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Humanized mouse models with endogenously developed human natural killer cells for *in vivo* immunogenicity testing of HLA class I-edited iPSC-derived cells

Charlotte Flahou [a], Tatsuya Morishima [b, c], Natsumi Higashi [a], Yoshikazu Hayashi [b, c], Huaigeng Xu [a, 1], Bo Wang [d], Chaoqi Zhang [d], Atsushi Ninomiya [a], Wei-Yin Qiu [a], Akinori Yuzuriha [a], Daisuke Suzuki [a], Sou Nakamura [a], Markus Manz [e], Shin Kaneko [d], Akitsu Hotta [a], Hitoshi Takizawa [b, f], Koji Eto [a, g, **], Naoshi Sugimoto [a, *]

[a] *Department of Clinical Application, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan*
[b] *Laboratory of Stem Cell Stress, Kumamoto University, Kumamoto, Japan*
[c] *Laboratory of Hematopoietic Stem Cell Engineering, International Research Center for Medical Sciences (IRCMS), Kumamoto University, Kumamoto, Japan*
[d] *Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan*
[e] *Department of Hematology, University and University Hospital Zurich, 8091, Switzerland*
[f] *Center for Metabolic Regulation of Healthy Aging, Kumamoto University, Kumamoto, Japan*
[g] *Department of Regenerative Medicine, Chiba University Graduate School of Medicine, Chiba, Japan*

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Human induced pluripotent stem cells (hiPSCs) genetically depleted of human leucocyte antigen (HLA) class I expression can bypass T cell alloimmunity and thus serve as a one-for-all source for cell therapies. However, these same therapies may elicit rejection by natural killer (NK) cells, since HLA class I molecules serve as inhibitory ligands of NK cells. Here, we focused on testing the capacity of endogenously developed human NK cells in humanized mice (hu-mice) using MTSRG and NSG-SGM3 strains to assay the tolerance of HLA-edited iPSC-derived cells. High NK cell reconstitution was achieved with the engraftment of cord blood-derived human hematopoietic stem cells (hHSCs) followed by the administration of human interleukin-15 (hIL-15) and IL-15 receptor alpha (hIL-15Rα). Such “hu-NK mice” rejected HLA class I-null hiPSC-derived hematopoietic progenitor cells (HPCs), megakaryocytes and T cells, but not HLA-A/B-knockout, HLA-C expressing HPCs. To our knowledge, this study is the first to recapitulate the potent endogenous NK cell response to non-tumor HLA class I-downregulated cells *in vivo*. Our hu-NK mouse models are suitable for the non-clinical evaluation of HLA-edited cells and will contribute to the development of universal off-the-shelf regenerative medicine.

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1. Introduction

Stem cell-derived therapies follow the same compatibility considerations between donors and recipients as regular transplantations and transfusions. In the case of human induced pluripotent stem cells (hiPSCs), clinical trials are adopting either fully compatible autologous cells or stocked iPSC-derived human leucocyte antigen (HLA)-homozygous cells, which have wide compatibility and are potentially readily available [1–3]. An alternative but experimental approach is depleting the HLA expression on donor iPSCs to make them universally compatible. In this way, the transplanted cells can circumvent the main barrier to transplantation, namely the mismatch of HLA [4,5]. Because natural killer (NK) cells possess a “missing-self” immunity, whereby they target cells with low or no expression of HLA class I [6–8], approaches to maintain inhibitory HLA class I molecules, such as the selective knockout of HLA-A/B while retaining HLA-C, are also under exploration [9]. Currently, the preclinical evaluation of such

* Corresponding author. Department of Clinical Application, Center for iPS Cell Research and Application (CiRA), Kyoto University, 606-8507, Japan.
** Corresponding author. Department of Clinical Application, Center for iPS Cell Research and Application (CiRA), Kyoto University, 606-8507, Japan.
E-mail addresses: kojieto@cira.kyoto-u.ac.jp (K. Eto), sugimoto.naoshi.4v@kyoto-u.jp (N. Sugimoto).
[1] Currently affiliated with the University of California San Francisco.

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strategies is insufficient due to the lack of assessment in *in vivo* models with endogenously developed human NK cells.

In our previous work [10], MSTRG mice, an immunodeficient mouse strain with improved human hematopoietic cell reconstitution by introducing human M-CSF, thrombopoietin and SIRPα genes [11], was further treated with exogenous hIL-15 and hIL-15Rα [12,13] after the engraftment of cord blood (CB)-derived CD34$^+$ human hematopoietic stem cells (hHSCs) to reconstitute high levels of human NK cells. HLA class I-depleted (hereafter referred to as "HLA-KO") hiPSC-derived hematopoietic progenitor cells (iHPCs) were infused and remained less abundant in hu-MSTRG mice with high levels of human NK cells. In the present study, we aimed to formally establish the appropriateness of hu-mice for *in vivo* immunogenicity testing against NK cells and further clarify the immunogenicity of HLA-edited hiPSC-derived cells from different blood lineages and differentiation stages in MSTRG mice and the newly developed and commercially available NSG-SGM3 strain, a mouse strain that is similarly enhanced for human hematopoietic cell reconstitution.

2. Material and methods

Ethics approval

The use of hiPSCs and the collection of CB from healthy volunteers with informed consent was approved by the ethics committees of Kyoto University and Kumamoto University. All experiments using human samples were conducted in accordance with the Declaration of Helsinki. All animal experiments were approved by university committees and conducted in accordance with the university regulations on animal experimentation.

