Resistance of KIR ligand-missing leukocytes to natural killer cells in vivo in patients with acquired aplastic anemia

Full-Length Article

Resistance of KIR ligand-missing leukocytes to natural killer cells *in vivo* in patients with acquired aplastic anemia

Mai Anh Thi Nguyen[1]*, Kohei Hosokawa[1]*, Takeshi Yoroidaka[1]*, Hiroyuki Maruyama[1], J. Luis Espinoza[2], Mahmoud I. Elbadry[1,3], Md Mohiuddin[4], Mikoto Tanabe[1], Takamasa Katagiri[2], Noriharu Nakagawa[1], Nobuyoshi Arima[5], Koichi Kashiwase[6], Hiroh Saji[7], Seishi Ogawa[8] and Shinji Nakao[1++]

[1] Department of Hematology, Kanazawa University, Kanazawa, Japan

[2] Clinical Laboratory Sciences, Kanazawa University Graduate School, Kanazawa, Japan

[3]Department of Internal Medicine, Division of Hematology, Faculty of Medicine, Sohag University, Egypt

[4] Department of Respiratory Medicine, Kanazawa University, Kanazawa, Japan

[5] Department of Hematology, Shinko Hospital, Kobe, Japan

[6] Japanese Red Cross Kanto-Koshinetsu Block Blood Center, Tokyo, Japan

[7] HLA laboratory, Kyoto, Japan

[8] Department of Pathology and Tumor Biology, Kyoto University, Kyoto, Japan

*These authors contributed equally to this work.

Key words

aplastic anemia, 6pLOH, killer cell immunoglobulin-like receptor

Short Title: Resistance of KIR-L(-) cells to NK cells in AA

Text word count: 4622

Abstract word count: 244

Number of figures: 4

Number of tables: 3

Number of references: 47

Number of supplemental files: 1

[++] Correspondence: Shinji Nakao, M.D, Ph.D.

Department of Hematology, Graduate School of Medical Sciences, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa, 920-8640, Japan.

E-mail: snakao8205@staff.kanazawa-u.ac.jp

Phone: +81-76-265-2274

FAX: +81-76-234-4252

Conflicts of Interest

The authors declare no conflicts of interest

Abstract

The loss of killer cell immunoglobulin-like receptor-ligands (KIR-Ls) due to the copy number neutral loss of heterozygosity of chromosome 6p (6pLOH) in leukocytes of patients with acquired aplastic anemia (AA) may alter the susceptibility of the affected leukocytes to NK cell killing *in vivo*. We studied 408 AA patients, including 261 who were heterozygous for KIR-Ls, namely C1/C2 or Bw6/Bw4, for the presence of KIR-L-missing (KIR-L[-]) leukocytes. KIR-L(-) leukocytes were found in 14 (5.4%, C1, n= 4, C2, n=3, and Bw4, n= 7) of the 261 patients, in whom corresponding KIR(+) licensed NK cells were detected. The incidence of 6pLOH in the 261 patients (18.0%) was comparable to that in 147 patients (13.6%) who were homozygous for KIR-L genes. The percentages of HLA-lacking granulocytes (0.8-50.3%, median 15.2%) in the total granulocytes of the patients with KIR-L(-) cells were significantly lower than those (1.2-99.4%, median 55.4%) in patients without KIR-L(-) cells. *KIR2DS1* and *KIR3DS1* were only possessed by three of the 14 patients, two of whom had C2/C2 leukocytes after losing C1 alleles. The expression of the KIR3DS1 ligand HLA-F was selectively lost on KIR-L(-) primitive hematopoietic stem cells (HSCs) derived from 6pLOH(+) iPS cells in one of the KIR3DS1(+) patients. These findings suggest that human NK cells are able to suppress the expansion of KIR-L(-) leukocytes but are unable to eliminate them partly due to the lack of activating KIRs on NK cells and the low HLA-F expression level on HSCs in AA patients.

Introduction

All normal blood leukocytes, including hematopoietic stem cells (HSCs), express killer cell immunoglobulin-like receptor (KIR) ligands (KIR-Ls) to protect themselves from autologous NK cell attacks, and malignant cells that lack KIR-Ls elicit NK cell-mediated killing of themselves (1). The engagement of inhibitory receptors by self MHC class I molecule leads to the transmission of an inhibitory signal to switch off the functions of NK cells. In contrast, target cells with the downregulation of major histocompatibility antigen molecules due to viral infection or malignant transformation are recognized and attacked by NK cells. There are four situations in predicting NK-cell activities that have been proposed based on the difference in the definition of KIR mismatches: the KIR-ligand missing model (2, 3), receptor-ligand mismatch model (4), missing-ligand model (3) and the presence of activating KIR model (5). The missing-self mechanism and the presence of activating KIR are believed to play essential roles in the elimination of malignant cells in the setting of allogeneic hematopoietic stem cell transplantation (5, 6). However, whether or not the killing of KIR-L-missing (KIR-L[-]) tumor cells by autologous NK cells occurs *in vivo* still remains unclear due to the lack of appropriate human models. The heterogeneity of tumor cells in terms of their proliferative capacity and the expression of various accessory molecules—other than KIR-Ls—that are involved in the NK cell attacks makes it challenging to understand the interaction between NK cells and KIR-L(-) tumor cells.

The lack of class I HLAs occurs not only in malignant cells but also in normal leukocytes of some patients with acquired aplastic anemia (AA). These HLA-lacking leukocytes, which are detectable in approximately 30% of patients with AA, are derived from HSCs that undergo copy number neutral loss of heterozygosity of the short arm of chromosome 6 (6pLOH) or loss-of-

function mutations of HLA class I genes, and thereby escape the cytotoxic T-lymphocyte (CTL) attack against HSCs (7, 8). Since the HLA haplotypes lost due to 6pLOH usually contain HLA-B and -C alleles, 6pLOH(+) HSCs and their progenies that lose KIR-Ls may suffer a change in susceptibility to NK cell-mediated cell killing. Unlike malignant cells, HLA-lacking leukocytes are essentially the same as their wild-type (WT) counterparts, except for their HLA expression. Therefore, studying 6pLOH(+) AA patients with KIR-L(-) leukocytes is expected to be useful for clarifying the interaction between NK cells and KIR-L(-) target cells *in vivo*.

To address this issue, we studied a large number of AA patients in remission for the presence of KIR-L(-) leukocytes as a result of 6pLOH and determined the influence of the KIR-L-missing status on the emergence and expansion of 6pLOH(+) leukocytes. This study revealed a paradoxical coexistence of KIR-L(-) leukocytes with licensed NK cells *in vivo* and provided evidence that the lack of activating NK receptors on NK cells and/or their ligands on target hematopoietic cells may contribute to the persistence of KIR-L(-) cells in AA patients.

