, PerkinElmer), counted on the LS6500 counter (Beckman, Indianapolis) and the percent specific lysis was calculated as (sample – average spontaneous release)/(average total lysis – average spontaneous release)\*100. Samples were done in triplicate.

In experiments testing neutralizing antibodies,  $^{51}\text{Cr}$ -labeled hematopoietic cells or NK cells were preincubated with 10  $\mu\text{g}/\text{ml}$  anti-human HLA-E (clone 3D12HLA-E, eBioscience) or 5  $\mu\text{g}/\text{ml}$  anti-NKG2A (clone Z199, Beckman), respectively, for 30 min at room temperature before performing the cocultures.

**Cytotoxicity assays with CD8<sup>+</sup> T cells.** Normal human CD8<sup>+</sup> T cells were primed by coculturing with single cells derived from IFN- $\gamma$  treated WT Elf-1 ESC-derived embryoid bodies ( $2 \times 10^6$  CD8<sup>+</sup> cells and  $5 \times 10^5$  embryoid body cells/2 ml) in RPMI 1640-HEPES supplemented with 10% human AB serum (Valley Biomedical), 50 U/ml IL-2, 25 ng/ml IFN- $\gamma$ , 1 mM sodium pyruvate, 2 mM L-glutamate, 50  $\mu\text{M}$  2-mercaptoethanol, 50 U/ml penicillin and 50  $\mu\text{g}/\text{ml}$  streptomycin. On day 7, cocultures were replenished with  $1.75 \times 10^6$  fresh single cells from IFN- $\gamma$  treated embryoid bodies (final CD8<sup>+</sup>/embryoid body cell ratio was 1). On day 14, these primed CD8<sup>+</sup> T-cells were collected by centrifugation and used in chromium release assays as described above for NK cells. Costimulatory molecule expression on both the embryoid body cells used for priming and the ESC-derived hematopoietic cells used for lysis was determined by flow cytometry. CD8<sup>+</sup> T cells primed by the same method were also used for *in vivo* studies. Chromium release assays were performed on RPE cultures by adding primed CD8<sup>+</sup> T cells to IFN- $\gamma$ -treated adherent RPE cultures on day 171 of differentiation after labeling with 20  $\mu\text{Ci}/\text{well}$  of  $^{51}\text{Cr}$  (PerkinElmer) overnight at 37 °C. The exact number of RPEs was determined by trypsin treatment of control wells that were not  $^{51}\text{Cr}$ -labeled. The CD8<sup>+</sup> T cell/RPE cocultures were incubated overnight at 37 °C and the chromium release was measured the next day, as described above.

**Recognition by anti-HLA antibodies.** Complement-dependent cytotoxicity (CDC) assays were performed to determine if human anti-HLA antibodies recognize ESC-derived hematopoietic cells. Assay plates were prepared by adding 1  $\mu\text{l}$  of a specific human serum containing previously characterized anti-HLA antibodies (or control serum) and 5  $\mu\text{l}$  of light mineral oil per well. IFN- $\gamma$ -treated ESC-derived CD45<sup>+</sup> cells isolated on day 32 of differentiation were resuspended in RPMI 1640 medium with 15% FBS at a concentration of  $1.5 \times 10^6$  cells/ml, and 1  $\mu\text{l}$  of each cell suspension was added per well. The reactions were incubated 30 min at room temperature (RT) and 5  $\mu\text{l}$  of rabbit serum (Immucor GTI Diagnostics, Inc.) was added as a source of complement per well. After 1 h of incubation at RT, 5  $\mu\text{l}$  of acridine orange/ethidium bromide solution (14.3  $\mu\text{g}/\text{ml}$  acridine orange, 47.6  $\mu\text{g}/\text{ml}$  ethidium bromide, 2.4% v/v India ink in Hanks Balanced Salt Solution) was added per well. Percent cytotoxicity was determined by counting dead and live cells visualized on a fluorescent microscope, subtracting spontaneous lysis values obtained in the absence of anti-HLA antibodies and scoring with the semiquantitative scale described by Terasaki *et al.*<sup>52</sup>. Each serum sample was derived from a single individual. Sera were obtained from multiparous blood donors or extracted from placentas post-partum. The positive control serum was generated as a pool of sera from 30 high 'panel reactive antibody' individuals. The panel was tested and prepared by Bloodworks Northwest (Seattle, WA).

***In vivo* reactivity with allogeneic CD8<sup>+</sup> T cells and NK-92 cells.** All animal experiments were approved by the University of Washington's Institutional Animal Care and Use Committee. "NSG-B2m" mice (NOD.Cg-B2m<sup>tm1UncPrkd<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ</sup>) were purchased from the Jackson Laboratory. ESCs were stably transduced with the luciferase-expressing Red-FLuc-Puromycin lentivirus vector (CLS96000, PerkinElmer) as per the manufacturer's protocol, and puromycin-resistant polyclonal populations were used to produce teratomas. Implants containing  $\sim 2 \times 10^6$  luciferase-expressing ESCs were made by suspending ESCs in a collagen gel in 0.5 cm-diameter Merocel polyvinyl alcohol sponges (Medtronic, Inc., Mystic, CT) as described<sup>53</sup>. Implants were cultured overnight at 37 °C, and on the next day they were inserted subdermally into NSG-B2m mice where they formed tri-lineage teratomas. Recipients were 3- to 4-month-old females. Randomization or blinding of recipients was not used. No recipients were excluded. Experimental group sizes were based on pilot studies.

