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DNMT1 silencing induces KIR2DL1/2/3 expression via methylation to alleviate graft-*versus*-host disease after allogeneic hematopoietic stem cell transplantation

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Summary. Natural killer (NK) cells are the promoters in graft-*versus*-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT), while demethylation can regulate NK cell function. We explored the mechanism of demethylation regulating NK cell function to affect GVHD after allo-HSCT. BALB/c mice were transfused with C57BL/6 mouse-derived NK and bone marrow cells to establish GVHD models, followed by isolation and *in vitro* expansion of NK cells. NK cell purity, cytokine levels, proliferation, and cytokine-producing NK cell levels were measured via flow cytometry. KIR2DL1/2/3 methylation was tested by Methylation-specific polymerase chain reaction (MSP), with determination of mouse survival and GVHD scores. KIR2DL1/2/3 and DNMT1 expression was detected through qRT-PCR and/or western blot. Methylation levels were upregulated and KIR2DL1/2/3 expression was downregulated in GVHD mouse model-derived NK cells following IL-2 stimulation. DNMT1 silencing promoted KIR2DL1/2/3 expression, proliferation, and the secretion of Granzyme, Perforin, and Interferon-γ (IFN-γ) in C57BL/6 mouse-derived NK cells. DNMT1 silencing also enhanced mouse survival, reduced GVHD scores, promoted KIR2DL1/2/3 expression on the NK cell surface, and increased the secretion of Granzyme, Perforin, IFN-γ, and the number of cytokine-producing NK cells in the spleen, liver, and lung tissues of the models. Collectively, DNMT1 silencing induced KIR2DL1/2/3 expression in NK cells through reducing methylation to alleviate GVHD after allo-HSCT.

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Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) plays an irreplaceable role in the treatment of hematologic diseases as a curative therapeutic option (Williamson et al., 2016; Saha and Blazar, 2022). However, some complications may occur, with graft-*versus*-host disease (GVHD) being a major one (Liu et al., 2017; Barzin et al., 2022). GVHD has been the major obstacle of allo-HSCT, the occurrence and severity of which directly affect the efficacy of allo-HSCT and patients' quality of survival (Zeiser and Blazar, 2017). Hence, given the poorly understood mechanism of GVHD, further investigations are imperative.

The role of natural killer (NK) cells in post-transplant immune reconstitution and the impact of their surface receptor killer immunoglobulin-like receptors (KIR) genotyping on the prognosis of allo-HSCT have drawn much attention in recent years. NK cells are cytotoxic granular lymphocytes (Kim et al., 2022), which have been regarded as important players in the natural immune system (Shi et al., 2016). Their cytotoxic activity has been proven to be regulated by KIRs (Dębska-Zielkowska et al., 2021). Importantly, NK cells have been perceived as the promoters in the progression of GVHD after allo-HSCT (Blazar et al., 2018). In other words, the occurrence of GVHD, to different degrees, may be stimulated by an increase in NK cells (Zhang et al., 2019). The *KIR* gene family is located on human chromosome 19q13.4, and its

expression on the surface of NK cells is highly random and diverse. NK cells are therefore able to recognize the ever-changing class I molecules of leukocyte antigens, thereby specifically clearing abnormal cells (Rascle et al., 2023). As an important means of epigenetic regulation of gene expression, methylation is involved in the silencing of multiple genes (Meng et al., 2015). The promoter region of the *KIR* gene is rich in CpG dinucleotides and is a potential target for methylation regulation; NK cells can maintain a clonally restricted expression of highly homologous *KIR* genes via DNA methylation (Chan et al., 2003). Further, it has been acknowledged that demethylation leads to upregulation of KIRs in NK cells (Li et al., 2019). However, whether demethylation can participate in the effects of NK cells on GVHD after allo-HSCT via regulating KIR expression is still incompletely illuminated.