2.1. Human hematopoietic stem cells (hHSCs)

CB-derived CD34$^+$ hHSCs from full-term deliveries were purchased from Takara Bio or purified from CB obtained at Kyoto University, Kumamoto University, or the Japanese Red Cross Kinki Cord Blood Bank. For purification, mononuclear cells were isolated using Lymphoprep (STEMCELL Technologies), and hHSCs were positively selected using a CD34$^+$ Cell Isolation Kit (Miltenyi Biotec). hHSCs were frozen in 90% fetal bovine serum (FBS; Cell Culture Bioscience) and 10% dimethyl sulfoxide in liquid nitrogen.

2.2. Human immune cell reconstitution in mice

MSTRG (*CSF1*$^{h/h}$*SIRPA*^{tg}*THPO*$^{h/h}$*Rag2*$^{-/-}$*Il2rg*$^{-/-}$) and NSG-SGM3 (NSGS; *NOD.CgPrkdcscidIl2rgtm1WjlTg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ*) mice (The Jackson Laboratory) [14,15] were housed in specific pathogen-free vivaria in the animal research facilities of Kumamoto University and Kyoto University.

For MSTRG mice, the reconstitution followed our previous report with minor modifications [10]. 2×10^5 hHSCs were intravenously injected into 7-14-week-old mice irradiated with 2.5 Gy. Four to seven weeks later, 0.5 μg recombinant human IL-15 (PeproTech) and 1.0 μg recombinant human IL-15 receptor alpha Fc chimera protein (R&D Systems) in 200 μL PBS were intraperitoneally injected three times.

For NSGS mice, 8-9-week-old female mice were irradiated with the indicated doses and intravenously injected with hHSCs in 100 μL PBS. Body weight, blood cell composition using a Sysmex XS-500i hematology analyzer, and human cell reconstitution were monitored at designated time points. To further support the development and maturation of human NK cells, the mice were treated with 0.5 μg rhIL-15 and 1 μg rhIL-15Rα-Fc by intraperitoneal injection four days before infusion of the target cells unless stated otherwise.

For the human immune cell reconstitution analysis, cells were stained with the antibodies listed in the supplementary tables. The data were obtained using FACSLyric, FACSVerse, FACSAria IIIu, or FACS Canto II (BD Bioscience) flow cytometers, and analyzed using FlowJo.

2.3. Cells

iHPCs were derived from the wild-type (WT), HLA-KO, or HLA-A/B-knockout, HLA-C-retaining hiPSC line Ff-XT28s05, as previously described [9]. HLA-KO hiPSCs were made by knocking out beta-2 microglobulin gene using CRISPR/Cas9 method. For CD43$^+$ hematopoietic cell differentiation, the hiPSCs were seeded into an ultra-low attachment 6-well plate and cultured with StemFit AK03 N medium (Ajinomoto) and 10 mM ROCK inhibitor Y-27632 (Wako) in a 5% CO_2 incubator for 2 days. From day 2, the medium was changed to StemPro-34 (Thermo Fisher) with 50 ng/mL bFGF (Oriental Kobo), 50 ng/mL VEGF (293-VE-050/CF; R&D Systems), 100 x ITS (Thermo Fisher), 100 x GlutaMax-I (Thermo Fisher), 50 mg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (AAP; Sigma), 100 nM MTG (Nacalai tesque), and 40 ng/mL BMP4 (R&D Systems) for 3 days. From day 5, BMP4 was removed, and 50 ng/mL SCF (R&D systems) was added for 2 days. From day 7, 10 ng/mL Fms-like tyrosine kinase 3 ligand (FLT3L) (R&D systems) and 30 ng/mL thrombopoietin (PeproTech) were added, and the cells were cultured in a normal 5% CO_2 incubator. Day 21–28 floating CD43$^+$ hematopoietic cells pretreated for 48 h with 50 ng/mL IFN-γ (PeproTech) were collected for the experiments.

WT and HLA-KO immortalized megakaryocyte cell lines (imMKCLs) were prepared as previously reported [16]. imMKCLs were cultured in IMDM medium (Sigma) with L-glutamine (Thermo Fisher), Insulin-transferrin-selenium (Thermo Fisher), 50 μg/mL AAP (Sigma), 450 μM 1-Thioglycerol (Sigma), 15% FBS, 50 ng/mL recombinant human thrombopoietin (PeproTech), and 50 ng/mL recombinant human Stem Cell Factor (rhSCF: R&D Systems) in a normal 5% CO_2 incubator.

HLA-KO and WT hiPSC-derived cytotoxic T cells (hiPSC-T cells) were prepared as previously described with some adaptation [9,17]. Briefly, the hiPSC line TKT3V1-7 was differentiated into CD4CD8 double-positive cells in a culture medium supplemented with 1 ng/mL recombinant hIL-7 (PeproTech) and 10 ng/mL recombinant human FLT3L (PeproTech), and then into CD8 single-positive cells by 2-day treatment with 1 μg/mL anti-CD3ε antibody, 10 ng/mL IL-7, 10 ng/mL FLT3L and ITS. The cells were then matured on RetroNectin-coated culture dishes. After maturation (CD1a down-regulation), they were sorted using a FACSAria II. hiPSC-T cells were then cultured in αMEM with 15% FBS, 50 μg/mL AAP (Sigma, A8960-5G), 5 ng/mL IL-7, and 5 ng/mL IL-15 (PeproTech) in a normal 5% CO_2 incubator.