Materials and methods

Patients

A total of 408 AA patients were enrolled in this study to determine the prevalence of HLA class I allele-lacking leukocytes, which was determined using GeneChip 500 K arrays (Affymetrix, Japan) and droplet digital polymerase chain reaction (PCR) using a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA), and flow cytometry (FCM), as previously described (7-9). **Table 1** shows the patients' characteristics. The male to female ratio was 210/198, and the median age was 54 years (range: 2-86 years). The diagnosis and severity of AA were determined according to the standard criteria (10). This study protocol was approved by the ethics committee of the Graduate school of Medical Science, Kanazawa University, and all patients provided their informed consent, in accordance with the Declaration of Helsinki, prior to their participation.

Determination of 6pLOH in peripheral blood leukocytes

The presence of 6pLOH(+) leukocytes and their percentages of total leukocytes were determined using either SNP arrays (7) or ddPCR using a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, USA) by comparing the copy number of each HLA allele in individuals heterozygous for the HLA allele. 6pLOH that involves HLA genes gives rise to a copy number imbalance between the two different alleles. The reaction mixtures were previously described in detail (9). The percentages of 6pLOH(+) leukocytes were calculated using the following equation:

$$\%\ 6pLOH(+) = \frac{(|A-B|)}{A+B} \times 100$$

where A and B represent the absolute copy number of two alleles estimated by the Poisson

statistic. The HLA alleles in each mixture (probes and primers) are shown in **Supplemental Table 1**.

Determination of the HLA class I allele expression by peripheral blood leukocytes

AA patients who were heterozygous for HLA-A alleles or for HLA-Bw6 and HLA-Bw4 were assessed for the presence of HLA(-) leukocytes using FCM, as previously described in detail (7-9). Four lineages of peripheral blood (PB) cells, including granulocytes, monocytes, T cells, and B cells were subjected to the analysis. PB samples after erythrocyte lysis were suspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and stained with anti-HLA-A allele-specific monoclonal antibodies (mAbs), and lineage marker mAbs specific for CD33, CD3, and CD19. The mAbs used for this study are provided in a previous report (7, 8). In summary, antibodies specific to HLA-A9/24 (FH0964; One Lambda), A2/28 (FH0037; One Lambda), A25/26 (BIH0048; One Lambda), A30/31 (BIH0087; One Lambda), A11 (BIH0084; One Lambda), Bw6-PE (130-099-835; Miltenyi), Bw6-FITC (FH0038; One Lambda), Bw4-PE (130-103-917; Miltenyi), and Bw4-FITC (FH0007; One Lambda) were used. Two non-classical HLA class I antigens were also examined using anti-HLA-E-PE (cat no.342603, clone 3D12; Biolegend) and anti-HLA-F-PE/APC, cat no. 373203/373207, clone 3D11; Biolegend).

Determination of the expression of NK-cell receptor ligands by hematopoietic stem cells (HSCs) and hematopoietic stem progenitor cells (HSPCs)

HSCs and hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (BM) from AA and healthy volunteers, and umbilical cord blood (UCB) were identified according to the previous reports (11, 12). Six different populations were examined for the expression of HLA-F:

HSCs, multipotent progenitors (MPPs), multilymphoid progenitors (MLPs), common myeloid progenitor (CMPs), granulocyte-monocyte progenitor (GMPs) and megakaryocyte-erythroid progenitors (MEPs). The expression levels of HLA-F on HSCs and HSPCs were determined using mAbs specific to anti-human lineages cocktail-APC (cat no.348803; Biolegend), anti-CD34-APC/Cy7 (cat no.343514; Biolegend), anti-CD38-PE/Cy7 (cat no.560677; Biolegend), anti-CD45RA-Pacific Blue (cat no.562885; Biolegend), anti-CD90-FITC (cat no.328108; Biolegend), anti-CD123-PerCP-Cy5.5 (cat no.45-1239-42; Invitrogen) and anti-CD49f-BV510 (cat no.563271; BD Horizon). The expression levels of all surface proteins were analyzed using a FACSCanto II® instrument (BD Biosciences) with the Flowjo 10.0 software program.

Differentiation of HSPCs from induced pluripotent stem cells (iPSCs)

Induced pluripotent stem cell (iPSC) clones with different phenotypes from Cases 1 and 8 were cultured and differentiated into HSPCs using the previously described method (13, 14). The HLA expression of each clone in Case 1 was as follows: the WT clone (*A02:01/A24:02, B35:01/B40:02, C08:01/C03:04)*, the $B^{*}40{:}02^{mut}$ ($B61^{-}$) clone (*A02:01/A24:02, B35:01/—, C08:01/C03:04*), and the 6pLOH clone (*A02:01/A02:01, B35:01/B35:01, C08:01/C08:01*) (14). The HLA alleles of each clone in Case 8 were as follows: WT clone (*A24:02/A01:01, B54:01/B37:01, C01:02/C06:02*), $B^{*}54{:}01^{mut}$ ($B54^{-}$) clone (*A24:02/A01:01, —/B37:01, C01:02/C06:02*), and 6pLOH clone (*A01:01/A01:01, B37:01/B37:01, C06:02/C06:02*) (13). The iPSCs from Case 1 and 8 were differentiated to HSPCs as described previously and collected after 21 days. The $CD34^{+}$ cells were purified using a CD34 MicroBead Kit (cat no.130-046-702; Miltenyi Biotec) and stained with the specific lineage markers described above.

HLA haplotypes and KIR genotyping

HLA haplotypes of 6pLOH(+) patients were determined by either an SNP analysis or estimation by taking advantage of common allelic combinations in the general Japanese population. HLA-C alleles were classified to the C1 or C2 ligand category based on the dimorphism (asparagine or lysine) at position 80 of the alpha-1 domain of the alpha helix (15, 16). HLA-B alleles were assigned as either Bw4 or Bw6, based on the amino acid positions spanning 77-83. HLA-A23, -A24, and -A32 were defined as belonging to the HLA-Bw4 group of serological epitopes.

Genomic DNA was extracted from 5 ml of EDTA anticoagulated PB samples with a Qiagen kit, according to the manufacturer's instructions and was stored at –20°C before use. Genomic DNA of patients and the healthy group were genotyped for the 16 KIR genes (*KIR2DP1, KIR3DP1, KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5,* and *KIR3DS1*) by PCR with sequence-specific primers (SSP)-PCR, as previous described (17, 18). Samples from 35 healthy individuals were subjected to the same KIR genotyping as well.

Definition of KIR and KIR-ligand mismatch, activation and inhibitory receptors of NK cells, KIR haplotypes assignment and KIR ligand combination

KIR and KIR-ligand missing-self were defined according to Ruggeri et al. (4), considering that the missing-self model requires the presence of the corresponding KIR to be detected. For example, a KIR2DL1-ligand missing self involves the presence of the *KIR2DL1* gene and the absence of an HLA-C group 2 allele (C2+) in the target cells.

Activating KIRs are encoded by *KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5,* and *KIR3DS1,* whereas inhibitory KIRs are encoded by *KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5,*

KIR3DL1, KIR3DL2, and *KIR3DL3.* It has been reported that *KIR2DL4* can exert both activating and inhibitory functions (19).