To date, demethylation as a treatment strategy has aroused widespread interest (Yang et al., 2024). For instance, demethylation has been proven to activate corresponding receptor genes to promote the expression of NK-cell receptors (Costello et al., 2015). High methylation of histones suppresses NK-cell activation by inhibiting NK-cell receptor (NKG2D) activity (Shi et al., 2016). Prior studies elucidated that *in vitro* culture of the NK92MI cell line with demethylation treatment causes an elevated expression of KIR in NK cells (Gao et al., 2009). As a vital methyltransferase, DNA methyltransferase-1 (DNMT1) is the mediator of DNA methylation, which is responsible for copying DNA methylation onto the newly synthesized DNA strand following the replication of relative DNAs (Tao et al., 2022). More importantly, DNMT1 inhibitors have been reported to relieve GVHD (Choi et al., 2014). Collectively, the present study aimed to investigate the underlying mechanism by which demethylation affects NK cells and thus attenuates GVHD after allo-HSCT.

Materials and methods

Ethics statement and animals

In the present study, all animal experiments were conducted in accordance with the guidelines of the China Council on Animal Care and Use, and the experimental protocol was approved by the Ethics Committee of Zhejiang Baiyue Biotech Co., Ltd for Experimental Animals Welfare (approval number: ZJBYLA-IACUC-20221020).

C57BL/6 mice (18-22 g; male; n=24) were obtained as the donors, and BALB/c mice (18-22 g; male; n=30) were procured as recipients. All mice were maintained in an environmentally controlled animal laboratory with a standard day-night cycle at 20-26°C and humidity of 40-70%.

NK cell isolation, in vitro expansion, and transfection

The experimental processes referred to a previous study (Wang et al., 2020). C57BL/6 mice (n=24) were sacrificed via cervical dislocation after anesthesia with 0.3% pentobarbital sodium (50 mg/kg, P3761, Haoran Biological Technology, Shanghai, China). The collected mouse spleens were milled, and filtered to obtain splenocytes. The splenocytes were incubated with CD90.1 MicroBeads (130-121-273, Miltenyi Biotec, San Diego, CA, USA) and DX5 MicroBeads (130-052-501, Miltenyi Biotec, USA), respectively, to produce CD3-DX5+ NK cells with high purity. Then the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, M012-500, VectorBuilder, Guangzhou, China) with 10% fetal bovine serum (FBS, S9030, Solarbio, Beijing, China) in a 5% CO_2 environment at 37°C, and flow cytometry was used to identify the cell purity.

For *in vitro* expansion of $CD3^-DX5^+$ NK cells, cells (1×10^4 cells/well) were cultured in a 96-well plate, and treated with 100 U/ml interleukin 2 (IL-2, I0523, Sigma-Aldrich, Darmstadt, Germany) in each well for 24h (37°C) (Becker et al., 2016).

For transfection, the short hairpin RNA (pGPU60 vector, C01001) targeting DNMT1 (shDNMT1, sense: 5'-GGTAGAGAGTTACGACGAA-3'; antisense, 5'-TTCGTCGTAACTCTCTACC-3') and its negative control SuperSilencing™ shRNA (shNC) were customized by GenePharma (Shanghai, China). Transfection of shDNMT1/shNC into NK cells was achieved by Lipofectamine 3000 transfection reagent (L3000015, Thermo Fisher Scientific, Waltham, MA, USA). In short, NK cells (1×10^4 cells/well) were seeded in each well of a 96-well plate until a 90% confluent monolayer was formed. Opti-MEM medium (31985062, Thermo Fisher Scientific, USA) and P3000™ reagent were used to dilute Lipofectamine™ 3000 reagent and shRNA, followed by a 15-minute incubation at 37°C. Finally, the gene-lipid complexes were added to each well and incubated for another 48h at 37°C.

Mouse models of GVHD and isolation of NK cells

Prior to the establishment of GVHD mouse models, femurs and shin bones from C57BL/6 mice were collected and washed in phosphate-buffered saline (PBS, abs961, Absin, China) to obtain bone marrow cells, followed by centrifugation at 300×g for 10 min and PBS washing again. Later, bone marrow cells were also purified following the processes mentioned above, and were prepared at a final concentration of 1×10^7 cells/ml for subsequent analyses.