The K562 cell line was cultured as per ATCC recommendations in RPMI1640 medium with 10% FBS (CCB) and L-glutamine (Gibco) in a normal 5% CO_2 incubator.

2.4. Cell infusion and survival analysis

For experiments involving iHPCs and imMKCLs, the cells were treated with a final concentration of 50 ng/mL interferon-γ one day prior to the infusion. WT, HLA-KO and K562 cells labeled using the CellTrace CFSE and Violet Cell Proliferation Kits (Invitrogen) were infused at a ratio of 1:1:1 by tail-vein injection. Mice received between 5×10^6 and 1×10^7 cells in total.

24 h (D1) and 72 h (D3) after the cell infusion, mice were put under anesthesia, then the lung and spleen were collected. Spleens were cut into 2–3 pieces and passed through a 70-μm nylon cell strainer. The lungs were cut into small pieces, then enzymatically

digested using Liberase (Roche) and passed through the strainer. Single cell suspensions were analyzed by flow cytometry, with a stop rule at either 10,000 events in the WT cells gate or for a maximum of 10 min. The cell populations were analyzed with FlowJo software. Single cells were gated for FITC (CFSE positive cells, WT), BV450 (Violet positive cells, K562) or both to select double-positive cells (CFSE and Violet, HLA-KO cells). HLA-KO and K562 cell levels were compared against the WT cell population.

2.5. Statistics

All statistical analyses were performed using GraphPad Prism software and are reported as individual points for each biological replicate. All data were evaluated for normality and variance to determine the appropriateness of parametric versus nonparametric statistical tests.

3. Results

3.1. Hu-MSTRG mice competently assess the NK cell immunogenicity of HLA class I-edited hiPSC-derived cells

MSTRG mice were humanized by the engraftment of hHSCs and reconstituted with high levels of NK cells in MSTRG by three intraperitoneal injections of hIL-15/hIL-15Rα (Fig. 1A). The reconstitution of human hematopoietic cells (Fig. S1A) was higher for female mice according to the percentage of human CD45 positive ($hCD45^{+}$) cells among total $CD45^{+}$ cells compared with male mice [18]. The predominant cell population was $CD19^{+}$ B cells for both male and female mice, and $CD3^{+}$ T cell levels were negligible (Fig. 1B). Myeloid cells ($hCD33^{+}$) were reconstituted with a higher percentage within the $hCD45^{+}$ cell population for female mice, and $CD56^{+}$ NK cells showed the predominant subtype being the $CD56^{dim}CD16^{+}$ mature subset (Fig. 1B).

Four days after the last injection of hIL-15/hIL-15Rα, we co-infused WT and HLA-KO imMKCLs together with a K562 myeloid leukemia cell line, a well-known target of NK cells, into MSTRG mice. We then compared the presence of the infused cells in the lung and spleen. To differentiate between spontaneous cell loss and NK cell-dependent rejection, the ratio of HLA-KO or K562 cells against the WT cells were plotted against the NK cell level at the time of infusion. The detection of HLA-KO imMKCLs on the next day was inversely logarithmically correlated to the NK cell level in the lung (Fig. 1C left) and spleen (Fig. S2A left). K562 cells were also rejected depending on the logarithmic human NK cell level in the lung (Fig. 1C right), and the spleen showed a similar tendency (Fig. S2A right).

We then co-infused WT, HLA-KO, and HLA-C-retaining iHPCs into hu-MSTRG mice and analyzed their levels (Fig. S1B). We confirmed that the ratio of HLA-KO iPSC-derived iHPCs relative to their co-infused WT counterpart was significantly and inversely logarithmically correlated to the human NK cell level in the lung (Fig. 1D left) and spleen (Fig. S2B left). However, HLA-C-retaining iHPC levels followed WT levels (Fig. 1D right), indicating their tolerance to NK cells *in vivo* in this model, as intended [9]. These results demonstrate the accuracy of hu-MSTRG mice to monitor the immunogenicity of HLA-edited hiPSC-derived cells in response to NK cells.

3.2. Hu-NSGS mice show high levels of human chimerism and support human circulating NK cell development with exogenous hIL-15/hIL-15Rα

We optimized our regimen to NSGS mice, which are immunodeficient NSG mice genetically modified to express human SCF, granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3). For this model, the hHSC graft was treated with anti-hCD3 antibody to purge contaminating T cells and suppress the development of xeno-graft-versus-host disease (xeno-GVHD). To minimize the strain of the regimen on the NSGS mice, we compared three different combinations: with low or no irradiation, and fewer hHSCs in the injection (Fig. S3). Body weight increased throughout the follow-up for 2×10^{5} hHSCs with 0 or 1 Gy radiation and for 1×10^{5} hHSCs with 1 Gy radiation, indicating a generally healthy status. The number of peripheral white blood cells (WBCs) was also stable. In contrast, red blood cells (RBCs), platelets, hemoglobin, and hematocrit tended to decrease with time. Regarding chimerism, the overall percentage of human hematopoietic cells detected as $hCD45^{+}$ cells among all $CD45^{+}$ cells in the peripheral blood 5 weeks after the hHSC transplantation were similar for all three conditions.