On the basis of gene content, KIRs are classified into group A and B haplotypes. Both groups A and B haplotypes are conserved with four framework genes (*KIR3DL3, KIR3DP1, KIR2DL4,* and *KIR3DL2).* The KIR B haplotype was assigned if one or more KIR B defining loci (activate KIR) were present: *KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR3DS1,* and the genes encoding inhibitory KIRs, *KIR2DL5,* and *KIR2DL2*. Group A haplotype consists of the four framework genes and *KIR2DL3, KIR2DP1, KIR2DL1, KIR3DL1,* and *KIR2DS4.* Due to difference in the gene contents and alleles, patients homozygous for KIR A haplotypes (KIR genotype AA) could be distinguished from patients heterozygous for KIR B haplotypes (KIR genotypes AB or BB, referred together as KIR genotype Bx) (15, 20).

Genotyping of NKG2D

NKG2D gene polymorphisms were genotyped using TaqMan-Allelic discrimination with a StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), as previously described (21, 22). We referred to the CC genotype as LNK/LNK, and the GC and GG genotypes as HNK/LNK and HNK/HNK, respectively.

NK cell phenotyping

Fresh PB or frozen peripheral blood mononuclear cell (PBMC) samples were stained and gated with mAbs as previously described (23-28). The cells were stained with the live and dead antibody (eBioscience™ Fixable Viability Dye eFluor™ 506, cat no. 65-0866-14, Thermo Fisher), anti-CD3-PerCP-Cy5.5 (cat no.340949; BD Biosciences), anti-CD56-APC/PE (cat no.555518/55516; BD Pharmingen), anti-CD158a-APC (KIR2DL1) (cat no.FAB1844A, clone

143211; R&D System), anti-CD158a/h-FITC (KIR2DL1/DS1) (cat no.130-119-150, clone 11PB6; Miltenyi Biotec), anti-CD158b-PE-Cy7 (KIR2DL2/2DL3/2DS2) (cat no.312609, clone DX27; Biolegend), anti-CD158e1-FITC (KIR3DL1) (cat no.555966, clone DX9, BD Pharmingen), and anti-CD158e1/e2-APC (KIR3DL1/3DS1) (cat no.A60795, clone Z27, Beckman Coulter). The cells were analyzed by FCM using a FACSCanto II® instrument (BD Biosciences) with the Flowjo 10.0 software program.

NK-cell degranulation and cytotoxicity assays

Fresh PBMCs from healthy donors who had the same KIR-haplotype as Case 1 and 8 were collected and subjected to an NK-cell degranulation assay and cytotoxicity assay using WT and 6pLOH(+) iPSC-HSPCs from Case 1 and 8 as targets, respectively. NK cells were isolated using a Dynabeads® Untouched™ Human NK Cells kit (cat no. 11349D; Invitrogen) and then co-cultured with target cells at a 5:1 ratio. The NK cells were stained with 7-AAD (cat no. 51-6981E; BD Bioscience), anti-CD3-PerCP-Cy^{TM}5.5 (cat no. 340949; BD Bioscience), anti-CD56-PE, and anti-CD107a-FITC (cat no. 328605, clone H4A3; Biolegend) after 2 h for a CD107a degranulation assay (29-31).

The target cells were collected after 6 h of co-culture with the NK cells to assess the cytotoxic activity. Target iPSC-HSCs were labeled with CFSE (CellTrace™ CFSE Cell Proliferation Kit, cat no. C34554; Thermo Fisher Scientific) and stained with 7-AAD after the co-culture. The K562 cell line phorbol-12-Myristate-13-acetate (PMA) and Ca2+ Ionophore were used as stimulants for positive controls. The assay was considered valid if the following control conditions were met: 1) only effector cells (NK cells; negative control for the target cell death) were negative for CFSE signals and the percentage of dead cells was <5%; 2) only target cells suffered <15% target cell

death; 3) the Tween-mediated killing of target cells (positive control for target cell death) killed >85% of cells. NK cells + target cells + PMA + Ca^{2+} ionophore condition served as a positive control for NK-cell cytotoxicity (29). Every assay was repeated three times. The assays were analyzed by FCM using a FACSCanto II® instrument (BD Biosciences) with the Flowjo 10.0 software program.

Statistical analysis

Categorical variables were analyzed using Fisher's exact test. The *t*-test was used for the comparison of non-paired variables. All statistical analyses were performed using the Stata 12.0 software program. Graphs were generated using GraphPad PRISM 7.0 (GraphPad Software Inc, CA, USA). *P* values of <0.05 (two-sided) were considered to indicate statistical significance.

Results

Persistence of KIR-L(-) leukocytes in patients with AA

Among 408 patients studied for the presence of 6pLOH(+) leukocytes, 261 patients were heterozygous for KIR-Ls, namely C1/C2 or Bw4/Bw6, while the other 147 were homozygous for KIR-L alleles. 6pLOH(+) leukocytes were detected in 47 (18.0%) of the 261 patients who were heterozygous for KIR-Ls. The incidence of 6pLOH(+) leukocytes was comparable to that detected in the other 147 patients (13.6%, 20 patients) who were homozygous for KIR-Ls (p=0.27), suggesting that the elimination of KIR-L(-) leukocytes by autologous NK cells is unlikely to occur in AA patients.

A KIR-L missing status due to 6pLOH, which involved both HLA-C and-B alleles, was found to occur in the leukocytes of 14 (5.4%) of the 261 patients, based on the results of the SNP array and droplet digital PCR (**Fig. 1A, B**); this affected all PB leukocyte lineages (**Fig. 1C**). The HLA-A24 lacking status in Case 1' leukocytes was thought to have occurred as a collateral result of 6pLOH, in order to delete *HLA-B*40:02* (9), an HLA class I allele that is most closely involved in the antigen presentation of AA to T cells, in the *A*24:02-B*40:02-C*03:04* haplotype. The lost KIR-L genes were C1 (n=4, Cases 6, 8, 9 and 14), C2 (n=3, Cases 7, 10, and 11), and Bw4 (n=7, Cases1, 2, 3, 4, 5, 12, and 13). The other 33 patients who were heterozygous for KIR-Ls lost Bw6 as a result of 6pLOH. The missing and retained HLA alleles of the 14 patients with KIR-L(-) leukocytes are shown in **Supplemental Table 2**. Of the 14 patients with KIR-L(-) leukocytes, fresh blood cells were available from 9 patients to examine the HLA-A allele or Bw4 expression levels—which were included in the lost haplotype—using FCM. Consistent with their genotypes, granulocytes that lacked HLA-A or B alleles, which were included in the lost haplotype, were detected in all 9 patients. **Figure 1D** shows representative

scattergrams of two cases (Cases 3 and 8), which indicated the presence of *A*24:02-B*40:01-C*07:02*-lacking granulocytes (Case 3) and *A*24:02-B*54:01[Bw6]-C*01:02*-lacking as well as B54-lacking granulocytes due to a loss-of-function mutation of *B*54:01* (Case 8).