To induce allo-HSCT, the recipient BALB/c mice were firstly irradiated (8.5 GY) using an X-ray irradiator (WBK-01, 350 kV, 11.4 mA, a Thoraeus filter [0.75 mm Tin (Sn), 0.25 mm Copper (Cu), and 1.5 mm Aluminum (Al), Dandong Tongda Science&Technology Co., Ltd, China), and then transfused with 1×10^7 bone marrow cells and 1×10^5 NK cells via the tail vein. The recipient mice in the Control, GVHD, GVHD+NK, GVHD+NK(shNC), and GVHD+NK(shDNMT1) groups (six mice per group) were measured weekly for

body weight and observed daily for survival and clinical signs of GVHD. The clinical indicators of GVHD included skin changes (hair loss, erythema, etc.), diarrhea, and hunchback syndrome. If the mice were in a near-death state (for example, loss of more than 20% of their body weight for two consecutive weeks), they were humanely euthanized according to the relevant regulations (Wang et al., 2020). After 40 days, all mice used in this study were sacrificed by cervical dislocation after anesthesia with 0.3% pentobarbital sodium (50 mg/kg). The spleen, liver, and lung tissues of the mice were collected and stored at -80°C for subsequent analyses (Snyder et al., 2020).

NK cells were also isolated from the spleens of mice in the GVHD+NK, GVHD+NK(shNC), and GVHD+NK(shDNMT1) groups (six mice per group), purified, and activated *in vitro* in line with the above processes, and then the collected cells were used in the following analysis.

Methylation-specific polymerase chain reaction (MSP)

KIR2DL1, KIR2DL2, and KIR2DL3 methylation levels in GVHD mouse model-derived NK cells were evaluated by MSP assay. Briefly, 500 ng total DNA was extracted from NK cells with the help of DNAzol® Reagent (10503027, Thermo Fisher Scientific, USA), and then exposed to sodium bisulfite from the EpiTect fast bisulfite kit (59826, Qiagen, Hilden, Germany). Later, CpG islands around the promoter regions of KIR2DL1, KIR2DL2, and KIR2DL3 were tested through the UCSC genome browser (http://genome.ucsc.edu/index.html), and SYBR green-based qMSP was employed to analyze KIR2DL1, KIR2DL2, and KIR2DL3 methylation levels in NK cells. Thereafter, methPrimer (http://www.urogene.org/methprimer/) was utilized to design two sets of qMSP primers. The DNA sample was mixed with a pair of primers and the KAPA SYBR Fast qPCR kit (07959362001, Roche, Basel, Switzerland), and polymerase chain reaction (PCR) was carried out using the following amplification profile: 5 min at 94°C; 30 s at 94°C, 30 s at 55°C and 30 s at 72°C for 40 cycles; 4 min at 72°C. Finally, the PCR product was electrophoresed on 2% agarose gels and visualized under a gel imager (Chemi Doc XRS, BIO-RAD, Irvine, CA, USA) with the help of ethidium bromide (15585011, Thermo Fisher Scientific, USA) staining (Zhao et al., 2019).

Primers were displayed as follows: KIR2DL1 (methylated): forward, 5'-TATAGATTTTAGGTATTTCGTGTTCG-3'; reverse, 5'-ACATAAAACCCAATAAATACTCGCT-3'; KIR2DL1 (unmethylated): forward, 5'-TATAGATTTTAGGTATTTTGTGTTTGG-3'; reverse, 5'-TTACATAAAACCCAATAAATACTCACT-3'. KIR2DL2 (methylated): forward, 5'-TTAGTTTTTTAAAGTGTTGGGATTAC-3'; reverse, 5'-ACACAAAACCATAAAATTACACGTA-3'; KIR2DL2 (unmethylated): forward, 5'-TTTTTTAAAGTGTTGGGATTATGG-3'; reverse, 5'-ACACAAAACCATAAAATTACACATA-3'; KIR2DL3 (methylated): forward, 5'-TTTTATGTAAGGTAGAAAGAGTTTGC-3'; reverse, 5'-ACAACGCACAAAATATTATTTAACG-3'; KIR2DL3 (unmethylated): 5'-GGTTTTATGTAAGGTAGAAAGAGTTTGT-3'; reverse, 5'- CAACACACAAAATATTATTTAACACC-3'.

Mouse survival and GVHD score determination

The GVHD score was measured following a previous study (Li et al., 2014). In short, five GVHD parameters to assess the degree of GVHD were assigned a score from 0 to 2, including weight loss, kyphosis, skin lesions, activity, and fur-ruffling. The clinical symptoms of GVHD included hunched posture, 40% weight loss, fur loss, decreased mobility, as well as tachypnea. The animals with the above symptoms were sacrificed by cervical dislocation after anesthesia with 0.3% pentobarbital sodium (50 mg/kg), during which the end point of survival was recorded.