We adopted the transplantation of 1×10^{5} hHSCs with 1 Gy preconditioning, since this number achieved stable reconstitution but had the advantage of less severe anemia and thrombocytopenia. We included mice with or without hIL-15/hIL-15Rα treatment at 7 or 8 weeks after the hHSC injection to compare different levels of human NK cell reconstitution. Four days after the single regimen IL-15 treatment, body weight and WBC, RBC, platelet, hemoglobin, and hematocrit levels were unchanged in hu-NSGS (Fig. 2A). However, spleen sizes were significantly increased in hu-mice regardless of whether they were treated with hIL-15/hIL-15Rα compared to non-humanized mice (Fig. 2B). Human immune cell reconstitution was also compared (Fig. 2C, S1C, and S4). Chimerism was constantly high but slightly more so following hIL-15/hIL-15Rα treatment. Similar to hu-MSTRG mice, $CD19^{+}$ B cells were dominant among $hCD45^{+}$ cells, and $CD33^{+}$ myeloid cells were next. The numbers of these cells did not significantly change with the hIL-15/hIL-15Rα treatment. $CD3^{+}$ T cell and $NKp46^{+}$ NK cell numbers were low, but the hIL-15/hIL-15Rα treatment dramatically increased NK cells for both $CD16^{+}$ and $CD16^{-}$ subsets and increased T cells to some extent. These results indicate that hIL-15/hIL-15Rα can significantly increase the reconstitution of human NK cells with negligible effect on other phenotypes in mice. Thus, hu-NSGS mice with low conditioning, the transplantation of a small number of hHSCs, and a single injection of hIL-15/hIL-15Rα provide a stable *in vivo* model with a high number of endogenously developed human NK cells.

3.3. Hu-NSGS mice reject HLA-KO cells depending on the reconstitution of human NK cells

We next examined the functional capacity of the reconstituted human NK cells to reject HLA-KO hiPSC-derived cells *in vivo* in hu-NSGS mice intraperitoneally injected with or without hIL-15/hIL-15Rα (Fig. 3A). We co-infused HLA-KO imMKCLs with their WT counterpart at a 1:1 ratio after labeling with fluorescent tracking probes and assessed the rate of rejection of the two cell types one (D1) and three (D3) days later by flow cytometry in the lung (Fig. 3B) and in the spleen (Fig. S5A). Similar to hu-MSTRG mice, we observed the rejection of HLA-KO cells observed in the lung at D1 is correlated to the reconstituted human NK cell logarithmic level and tended that way at D3. In the spleen, the same tendency was seen for both days.

We next performed similar experiments using a terminally differentiated cell type, hiPSC-derived T cells. Like the imMKCL experiment, we observed the rejection of HLA-KO cells is correlated to logarithmic NK cell levels in the lungs on D1 and D3 (Fig. 3C) and in the spleen on D3 (Fig. S5B). Taken together, these data show that hu-NSGS mice, especially with hIL-15/hIL-15Rα administration, recapitulate human NK cell immunity, thus enabling monitoring of