Influence of the KIR-L missing status on the 6pLOH(+) clone size

Of 67 patients with 6pLOH(+) leukocytes, the percentages of 6pLOH(+) cells in the PB leukocytes could be estimated in 40 patients based on the SNP array results. The percentages in 6 patients with the KIR-L missing status (0.2-10.3%, median 5.6%) were significantly lower than those in 34 patients without the KIR-L missing status (0.7-53.9%, median 19.1%) ($p<0.05$, **Fig. 1E, left**). Likewise, the percentages of HLA allele-lacking granulocytes could be determined using FCM for fresh PB samples in 45 patients (including 18 of the above 40 patients). The percentages were significantly lower in 11 patients with the KIR-L missing status (0.8-50.3%, median 15.2%) than in 34 patients without the KIR-L missing status (1.2-99.4%, median 55.4%) ($p<0.05$, **Fig. 1E, right**), suggesting that although KIR-L HSPCs and their progenies survive the NK cell attack, they may suffer from some inhibitory pressure from NK cells.

NK cell subsets and KIR gene repertoires in patients possessing KIR-L(-) leukocytes

To clarify the mechanisms underlying the resistance of KIR-L(-) leukocytes to NK cells, we analyzed NK cell subsets that are expected to attack corresponding KIR-L(-) blood cells through the “missing-self” mechanism in the 9 patients whose PB samples were available. The gating strategy for the eight NK-cell subsets and representative results of the subset identification are shown in **Figure 2A**. Phenotypic analysis of the NK cell subsets defined by anti-2DL1, anti-2DL2/2DL3, and anti-3DL1 mAbs showed that all 9 patients had a similar percentage of the

eight different NK cell subsets, which included 0.2 to 8% effector NK cells capable of killing leukocytes that lacked corresponding KIR-Ls (**Table 2**), suggesting that licensed NK cells capable of killing KIR-L(-) target cells are present in their PB.

KIR genotyping showed that all 14 patients possessed inhibitory KIR genes (*2DL1*, *2DL3*, and *3DL1*) responsive to the corresponding KIR-Ls, as expected (**Table 3**). An activating KIR gene *KIR2DS1*, which is known to be associated with potent graft-versus-leukemia effects in allogeneic hematopoietic stem cell transplant recipients, was possessed by 3 (21.4%) of the 14 patients, a lower percentage than that (40.8%) in a general Japanese population (17). In the three patients (Cases 3, 8, and 9) with *KIR2DS1*, Case 3 who lacked HLA-A24 (Bw4) did not possess C2, suggesting that the lack of the KIR2DS1's ligand on 6pLOH(+) cells may be a reason for the persistence of KIR-L(-) leukocytes in this patient. In contrast, the remaining two patients (Cases 8 and 9) whose 6pLOH(+) leukocytes lacked C1, causing them to become the C2/C2 phenotype, had the *KIR2DS1* gene as well as 2DS1(+) NK-cell subsets in their PB (**Fig. 2B**).

NKG2D polymorphism in patients possessing KIR-L(-) leukocytes

NKG2D, an active and co-stimulatory receptor expressed on NK cells, has two gene polymorphisms, HNK and LNK; the former is associated with higher NK cell activity than the latter (21, 32). The frequencies of HNK/HNK, HNK/LNK, and LNK/LNK alleles in the 14 patients (35.7%, 42.9%, and 21.4%) were similar to those in healthy Japanese individuals (34%, 42%, and 24%) (32) (**Table 3**). Of note, the two patients (Cases 8 and 9) whose C2/C2 leukocytes that lost C1 due to 6pLOH coexisted with 2DS1(+) NK cells, had an LNK/LNK combination, which is characterized by weak NK cell activity.

The expression of HLA-E and HLA-F by peripheral blood leukocytes in KIR-L(-) AA patients

High expression levels of HLA-E, a primary ligand of the inhibitory NK receptor CD94/NKG2A, has been shown to reduce the sensitivity of KIR-L(-) target cells to NK-cell killing (33). In the 9 patients with KIR-L(-) leukocytes, HLA-E was strongly expressed by KIR-L(-) leukocytes, to a similar degree to KIR-L-retaining (normal) granulocytes (**Supplemental Figure S1A**). On the other hand, in keeping with previous reports (34), HLA-F, a ligand of activating receptor KIR3DS1, was not expressed by granulocytes and monocytes, regardless of the presence or absence of KIR-Ls (**Supplemental Figure S1B**).

The expression of HLA-F by iPS cell (iPSC)-derived HSCs with or without the KIR-L missing status

In addition to *KIR2DS1*, Cases 8 and 9 possessed *KIR3DS1*, which is known to activate NK cells through binding to its cognate receptor of HLA-F on target cells. KIR3DL1^{+}/KIR3DS1^{+} NK cells in the two patients accounted for 14.4/18.7% and 10.7%/14%, respectively (**Fig. 2C**).

The HLA-F expression may affect the susceptibility of HSCs to killing by NK cells in individuals possessing *KIR3DS1,* given that the HLA-F gene expression in HSCs has been shown by previous studies (35), although little is known about the HLA-F expression on HSCs. We examined the HLA-F expression on HSCs and hematopoietic progenitor cells (HPCs) from healthy BM, UCB, and BM from an AA patient. The expression of HLA-F in HSCs was observed to be higher than that in HPCs in both BM and UCB from healthy individuals, suggesting that HSCs may become sensitive to killing by 3DS1^{+} NK cells (**Fig. 3A**). Similarly, the expression of HLA-F in HSCs was observed to be higher than that in HPCs in BM from an AA patient who did not possess KIR-L(-) leukocytes (data not shown**)**.

As Case 8's BM cells were unavailable for the examination of HLA-F, the iPSC-derived $CD34^+$ cells were collected from three different iPSCs, including WT, *HLA-B*54:01*-mutated, and 6pLOH(+) iPSCs from Case 8, which had been established in our previous study (13). The iPSC-derived $CD34^+$ cells showed different phenotypes ($A24^-Bw6^-$ [6pLOH], $A24^+Bw6^-$, and $A24^+Bw6^+$ [WT]) compatible with HLA genotypes of each iPSC clone (**Fig. 3B)**. The HLA-F expression level in iPSC-derived HSPCs (iPSC-HSPCs) was generally higher rather than that in HSPCs in primary BM or UCB (**Fig. 3A, 3C**). The 6pLOH(+) HSPCs virtually lacked the expression of HLA-F while KIR-L(+) $A24^+Bw6^-$ HSPCs expressed HLA-F at a similar level to WT HSPCs (**Fig. 3C**).

Sensitivity of iPSC-HSPCs to NK cells expressing 3DS1

To determine whether or not the difference in the expression of HLA-F on HSPCs affected the sensitivity to NK-cell cytotoxicity, we compared the CD107a expression on NK cells induced by co-culture with different iPSC-HSPCs. CD107a was induced on NK cells assays to a lesser degree by 6pLOH(+) iPSC-HSPCs (5.6% ± 2.31% and 8% ± 3.05%) than by WT iPSC-HSPCs (13.3% ± 4.15% and 18.6% ± 2.34%) in Cases 8 and 1, respectively ($p<0.05$, **Fig. 4A and Supplemental Figure 1D and 1F**). When the cytotoxicity of NK cells from the healthy individual was assessed using the 7-AAD positivity in CFSE-labelled target cells, the NK cells killed 42.7% ± 5.95% of K562 cells (**Supplemental Figure 1C**). In accordance with the results of the NK-cell degranulation assay, 6pLOH(+) iPSC-HSPCs from Cases 1 and 8 were killed by NK cells less efficiently than their WT counterparts (18.8% ± 5.67% vs. 28.9% ± 6.89%, $p<0.05$, 12.5% ± 6.75% vs. 31.5% ± 7.89%, $p<0.05$), respectively (**Fig. 4B and Supplemental Figure 1E and 1F**).