Quantitative real-time reverse transcription PCR (qRT-PCR)

The total RNA in transplant donor C57BL/6 mouse-derived NK cells was first isolated with the TRIeasy total RNA extraction kit (10606ES60, Yeason, Shanghai, China) as per the instructions, and the concentration was determined via a spectrophotometer (Mettler Toledo, Columbus, OH, USA). Afterward, a one-step qRT-PCR assay kit (T2210, Solarbio, China) was used to carry out the PCR with the following conditions: reverse-transcription (50°C for 20 min), pre-denaturation (95°C for 3 min), and 40 cycles of denaturation (95°C for 20 s), annealing (60°C for 30 s), and extension (72°C for 30 s). Relative mRNA expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method (Li et al., 2018).

The primer sequences used in this assay were: DNMT1: forward, 5'-CAGGAGAAGCAAGTCGGACA-3'; reverse, 5'-TCCTGGTCTCTCTCCTCTGC-3'. KIR2DL1: forward, 5'-ATGTCGCTCTTGGTCGTCAG-3'; reverse, 5'-AGAAGTTGGCCTTGGAGACC-3'. KIR2DL2: forward, 5'-TTCTCCTTCATCGCTGGTGC-3'; reverse, 5'-TGGGCAGGAGACAACTTTGG-3'. KIR2DL3: forward, 5'-ACCATCTATCCAGGGAGGGG-3'; reverse, 5'-CATGCAGGTGTCTGGGGTTA-3'. GAPDH (internal controls): forward, 5'-CCCATCTATGAGGGTTACGC-3'; reverse, 5'- TTTAATGTCACGCACGATTTC-3'.

Western blot

Total protein was isolated from C57BL/6 mouse-derived NK cells with the RIPA Lysis Buffer (E-BC-R327, Elabscience, Wuhan, China), and the BCA protein assay kit (C503021, Sangon Biotech, Shanghai, China) was applied to determine protein concentration. Later, the protein samples were separated via 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) gels (P0672, Beyotime, China), and transferred onto polyvinylidene fluoride (PVDF) membranes (F619536, Sangon Biotech, China). The membranes were blocked with 5% non-fat skim milk (D8340, Solarbio, China) diluted in Tris Buffered Saline with Tween-20 (TBST, T1085, Solarbio, China) for 2h at room temperature, and incubated at 4°C overnight with primary antibodies DNMT1 (1:1000, ab188453, 183 kDa, Abcam, Cambridge, UK) and GAPDH (1:1000, ab8245, 36 kDa, Abcam, UK), followed by 1-h culture with horseradish peroxidase (HRP)-labeled anti-Rabbit IgG (1:1000, ab99697, Abcam, UK) and anti-Mouse IgG (1:2000, ab6728, Abcam, UK) secondary antibodies at room temperature.

Finally, the band signals were analyzed by ECL reagent kits (FP300, ABP Biosciences, Rockville, MD, USA) on the Tanon 5200 imaging system (Shanghai, China). Quantitative analysis was performed using ImageJ software (1.52s version, National Institutes of Health, Bethesda, MD, USA).

Flow cytometry

Transplant donor C57BL/6 mouse-derived NK cell purity was determined following staining with 0.25 μg/5 μl CD56 antibody (ab220360, Abcam, UK) and 0.25 μg/20 μl CD3 antibody (ab16669, Abcam, UK) at room temperature for 20 min. A flow cytometer (1026, Beamdiag, Changzhou, China) was adopted to detect the cells, which was analyzed by Cell Quest software (BD Biosciences, San Diego, CA, USA) (Eaton et al., 2018).

The levels of Granzyme, Perforin, and Interferon-γ (IFN-γ) in GVHD mouse model/C57BL/6 mouse-derived NK cells were tested following the above processes using IFN-γ (#62675, Cell Signaling Technology, Danvers, MA, USA), Granzyme B (ab255599, Abcam, UK), and Perforin antibodies (17-9392-80, Thermo Fisher Scientific, USA).