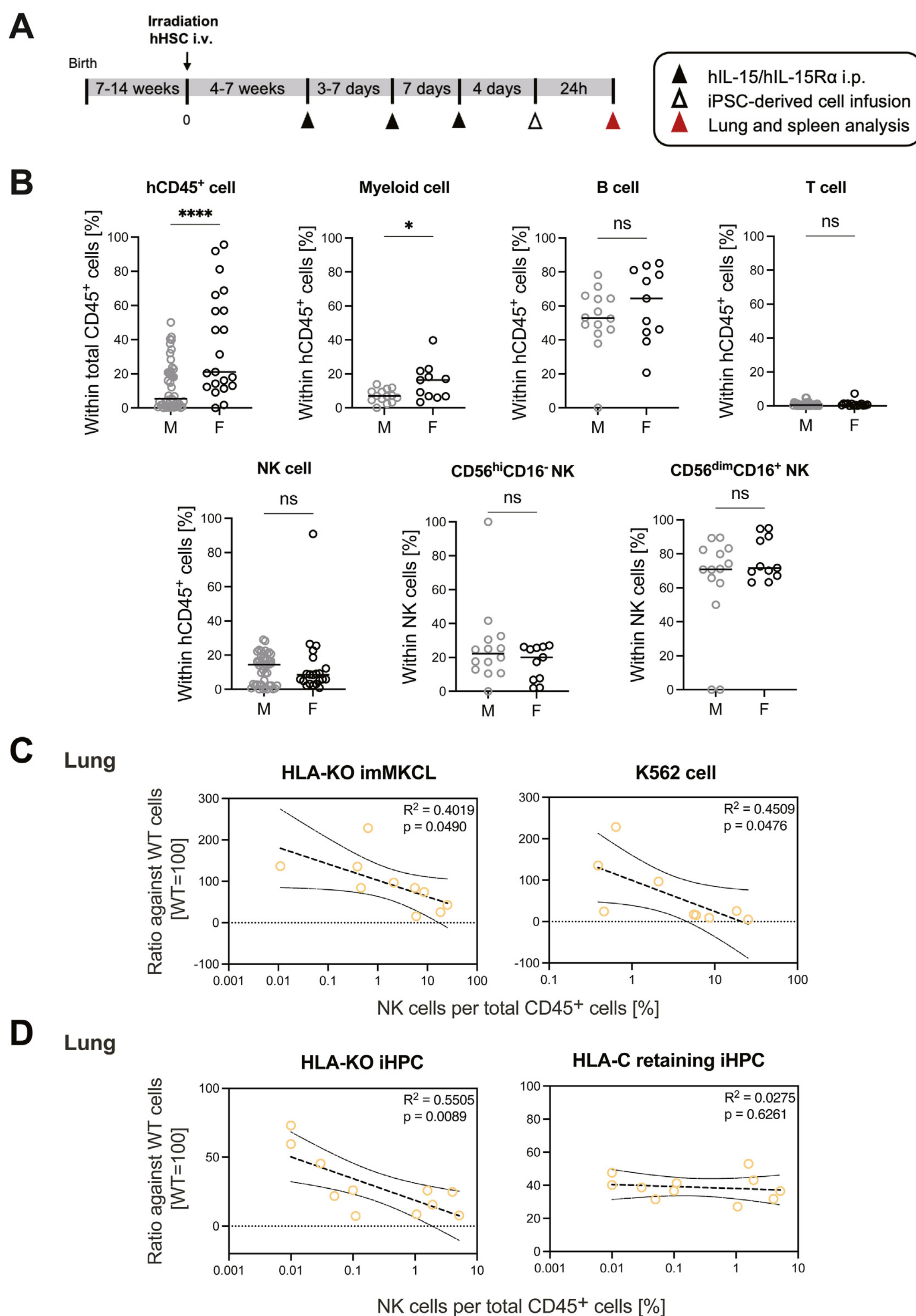

Fig. 1. hu-MSTRG mice reject HLA-KO hiPSC-derived cells but not HLA-C-retaining iHPCs depending on human NK cells
A. Experimental design of the hiPSC-derived cell rejection assay in hu-NK-MSTRG mice.
B. Reconstitution of human immune cells in hu-NK-MSTRG mice. Chimerism within $CD45^+$ hematopoietic cells, myeloid cells, B cells, T cells and NK cells, including subsets at the time of hiPSC-derived cell infusion, for male (M) and female (F) mice is shown.
C. Correlation between the human NK cell reconstitution and rejection of HLA class I–KO imMKCLs and K562 cells relative to WT imMKCLs in the lung.
D. Correlation between the human NK cell reconstitution and rejection of HLA class I–KO and HLA-C-retaining iHPCs relative to WT iHPCs in the lung.
Statistical analyses were performed using the Mann-Whitney test or unpaired two-tailed *t*-test for two-group comparisons and a simple linear regression with 95% confidence intervals (ns: not significant, *$p < 0.05$, ****$p < 0.00001$). Each circle represents one mouse; data are from 3 independent experiments for iHPCs and 2 independent experiments for imMKCLs and K562. i.v., intravenous injection; i.p., intraperitoneal injection.