Discussion

This study identified 14 AA patients in remission whose leukocytes lost KIR-L genes, and which may thereby become vulnerable to autologous NK-cell attack in 67 6pLOH(+) patients. Indeed, KIR-L(-) leukocytes were detected in all 9 patients whose PB leukocytes were available for an FCM analysis. All of these patients were found to possess KIR^+ licensed NK cells that are capable of killing corresponding KIR-L(-) blood leukocytes. Although the persistence of KIR-L(-) leukemia cells due to 6pLOH has been shown in patients after HLA-mismatched allogeneic hematopoietic stem cell transplantation (36, 37), this study is the first to demonstrate the paradoxical coexistence of functional NK cells and KIR-L(-) target cells that do not have a proliferative advantage like leukemia cells.

HSPCs that spontaneously suffer 6pLOH are thought to be present in a dormant state in the BM of healthy individuals. Some 6pLOH(+) HSPCs acquire a survival advantage over WT HSPCs when CTLs specific to autoantigens presented by particular HLA class I alleles were generated and attack HSPCs at the onset of AA, because the 6pLOH(+) cells lack the HLA alleles that are required for antigen presentation. This escape of 6pLOH(+) HSPCs was expected to occur less frequently in individuals who are heterozygous for C1/C2, Bw4/Bw6, or HLA-A24/HLA-Ax and therefore have a lower risk of becoming NK-cell-sensitive as a result of the KIR-L missing status than those who were homozygous for C1, C2, Bw4, Bw6, or A24, or who lacked A24. However, the incidence of 6pLOH(+) cells (18.0%) in the former group was comparable to that (13.6%) in the latter group. This finding, and the persistence of KIR-L(-) leukocytes clearly indicate that the KIR-L missing status is not enough for the target cells to be killed by autologous NK cells *in vivo*.

Then how do KIR-L(-) leukocytes protect themselves from the NK-cell attack? Mechanisms

underlying the resistance of KIR-L(-) leukemia cells to NK cells after HLA-mismatched stem cell transplantation have been a subject of extensive studies. High HLA-E expression levels were shown to inhibit NKG2A$^+$ NK cells from killing KIR-L(-) leukemia cells (33). Although the HLA-E expression level of KIR-L(-) granulocytes was comparable to that of WT granulocytes, the high expression of HLA-E may play a role in protecting KIR-L(-) granulocytes from the NK-cell attack. It is also possible that an augmented NKG2A expression may prevent missing-self-reactive NK cells from killing KIR-L(-) leukocytes. However, we were unable to find evidence showing that a greater expression of NKG2A by patients' NK cells than by healthy individuals' NK cells attenuates the NK-cell cytotoxicity. Conversely, a previous study by Chen et al. showed that the NKG2A expression by NK cells was significantly lower in AA patients than in healthy controls (38). It is therefore unlikely that such an augmented NKG2A expression by NK cells was involved in the sparing of KIR-L(-) leukocytes.

According to the "missing-self" hypothesis, the activation of NK cells occurs in contact with malignantly transformed cells that have lost MHC class I molecules and that have additionally acquired stress-induced ligands to activate NK cell receptors. The activation of receptors on NK cells such as 2DS1 and 3DS1 has been shown to play essential roles in the killing of target cells (39, 40). The lack of the *KIR2DS1* and *KIR3DS1* genes in 11 patients (Cases 1, 2, 4, 5, 6, 7, 10, 11, 12, 13, and 14) and the lack of 2DS1 ligand C2 on leukocytes in Case 3 may in part explain the persistence of KIR-L(-) cells in these patients.

On the other hand, Cases 8 and 9 possessed both 2DS1(+) and 3DS1(+) NK cells, and their 6pLOH(+) leukocytes were homozygous for the 2DS1 ligand C2. One possible reason for the resistance of their leukocytes to NK cells is that they had an NKG2D allele combination LNK/LNK, which is known to be associated with lower NK cell activity. A current report

highlighted the role of NKG2D-L in the susceptibility of leukemic stem cells to NK-cell killing (41). The lower NK cell activity may be involved in the sparing of KIR-L(-) leukocytes by the two patients' NK cells. Another plausible explanation is the disarming of NK cells in the two patients. $2DS1^+$ NK cells in individuals with KIR-L haplotypes C1/C2 and C2/C2 have been shown to exert lower NK cell activity against KIR-L(-) target cells than in those in individuals with C1/C1 (24). NK cell disarming may have affected the persistence of KIR-L(-) leukocytes in Cases 8 and 9, who originally had C1/C2 haplotypes.

While HLA-E and -G have been well characterized as antigen-presenting molecules, both functionally and structurally, the role of HLA-F in regulating the immune system has remained uncovered until recently. The expression of HLA-F is strictly controlled and tissue-specific, with higher levels in lymphoid cells in comparison to non-lymphoid cells and is predominantly localized to the endoplasmic reticulum (42-44). A previous study showed that $KIR3DS1^+$ NK cells exhibited the highest affinity to HLA-F and induced the most potent functional signaling upon engagement of HLA-F open conformers (OCs) on target cells (45, 46). We found that HLA-F was predominantly expressed on primary HSCs, and its expression decreased in other HSPCs during the differentiation of HSCs, a finding compatible with the available RNA seq dataset in HSPCs (35). Of note, 6pLOH(+) iPSC-HSPCs, which are considered to reflect the phenotype of the patient's original HSPCs due to the epigenetic memory (47), showed lower HLA-F expression levels than WT HSPCs. Moreover, our NK-cell degranulation and cytotoxicity assays showed a decreased sensitivity of 6pLOH(+) iPSC-HSPCs from Cases 1 and 8 to NK cells compared to their WT counterparts. These data suggest that a decreased HLA-F expression may be partially responsible for the persistence of KIR-L(-) HSCs.

In conclusion, NK cells were able to inhibit the expansion of KIR-L(-) leukocytes, but were

unable to eliminate them through various mechanisms that weaken the NK cell activity (the lack of activating KIRs on NK cells, the LNK/LNK NKG2D alleles, and disarming of NK cells) or confer resistance to target cells (a decrease in the HLA-F expression level on HSCs) (**Supplemental Figure S2**). These findings indicate the limitation of the NK cells' ability to control malignant cells or virally infected cells that lose KIR-Ls to escape the CTL attack *in vivo*.

Acknowledgments

We thank Toshio Yabe for careful reading of an earlier version of the manuscript, and Rie Ohmi for their excellent technical assistance. iPSCs were provided by Kazuhisa Chonabayashi and Prof. Yoshinori Yoshida at Kyoto University. The authors thank the patients, donors, and their physicians for contributing to this study and the Advanced Preventive Medical Sciences Research Center, Kanazawa University for the use of their facilities. M.A.T.N. is a Ph.D. candidate at Kanazawa University, and this work is submitted in partial fulfillment of the requirements for the Ph.D.