Allogeneic human CD8<sup>+</sup> T cells primed against B2M<sup>+/+</sup> Elf-1 embryoid body cells (described above) were labeled with VivoTrack 680 (NEV12001, PerkinElmer) as per the manufacturer's instructions. Cell labeling was >99% by flow cytometry (data not shown). On day 35 after implant placement,  $8.5 \times 10^5$  labeled CD8<sup>+</sup> T cells were injected intravenously into each mouse via the retro-orbital sinus. Control mice containing implants did not receive labeled CD8<sup>+</sup> cells. The experiments in **Supplementary Figure 6b,c** were performed with CD8<sup>+</sup> T cells from three different donors (non-HLA-typed) that were not labeled with VivoTrack 680. Teratoma size was measured weekly after implant placement by intra-peritoneal injection of 150 mg/kg sterile-filtered D-luciferin substrate in DPBS (Cat. #122799, PerkinElmer) and recording the peak bioluminescence in a Lumina II IVIS imaging system (Caliper Life Sciences). *In vivo* tracking of labeled CD8<sup>+</sup> T cells was done by recording fluorescence at an excitation wavelength of 675 nm and emission filter of 690–770 nm. Accurate quantitative measurement of CD8<sup>+</sup> T cells was difficult in live mice due to background signal from the skin and hair, so CD8<sup>+</sup> cell quantitation was determined by imaging harvested teratomas after euthanization and normalizing for teratoma weights. In all cases, data analysis was done using the Living Image software v. 4.2 (Caliper Life Sciences). Harvested teratomas were also analyzed by hematoxylin and eosin (H&E) staining of paraffin-embedded sections and by anti-human CD8 antibody staining of frozen sections (cat no. NCL-L-CD8-295, Leica Biosystems) using the Leica Bond Polymer DAB system, with hematoxylin blue counterstain. Histological procedures were conducted in the Histology and Imaging Core/Comparative Pathology Program and the Pathology Research Services Laboratory at the University of Washington. CD8<sup>+</sup> T cell infiltration in histological sections was calculated with ImageJ software (version 1.50i) as a % of area with CD8<sup>+</sup> cell color signal. Data were obtained from at least 12 separate non-overlapping fields of the same cross-sectional area for each teratoma genotype ( $n = 5$  teratomas with each genotype) and are presented as an average for each mouse.

Teratoma studies with NK-92 cells (**Supplementary Fig. 4g,h**) were performed as described above except that  $10^7$  NK-92 cells were delivered intravenously into each recipient on days 13, 15 and 17 after teratoma placement, and IVIS imaging was performed, before NK-92 injection on day 13 (pre) and on day 19 (post).

*In vivo* studies of luciferase-expressing, ESC-derived CD45<sup>+</sup> cells were performed by intraperitoneal (IP) injection of  $2.5 \times 10^5$  CD45<sup>+</sup> cells of each B2M genotype into NSG-B2m mice and IVIS-based imaging of pre-NK-92 luciferase levels, followed 4 h later by the IP injection of  $6.25 \times 10^6$  NK-92 cells into half of the mice and re-imaging 1 d and 3 d later. The average change in luminescence in the NK-92 non-injected mice was used to normalize the data shown in **Figure 3h**.

**Statistics.** Statistical analysis was carried out with GraphPad Prism software v. 5.03 and Microsoft Excel 2007. All numerical data shown in the chromium release assays are presented as mean  $\pm$  s.d. Statistically significant results were considered when  $P < 0.05$ . One-way ANOVA tests were used to analyze the data derived from NK and CD8 chromium release assays. In **Figure 3e,f,h** and **Supplementary Figure 4f**, pair-wise comparisons used the *post hoc* Tukey HSD test. Data shown in **Figures 4c** and **3h** and the comparison between groups (with vs. without NK-92 cells) shown in **Supplementary Figure 4g** were analyzed by using the unpaired Student's *t*-test. Data shown in **Figure 4e-g**, the analysis within groups shown in **Supplementary Figure 4g** and the data shown in **Supplementary Figure 6b,c** were analyzed by using the paired Student's *t*-test.

**Data availability.** The DNA sequences of the vectors and plasmids used in this study are available upon request.

- Ware, C.B. *et al.* Derivation of naive human embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **111**, 4484–4489 (2014).
- Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
- Kotini, A.G. *et al.* Functional analysis of a chromosomal deletion associated with myelodysplastic syndromes using isogenic human induced pluripotent stem cells. *Nat. Biotechnol.* **33**, 646–655 (2015).

47. Leach, L.L., Buchholz, D.E., Nadar, V.P., Lowenstein, S.E. & Clegg, D.O. Canonical/ $\beta$ -catenin Wnt pathway activation improves retinal pigmented epithelium derivation from human embryonic stem cells. *Invest. Ophthalmol. Vis. Sci.* **56**, 1002–1013 (2015).
48. Sambrook, J. & Russell, D.W. *Molecular Cloning: a Laboratory Manual* 3rd edn. (Cold Spring Harbor Laboratory Press, 2001).
49. Papapetrou, E.P. & Sadelain, M. Generation of transgene-free human induced pluripotent stem cells with an excisable single polycistronic vector. *Nat. Protoc.* **6**, 1251–1273 (2011).
50. Lauterbach, N., Voorter, C.E.M. & Tilanus, M.G.J. Molecular typing of HLA-E. *Methods Mol. Biol.* **882**, 143–158 (2012).
51. Aicheler, R.J. & Stanton, R.J. Functional NK cell cytotoxicity assays against virus infected cells. *Methods Mol. Biol.* **1064**, 275–287 (2013).
52. Terasaki, P.I. & McClelland, J.D. Mirodroplet assay of human serum cytotoxins. *Nature* **204**, 998–1000 (1964).
53. Vernon, R.B. *et al.* Reversal of diabetes in mice with a bioengineered islet implant incorporating a type I collagen hydrogel and sustained release of vascular endothelial growth factor. *Cell Transplant.* **21**, 2099–2110 (2012).