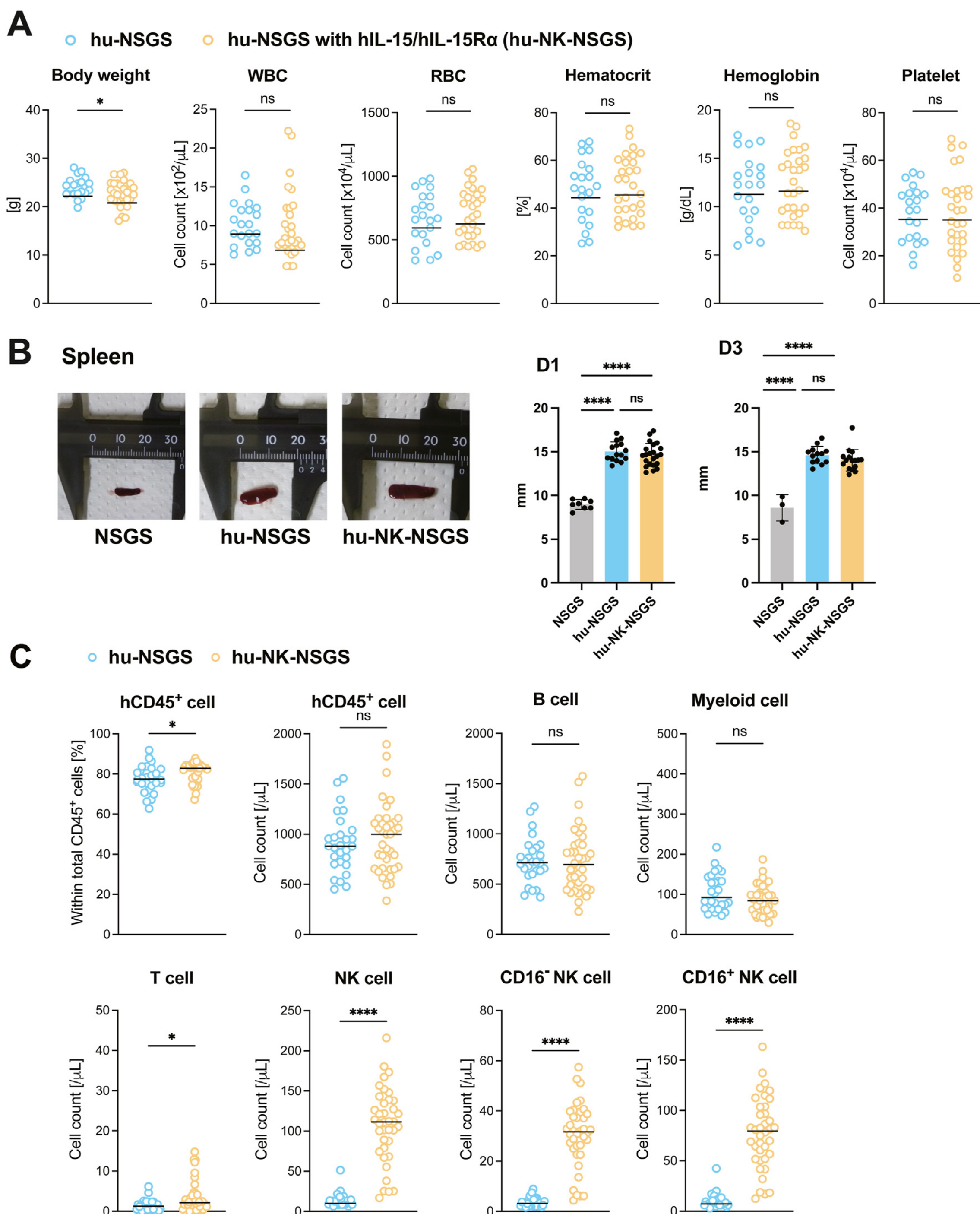

Fig. 2. Enhanced reconstitution of human NK cells in NSGS mice

A. Body weight and complete blood count in hu-NSGS mice at 8 weeks post infusion of hHSCs. WBC: white blood cells, RBC: red blood cells.

B. Representative photographs of spleens from NSGS and hu-NSGS 8 weeks post infusion of hHSCs with or without human IL-15/IL-15Rα treatment (left). Spleen size at 1 (D1) and 3 (D3) days after the infusion (right).

C. Reconstitution of human immune cells in hu-NSGS mice, 8 weeks post infusion of hHSCs. Chimerism within $CD45^+$ hematopoietic cells and cell numbers in the peripheral blood for $hCD45^+$ cells, myeloid cells, B cells, T cells and NK cells, including subsets at the time of the hiPSC-derived cell infusion, in hu-NSGS mice treated with or without human IL-15/IL-15Rα are shown.

Statistical analyses were performed using the Mann-Whitney test or unpaired two-tailed *t*-test for two-group comparisons and ordinary one-way ANOVA with Tukey multiple comparison test or Kruskal-Wallis with Dunn's multiple comparisons test for more than two-group comparisons (ns: not significant, *p < 0.05, ****p < 0.00001). Each circle represents one mouse. Blue and orange circles represent hu-NSGS mice without or with human IL-15/IL-15Rα treatment, respectively. Data are from three independent experiments for D1 imMKCLs, two for D3 imMKCLs, two for D1 hiPSC-T cells and for D3 hiPSC-T cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