Footnote

This work was supported by MEXT KAKENHI (Grant-in-Aid for Scientific Research (B), Grant Number: 24390243) to S.N., MEXT KAKENHI (Grant-in-Aid for Scientific Research (C), Grant Number: 17K09007) to T.K., MEXT KAKENHI (Grant-in-Aid for Young Scientists (B), Grant Number: 17K16184, 19K17823) to K.H., Hokkoku Foundation for Cancer Research to T.K and K.H., SENSHIN Medical Research Foundation to K.H.

Authorship Contribution

K.H., T.Y., H.M., M.T., T.K., N.N., and S.N. collected clinical data and blood samples. M.A.T.N. and T.Y. performed flow cytometry. M.A.T.N. and J.L.E. performed the KIR genotyping. M.A.T.N., M.I.E., and M.M. performed the iPSC culture. K.K. and H.S. performed HLA genotyping. S.O. performed SNP array analysis. M.A.T.N., K.H., N.A., and S.N. designed the research and wrote the manuscript. All authors critically reviewed the manuscript and checked the final version. M.A.T.N., K.H., and T.Y contributed equally to this work.

Conflict of Interest

The authors declare no conflicts of interest in association with the present study.

References

1. Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436: 709-713.
2. Ljunggren, H. G., and K. Karre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology today* 11: 237-244.
3. Symons, H. J., and E. J. Fuchs. 2008. Hematopoietic SCT from partially HLA-mismatched (HLA-haploidentical) related donors. *Bone Marrow Transpl* 42: 365-377.
4. Ruggeri, L., A. Mancusi, M. Capanni, E. Urbani, A. Carotti, T. Aloisi, M. Stern, D. Pende, K. Perruccio, E. Burchielli, F. Topini, E. Bianchi, F. Aversa, M. F. Martelli, and A. Velardi. 2007. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood* 110: 433-440.
5. Chewning, J. H., C. N. Gudme, K. C. Hsu, A. Selvakumar, and B. Dupont. 2007. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group in vitro. *Journal of immunology* 179: 854-868.
6. Ljunggren, H. G., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *The Journal of experimental medicine* 162: 1745-1759.
7. Katagiri, T., A. Sato-Otsubo, K. Kashiwase, S. Morishima, Y. Sato, Y. Mori, M. Kato, M. Sanada, Y. Morishima, K. Hosokawa, Y. Sasaki, S. Ohtake, S. Ogawa, S. Nakao, and P. Japan Marrow Donor. 2011. Frequent loss of HLA alleles associated with copy number-neutral 6pLOH in acquired aplastic anemia. *Blood* 118: 6601-6609.
8. Maruyama, H., T. Katagiri, K. Kashiwase, T. Shiina, A. Sato-Otsubo, Y. Zaimoku, K. Maruyama, K. Hosokawa, K. Ishiyama, H. Yamazaki, H. Inoko, S. Ogawa, and S. Nakao. 2016. Clinical significance and origin of leukocytes that lack HLA-A allele expression in patients with acquired aplastic anemia. *Experimental hematology* 44: 931-939.
9. Zaimoku, Y., H. Takamatsu, K. Hosomichi, T. Ozawa, N. Nakagawa, T. Imi, H. Maruyama, T. Katagiri, H. Kishi, A. Tajima, A. Muraguchi, K. Kashiwase, and S. Nakao. 2017. Identification of an HLA class I allele closely involved in the autoantigen presentation in acquired aplastic anemia. *Blood* 129: 2908-2916.
10. Camitta, B. M., and K. Doney. 1990. Immunosuppressive therapy for aplastic anemia: indications, agents, mechanisms, and results. *Am J Pediatr Hematol Oncol* 12: 411-424.
11. Notta, F., S. Zandi, N. Takayama, S. Dobson, O. I. Gan, G. Wilson, K. B. Kaufmann, J. McLeod, E. Laurenti, C. F. Dunant, J. D. McPherson, L. D. Stein, Y. Dror, and J. E. Dick. 2016. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science* 351: 139-+.
12. Notta, F., S. Doulatov, E. Laurenti, A. Poeppl, I. Jurisica, and J. E. Dick. 2011. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 333: 218-221.
13. Elbadry, M. I., H. Mizumaki, K. Hosokawa, J. L. Espinoza, N. Nakagawa, K. Chonabayashi, Y. Yoshida, T. Katagiri, K. Hosomichi, Y. Zaimoku, T. Imi, M. A. T. Nguyen, Y. Fujii, A. Tajima, S. Ogawa, K. Takenaka, K. Akashi, and S. Nakao. 2019. Escape hematopoiesis by HLA-B5401-lacking hematopoietic stem progenitor cells in men with acquired aplastic anemia. *Haematologica* 104: E447-E450.
14. Espinoza, J. L., M. I. Elbadry, K. Chonabayashi, Y. Yoshida, T. Katagiri, K. Harada, N. Nakagawa, Y. Zaimoku, T. Imi, H. Takamatsu, T. Ozawa, H. Maruyama, H. A. Hassanein, A. N. A. Khalifa, K. Takenaka, K. Akashi, H. Hamana, H. Kishi, Y. Akatsuka, and S. Nakao. 2018. Hematopoiesis by iPSC-derived hematopoietic stem cells of aplastic anemia that escape cytotoxic T-cell attack. *Blood advances* 2: 390-400.
15. Uhrberg, M., N. M. Valiante, B. P. Shum, H. G. Shilling, K. Lienert-Weidenbach, B. Corliss, D. Tyan, L. L. Lanier, and P. Parham. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity* 7: 753-763.
16. Cerwenka, A., and L. L. Lanier. 2001. Ligands for natural killer cell receptors: redundancy or specificity. *Immunological reviews* 181: 158-169.
17. Mogami, S., G. Hasegawa, I. Nakayama, M. Asano, H. Hosoda, M. Kadono, M. Fukui, Y. Kitagawa, K. Nakano, M. Ohta, H. Obayashi, T. Yoshikawa, and N. Nakamura. 2007. Killer cell immunoglobulin-like receptor genotypes in Japanese patients with type 1 diabetes. *Tissue antigens* 70: 506-510.
18. Vilches, C., J. Castano, N. Gomez-Lozano, and E. Estefania. 2007. Facilitation of KIR genotyping by a PCR-SSP method that amplifies short DNA fragments. *Tissue antigens* 70: 415-422.