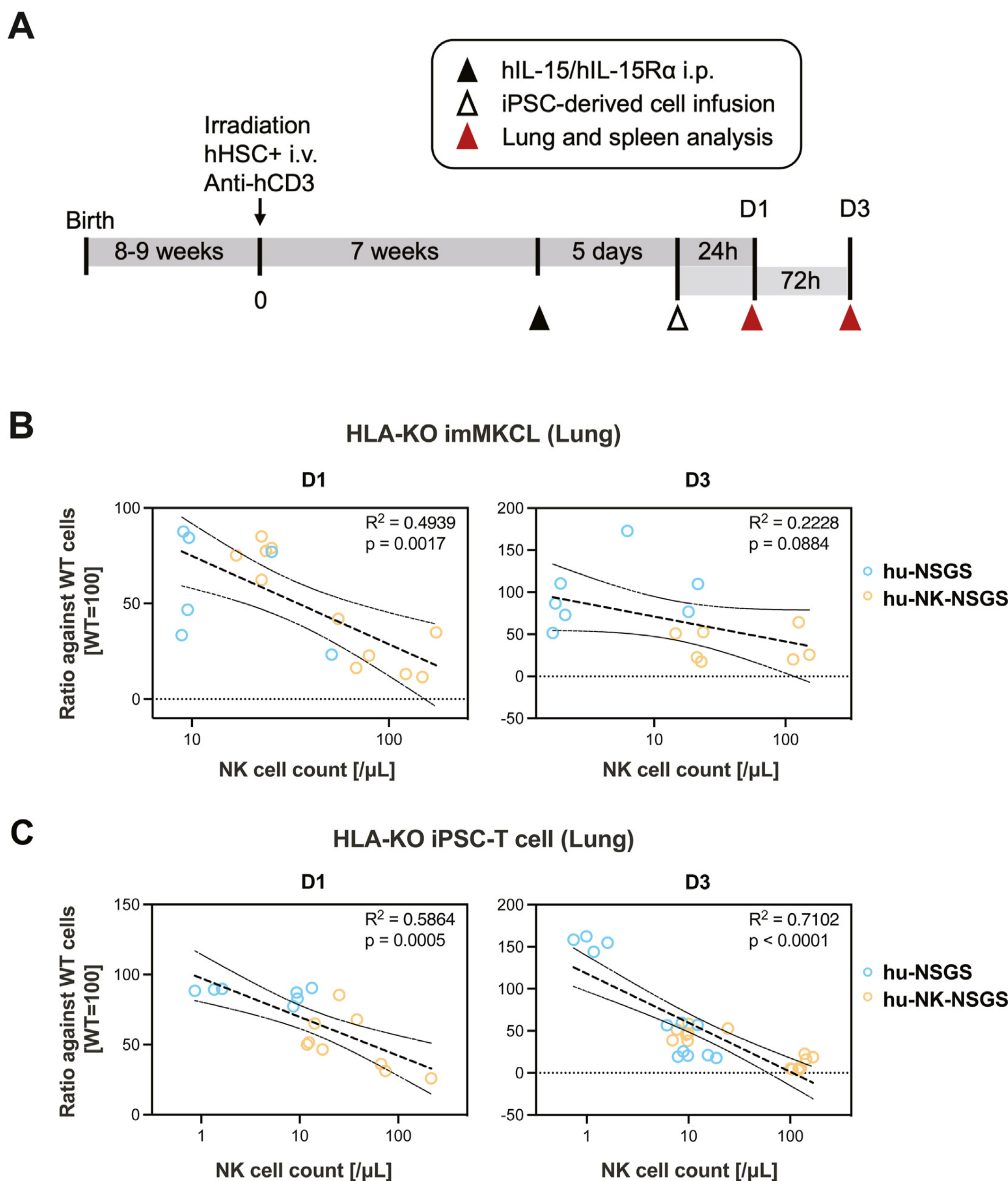

Fig. 3. Hu-NSGS mice reject HLA-KO hiPSC-derived cells depending on human NK cell reconstitution.
A. Experimental design of the hiPSC-derived cell rejection assay in the hu-NSGS model.
B. Correlation between the human NK cell reconstitution and rejection of HLA-KO imMKCLs in the lung.
C. Correlation between the human NK cell reconstitution and rejection of HLA-KO hiPSC-T cells in the lung.
Statistical analyses were performed with a simple linear regression and 95% confidence intervals. Each circle represents one mouse.

the rejection of HLA class I-depleted cells derived from hiPSCs, particularly in the lung on the day after the infusion. At the same time, the rejection of HLA-KO hiPSC-derived cells in hu-NK mice was shown with imMKCLs and hiPSC-derived T cells.

4. Discussion

The main goal of this study was to evaluate if our endogenous human NK cells reconstitution strategy is suitable to model the “missing self” immunity against HLA class I-edited hiPSC-derived cells. Recently developed immunodeficient mouse strains with the transgenic expression of human cytokines, such as MSTRG and NSGS, have enhanced the reconstitution of human hematopoietic cells upon hHSC transplantation. The transgenic expression of human M-CSF and GM-CSF enhances the reconstitution of human myeloid cells, which present hIL-15/hIL-15Rα complex on the cellular membrane to β and γ subunits of hIL-15 receptor on NK cells to support the expansion of NK cells. However, while MISTRG mice, which have additional human IL-3 and GM-CSF transgenes compared with MSTRG, were reported to highly reconstitute human NK cells [11,19], MSTRG mice did not show this reconstitution. However, by treating MSTRG mice with or without hIL-15/hIL-15Rα, we were able to reconstitute varying levels of human NK cells, thereby to observe the correlation of human NK cell reconstitution and the rejection of HLA-KO cells. A similar observation was made with NSGS mice, which harbor human IL-3 and GM-CSF transgenes

but not human M-CSF transgene. Other mouse strains expressing human IL-2 or IL-15 transgenes [20–24] may have similar utility without the need of administering exogenous cytokines, although the level of control provided by exogenous cytokines allows for more flexibility in NK cell reconstitution. The assessment of *in vivo* immunity, including human NK cells against HLA class I-downregulated grafts, has also been performed in mice adoptively transferred with human peripheral blood mononuclear cells, human peripheral blood-derived NK cells [25], and NK cell lines [26] using immunodeficient NOG and NSG strains. However, the reconstitution is transient and does not reflect the endogenously developed NK cell immune response. As for endogenously developed human NK cells in hu-mice with hHSC transplantation, the functionality of human NK cells to reject HLA class I-downregulated cells had only been confirmed against highly immunogenic malignant cells and not for non-tumor cells, including HLA-KO hiPSC-derived cells, until our previous study, which was still preliminary [10].

Human hematopoietic cell reconstitution was higher in female MSTRG mice than in male mice, similar to a previous report on NSG mice [18]. As such, we used only female mice in the NSGS mice experiments in this study. However, since NSGS mice showed higher human leucocyte reconstitution, male mice might also be useful *in vivo* models. When assessing human NK cell reconstitution in hu-MSTRG mice, we used CD56 as a marker to gate NK cells, which included immature $CD56^{high}CD16^{-}$ and mature $CD56^{dim}CD16^{+}$ subsets [27] as well as a $CD56^{low}CD16^{-}$ population, suggesting contaminating non-NK cells (Fig. S1A). For NSGS mice, we used NKp46 to identify NK cells. In this setting, we noticed that the expression of CD56 was not high and that the expression of NKp46 on the $CD16^{+}$ subset was lower than on the $CD16^{-}$ subset (Fig. S1C). We later confirmed that the difference in the CD56 expression level was likely due to differences in the antibodies used for the two strains. Nevertheless, our analysis showed that the *in vivo* rejection capacity correlated with the NK cell reconstitution level as a percentage or absolute number in the lung (Fig. 1C, D, 3B and 3C). In contrast, spleen samples showed more variable results (Figs. S2 and S4), likely due to the lower accumulation of infused cells in the spleens compared to the lungs.