19. Kikuchi-Maki, A., S. Yusa, T. L. Catina, and K. S. Campbell. 2003. KIR2DL4 is an IL-2-regulated NK cell receptor that exhibits limited expression in humans but triggers strong IFN-gamma production. *Journal of immunology* 171: 3415-3425.
20. Bashirova, A. A., M. P. Martin, D. W. McVicar, and M. Carrington. 2006. The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense. *Annual review of genomics and human genetics* 7: 277-300.
21. Hayashi, T., K. Imai, Y. Morishita, I. Hayashi, Y. Kusunoki, and K. Nakachi. 2006. Identification of the NKG2D haplotypes associated with natural cytotoxic activity of peripheral blood lymphocytes and cancer immunosurveillance. *Cancer research* 66: 563-570.
22. Furue, H., H. Kumimoto, K. Matsuo, T. Suzuki, Y. Hasegawa, M. Shinoda, T. Sugimura, K. Mitsudo, I. Tohnai, M. Ueda, K. Tajima, and K. Ishizaki. 2008. Opposite impact of NKG2D genotype by lifestyle exposure to risk of aerodigestive tract cancer among Japanese. *International journal of cancer* 123: 181-186.
23. Malnati, M. S., E. Ugolotti, M. C. Monti, D. Battista, I. Vanni, D. Bordo, F. Sironi, P. Larghero, E. D. Marco, P. Biswas, G. Poli, E. Vicenzi, A. Riva, M. Tarkowski, G. Tambussi, S. Nozza, G. Tripodi, F. Marras, A. De Maria, A. Pistorio, and R. Biassoni. 2017. Activating Killer Immunoglobulin Receptors and HLA-C: a successful combination providing HIV-1 control. *Scientific reports* 7: 42470.
24. Pittari, G., X. R. Liu, A. Selvakumar, Z. Zhao, E. Merino, M. Huse, J. H. Chewning, K. C. Hsu, and B. Dupont. 2013. NK cell tolerance of self-specific activating receptor KIR2DS1 in individuals with cognate HLA-C2 ligand. *Journal of immunology* 190: 4650-4660.
25. Pascal, V., E. Yamada, M. P. Martin, G. Alter, M. Altfeld, J. A. Metcalf, M. W. Baseler, J. W. Adelsberger, M. Carrington, S. K. Anderson, and D. W. McVicar. 2007. Detection of KIR3DS1 on the cell surface of peripheral blood NK cells facilitates identification of a novel null allele and assessment of KIR3DS1 expression during HIV-1 infection. *J Immunol* 179: 1625-1633.
26. Wang, W., A. K. Erbe, K. A. Alderson, E. Phillips, M. Gallenberger, J. Gan, D. Campana, J. A. Hank, and P. M. Sondel. 2016. Human NK cells maintain licensing status and are subject to killer immunoglobulin-like receptor (KIR) and KIR-ligand inhibition following ex vivo expansion. *Cancer immunology, immunotherapy : CII* 65: 1047-1059.
27. Zhang, X., X. Lu, C. Moog, L. Yuan, Z. Liu, Z. Li, W. Xia, Y. Zhou, H. Wu, T. Zhang, and B. Su. 2018. KIR3DL1-Negative CD8 T Cells and KIR3DL1-Negative Natural Killer Cells Contribute to the Advantageous Control of Early Human Immunodeficiency Virus Type 1 Infection in HLA-B Bw4 Homozygous Individuals. *Frontiers in immunology* 9.
28. Trundley, A., H. Frebel, D. Jones, C. Chang, and J. Trowsdale. 2007. Allelic expression patterns of KIR3DS1 and 3DL1 using the Z27 and DX9 antibodies. *European journal of immunology* 37: 780-787.
29. Kandarian, F., G. M. Sunga, D. Arango-Saenz, and M. Rossetti. 2017. A Flow Cytometry-Based Cytotoxicity Assay for the Assessment of Human NK Cell Activity. *Journal of visualized experiments : JoVE*.
30. Shabrish, S., M. Gupta, and M. Madkaikar. 2016. A Modified NK Cell Degranulation Assay Applicable for Routine Evaluation of NK Cell Function. *Journal of immunology research* 2016: 3769590.
31. Bryceson, Y. T., C. Fauriat, J. M. Nunes, S. M. Wood, N. K. Bjorkstrom, E. O. Long, and H. G. Ljunggren. 2010. Functional analysis of human NK cells by flow cytometry. *Methods in molecular biology* 612: 335-352.
32. Imai, K., S. Matsuyama, S. Miyake, K. Suga, and K. Nakachi. 2000. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* 356: 1795-1799.
33. Lo Monaco, E., E. Tremante, C. Cerboni, E. Melucci, L. Sibilio, A. Zingoni, M. R. Nicotra, P. G. Natali, and P. Giacomini. 2011. Human leukocyte antigen E contributes to protect tumor cells from lysis by natural killer cells. *Neoplasia* 13: 822-830.
34. Lee, N., A. Ishitani, and D. E. Geraghty. 2010. HLA-F is a surface marker on activated lymphocytes. *European journal of immunology* 40: 2308-2318.
35. Boegel, S., M. Lower, T. Bukur, P. Sorn, J. C. Castle, and U. Sahin. 2018. HLA and proteasome expression body map. *BMC medical genomics* 11: 36.
36. Toffalori, C., M. Riba, L. Zito, M. Barcella, O. Spinelli, L. Crucitti, N. Cieri, J. Peccatori, M. Bernardi, C. Bonini, D. Cittaro, D. Lazarevic, A. Rambaldi, C. Barlassina, E. Stupka, F. Ciceri, K. Fleischhauer, and L. Vago. 2014. Acute Myeloid Leukemia Relapses after Allogenenic HSCT Display a Distinctive Immune-Related Signature, with Frequent and Functionally Relevant Alterations in HLA Class II Antigen Presentation and T Cell

Costimulation. *Blood* 124: 427-427.
37. Christopher, M. J., A. A. Petti, M. P. Rettig, C. A. Miller, E. Chendamarai, E. J. Duncavage, J. M. Klco, N. M. Helton, M. O'Laughlin, C. C. Fronick, R. S. Fulton, R. K. Wilson, L. D. Wartman, J. S. Welch, S. E. Heath, J. D. Baty, J. E. Payton, T. A. Graubert, D. C. Link, M. J. Walter, P. Westervelt, T. J. Ley, and J. F. DiPersio. 2018. Immune Escape of Relapsed AML Cells after Allogeneic Transplantation. *The New England journal of medicine* 379: 2330-2341.
38. Chen, T., T. Zhang, C. Liu, C. Wang, S. Ding, Z. Shao, and R. Fu. 2019. NK cells suppress CD8(+) T cell immunity via NKG2D in severe aplastic anemia. *Cell Immunol* 335: 6-14.
39. Carlomagno, S., M. Falco, M. Bono, C. Alicata, L. Garbarino, M. Mazzocco, L. Moretta, A. Moretta, and S. Sivori. 2017. KIR3DS1-Mediated Recognition of HLA-*B51: Modulation of KIR3DS1 Responsiveness by Self HLA-B Allotypes and Effect on NK Cell Licensing. *Frontiers in immunology* 8: 581.
40. Fauriat, C., M. A. Ivarsson, H. G. Ljunggren, K. J. Malmberg, and J. Michaelsson. 2010. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood* 115: 1166-1174.
41. Paczulla, A. M., K. Rothfelder, S. Raffel, M. Konantz, J. Steinbacher, H. Wang, C. Tandler, M. Mbarga, T. Schaefer, M. Falcone, E. Nievergall, D. Dorfel, P. Hanns, J. R. Passweg, C. Lutz, J. Schwaller, R. Zeiser, B. R. Blazar, M. A. Caligiuri, S. Dirnhofer, P. Lundberg, L. Kanz, L. Quintanilla-Martinez, A. Steinle, A. Trumpp, H. R. Salih, and C. Lengerke. 2019. Absence of NKG2D ligands defines leukaemia stem cells and mediates their immune evasion. *Nature* 572: 254-259.
42. Boyle, L. H., A. K. Gillingham, S. Munro, and J. Trowsdale. 2006. Selective export of HLA-F by its cytoplasmic tail. *Journal of immunology* 176: 6464-6472.
43. Lee, N., and D. E. Geraghty. 2003. HLA-F surface expression on B cell and monocyte cell lines is partially independent from tapasin and completely independent from TAP. *Journal of immunology* 171: 5264-5271.
44. Lepin, E. J., J. M. Bastin, D. S. Allan, G. Roncador, V. M. Braud, D. Y. Mason, P. A. van der Merwe, A. J. McMichael, J. I. Bell, S. H. Powis, and C. A. O'Callaghan. 2000. Functional characterization of HLA-F and binding of HLA-F tetramers to ILT2 and ILT4 receptors. *European journal of immunology* 30: 3552-3561.
45. Garcia-Beltran, W. F., A. Holzemer, G. Martrus, A. W. Chung, Y. Pacheco, C. R. Simoneau, M. Rucevic, P. A. Lamothe-Molina, T. Pertel, T. E. Kim, H. Dugan, G. Alter, J. Dechanet-Merville, S. Jost, M. Carrington, and M. Altfeld. 2016. Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1. *Nature immunology* 17: 1067-1074.
46. Goodridge, J. P., A. Burian, N. Lee, and D. E. Geraghty. 2013. HLA-F and MHC class I open conformers are ligands for NK cell Ig-like receptors. *Journal of immunology* 191: 3553-3562.
47. Kim, K., A. Doi, B. Wen, K. Ng, R. Zhao, P. Cahan, J. Kim, M. J. Aryee, H. Ji, L. I. Ehrlich, A. Yabuuchi, A. Takeuchi, K. C. Cunniff, H. Hongguang, S. McKinney-Freeman, O. Naveiras, T. J. Yoon, R. A. Irizarry, N. Jung, J. Seita, J. Hanna, P. Murakami, R. Jaenisch, R. Weissleder, S. H. Orkin, I. L. Weissman, A. P. Feinberg, and G. Q. Daley. 2010. Epigenetic memory in induced pluripotent stem cells. *Nature* 467: 285-290.

Figure legends

FIGURE 1. **KIR-L-missing (KIR-L[-]) leukocytes due to 6pLOH in AA patients.**

A KIR-L-missing status due to 6pLOH, which involved both the HLA-C and-B alleles, was found to occur in the leukocytes of 14 of the 261 patients who were heterozygous for KIR-Ls. (A) Representative results (Case 1) of 6pLOH detected by SNP array. (B) Representative ddPCR plots are showing the presence of 6pLOH, which involved HLA-C alleles (Case 1) or HLA-B alleles (Case 9). 6-FAM-positive blue dots are detected if the following alleles were present in droplets: *HLA-C03:04* for C3 mixture (Case 1) and *HLA-B48:01* for B2 mixture (Case 9); while VIC-positive green dots were detected if the droplets contained other alleles. Orange dots represent the droplets carrying both two alleles. The percentage of 6pLOH in HLA-C (Case 1) and HLA-B (case 9) was 67.1% and 6.8%, respectively. (C) Representative flow cytometry (FCM) scattergrams are showing HLA-A24-lacking cells in the peripheral blood granulocytes, monocytes, B cells, and T cells of Case 1. (D) Representative scattergrams of two patients (Cases 3 and 8) that indicated the presence of *A*24:02-B*40:01-C*07:02* haplotype-lacking granulocytes (Case 3) and *A*24:02-B*54:01[Bw6]-C*01:02* haplotype-lacking granulocytes, as well as B54-lacking granulocytes due to a loss-of-function mutation of *B*54:01* (Case 8). (E) The percentage of 6pLOH fractions determined by the SNP array analysis (left) and the percentage of HLA-lacking granulocytes detected by FCM (right) in 6pLOH(+) AA patients with or without a missing KIR-L. The asterisk indicates statistical significance ($p<0.05$).

FIGURE 2. NK cell subsets defined by the KIR expression in patients possessing KIR-L(-) leukocytes.

(A) The identification of the eight different NK-cell subsets defined by the KIR expression in Case 8. (B) Representative dot plots showing the absence (Healthy 1) and the presence (Healthy 2 and Cases 8 and 9) of KIR2DL1^{-}KIR2DS1^{+} NK cells. (C) Representative dot plots showing the absence (Healthy 3) and presence (Healthy 4 and Cases 3, 8, and 9) of KIR3DL1^{-}KIR3DS1^{+} NK cells.

FIGURE 3. The HLA-F expression on bone marrow and cord blood HSPCs and iPSC-derived HSPCs.

(A) A gating strategy for HSCs and HSPCs in the bone marrow (BM) and the umbilical cord blood (UCB) (left), and HLA-F expression on healthy BM and UCB (right). HSC (hematopoietic stem cell; red line), MPP (multipotent progenitor; blue dotted line), MLP (multilymphoid progenitor; light green dashed line), CMP (common myeloid progenitor; dark green complex line), GMP (granulocyte-monocyte progenitor; violet long dashed line), MEP (megakaryocyte-erythroid progenitors; pink dot-dashed line), and isotype control (grey shadow). (B) Scattergrams showing three different HLA phenotypes (A24^{-}Bw6^{-} [6pLOH], A24^{+}Bw6^{-}, and A24^{+}Bw6^{+} [WT]) of iPSC-HSPCs from Case 8. (C) Histograms showing the different HLA-F expression levels between WT, HLA-B(-), and 6pLOH(+) iPSC-HSPCs from Case 8.

FIGURE 4. Sensitivity of different iPSC-HSPCs to NK cells expressing 3DS1.

(A) Degranulation of NK cells in response to different target cells. $CD3^{-}CD56^{+}$ NK cells that express CD107a in response to different target cells, including K562 cells, WT iPSC-HSPCs, and 6pLOH(+) HSPCs, are shown. The left upper scattergram shows a gating method for $CD3^{-}CD56^{+}$ cells that are derived from mononuclear cells compatible with NK cells on the side scatter (SSC-A) versus forward scatter (FSC-A) diagram. The bar plot represents the percentage of $CD107a^{+}$ NK cells among total NK cells that were co-cultured with the indicated target cells. (B) Cytotoxicity against HSPCs from Case 8 by $3DS1^{+}$ NK cells. 7-AAD^{+} dead cells among different target cells, including K562 cells, WT iPSC-HSPCs, and 6pLOH(+) HSPCs, induced by NK cells are shown. The right bar plot represents the percentage of 7-AAD^{+} cells in each target cell population. Graph bars show the mean ± SEM. *indicates a significant difference $p<0.05$.