Humanized mice can suffer from deteriorating conditions [28,29]. Because this effect could be due to the preconditioning by radiation, human to mouse xeno-GVHD, and potential toxic effects from the introduction of the repeatedly high systemic circulation of cytokines, we tested lower and no radiation levels, fewer hHSCs in the transplantation, pretreatment of the hHSCs with anti-hCD3 to deplete contaminating T cells, and a single hIL-15/hIL-15Rα treatment in NSGS mice. However, we observed progressive anemia and thrombocytopenia (Fig. S3) and an enlarged spleen size at 8 weeks after the cell transplantation (Fig. 2B), suggesting the reconstituted human macrophages phagocytose mouse erythrocytes and platelets. Furthermore, hu-mice started to show indications of wasting with human T cell expansion from 10 weeks after the hHSC transplantation, suggesting the need to better control xeno-GVHD, for instance, by the knockout of mouse MHC [30].

Our finding that HLA-KO iHPCs are rejected but HLA-C-retaining iHPCs are tolerated (Fig. 1D) is consistent with the expected role of HLA-C as the inhibitory ligand against human NK cells [9]. Diverse strategies to bypass the "missing-self" NK cell immunity against HLA-KO cells have been described, but only a few studies have tested *in vivo* the tolerance to human NK cell immunity, such as HLA-E expressing cells against a peripherally injected NK-92 cell line [26]. Our hu-NK mouse models may contribute to assessing the *in vivo* immunogenicity of diverse HLA-KO cells against endogenously developed human NK cells.

Ex vivo expanded T cells, such as CAR-T cells, are already being used in clinical settings [31]. The field is further looking into using hiPSC-derived T cells as an off-the-shelf product with rejuvenated properties [32–34]. In this setting, HLA-homozygous and HLA-edited cells are being studied to avoid allogeneic responses. Our hu-NK mice models may contribute to preclinical immunogenicity testing *in vivo* not only for the HLA-KO cells shown in this study but also for HLA-haplotype homozygous cells [35,36], which lack half the set of HLA alleles and thus may be vulnerable to NK cell immunity [37].

We previously showed that HLA-KO platelets can circulate similar to WT platelets in hu-NK-MSTRG [10], while here we show the rejection of their precursor cells, HLA-KO imMKCLs, in both hu-mice models by NK cells. These results provide grounds to prepare hiPSC-derived platelets with HLA class I depletion without HLA-C retention or any other measure to avoid NK cell immunity, but to complement this strategy with a fail-safe for the rejection of contaminating imMKCLs, which express oncogenic c-MYC and BCL-XL transgenes [38,39]. We are currently investigating the immune escape mechanism of such platelets.

The NK cell-dependent rejection response we observed in the lungs and spleens of three different cell types, including progenitor cells to terminally differentiated cells, suggest that our hu-NK mouse models are suitable for the intravenous infusion of a wide range of cells. Tissue transplantation studies using HLA-KO hiPSC-derived cells might also be feasible in the hu-NK mice models we describe here given that the NK cells are endogenously developed so their response is more likely to resemble the *in vivo* response in human tissues. However, this hypothesis should be confirmed with the further characterization of resident human NK cell phenotypes and graft infiltration.

HLA-edited hiPSCs may promote immunologically compatible off-the-shelf products but first require solid preclinical testing. This study provides an *in vivo* evaluation of NK cell rejection/tolerance of HLA-KO and HLA-A/B–KO, HLA-C-retaining hiPSC-derived cells, thus giving a more reliable basis for applying HLA-edited hiPSC-derived cells to clinical application. It also contributes to the development of regenerative medicine by providing a novel strategy for using hu-NK mice in the preclinical *in vivo* testing of HLA-edited cell therapies.

Author contributions

C.F. Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft; T.M. Investigation, Methodology, Writing – review & editing; N.H. Conceptualization, Data curation, Formal analysis, Investigation, Validation; Y.H. Investigation, Methodology; H.X. Investigation, Resources; B.W. Resources; C.Z. Resources; A.N. Investigation, Validation; WY.Q. Resources; A.Y. Resources, Methodology; D.S. Resources, Methodology; S.N. Resources, Methodology; M.M. Methodology, Resources; S.K Resources; A.H Resources; H.T Methodology, Resources, Writing – review & editing; K.E Funding acquisition, Supervision; N.S Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – original draft.

Declaration of competing interest

H.X., S.N., A.H., K.E., and N.S. have applied for patents related to this manuscript. K.E. is a founder of Megakaryon and a member of its scientific advisory board without salary. N.S. serves as an advisory for Megakaryon. S. Kaneko is a founder, shareholder, and director at Thyas Co., Ltd., and received research funding from Takeda Pharmaceutical Co., Ltd., Thyas Co., Ltd., Astellas Co., Ltd., KOTAI biotechnologies Co., Ltd., and Terumo Co., Ltd. This work was

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Appendix A. Supplementary data

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