As for C57BL/6 mouse-derived NK cell proliferation, the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) was used. In detail, NK cells were stained with 5 μmol/L CFSE (ab113853, Abcam, UK) for 15 min at room temperature. An equal volume of culture medium was added, with cells standing for another 5 min. Following culture medium washing, these cells were immediately examined via flow cytometry to evaluate the percentage of cells with lost or decreased CFSE fluorescence, and were quantified using ImageJ software (Kust et al., 2021).

Statistical analyses

GraphPad Prism 8.0 statistical software (GraphPad Software Inc., San Diego, CA, USA) was employed to analyze all data. Measurement data are expressed as mean ± standard deviation. The data in Fig. 1C-E were compared using an independent samples *t*-test, and comparison among multiple groups was conducted with a one-way analysis of variance. The data were statistically significant when the *p*-value was <0.05.

Results

Methylation of KIR2DL1/2/3 was increased, and KIR2DL1/2/3 expression was decreased in GVHD mouse model-derived NK cells

Mouse NK cells are widely defined as CD3-DX5+ lymphocytes (Li et al., 2015). After recipient BALB/c mice were transfused with NK and bone marrow cells via the tail vein to establish GVHD mouse models and NK cells were isolated from the models and purified, we measured NK-cell purity, and obtaining a high purity of 97.95% (Fig. 1A). IL-2 is often used to induce the expansion of NK cells purified from allogeneic donors (Becker et al., 2016). Here, we also employed IL-2 to activate the purified NK cells from the GVHD mouse models. According to the MSP results, the KIR2DL1/2/3 methylation levels were elevated following stimulation with IL-2 in GVHD mouse model-derived NK cells (Fig. 1B). Also, as shown in Fig. 1C-E, decreased expression of KIR2DL1/2/3 on the surface of GVHD mouse model-derived NK cells was observed after IL-2 induction ($p<0.01$).

DNMT1 silencing increased KIR2DL1/2/3 expression and enhanced secretion of Granzyme B, Perforin, and IFN-γ in C57BL/6 mouse-derived NK cells

Next, this study focused on probing the roles of DNMT1 in NK cells. After NK cells were isolated from C57BL/6 mice and purified, we successfully transfected shDNMT1 into these cells, which was verified by the reduced mRNA or protein expression of DNMT1 (Fig. 2A-C, $p<0.05$). Following transfection, we measured the expression of KIR2DL1/2/3 in C57BL/6 mouse-derived NK cells by dint of qRT-PCR. In accordance with the results, KIR2DL1/2/3 expression was upregulated in shDNMT1-transfected NK cells from C57BL/6 mice following IL-2 activation (Fig. 2D-F, $p<0.01$). More importantly, shDNMT1 led to increased KIR2DL1/2/3 expression in IL-2-activated NK cells from C57BL/6 mice (Fig. 2D-F, $p<0.001$). Besides, it has been documented that NK cells have cytotoxic effects on virus-infected cells, and kill target cells by secreting cytokines such as granulysin, perforin, IFN-γ and Granzymes (Feehan et al., 2022; Wu et al., 2022). Based on the results in Fig. 2G-I, an elevated positive ratio of Granzyme B or IFN-γ was detected in C57BL/6 mouse-derived NK cells transfected with/without shDNMT1 following IL-2 treatment (Fig. 2G,J, $p<0.01$), and shDNMT1 increased both the positive ratios of Granzyme B and IFN-γ in IL-2-activated NK cells (Fig. 2G,J, $p<0.05$). The positive ratio of Perforin in C57BL/6 mouse-derived NK cells was reduced following IL-2 stimulation (Fig. 2H, $p<0.05$), which was later augmented after transfection with shDNMT1 (Fig. 2H, $p<0.001$). Also, shDNMT1 increased the positive ratio

of Perforin in IL-2-activated NK cells from C57BL/6 mice (Fig. 2H, $p<0.001$). Taken together, we confirmed that shDNMT1 could promote the secretion of Granzyme B, Perforin, and IFN-γ in C57BL/6 mouse-derived NK cells. Moreover, as exhibited in Fig. 2J-K, the number of CFSE-low cells was increased in C57BL/6 mouse-derived NK cells transfected with/without shDNMT1 after IL-2 stimulation ($p<0.001$), and shDNMT1 increased the number of CFSE-low cells post IL-2 induction ($p<0.001$), suggesting that shDNMT1 boosted the proliferation of C57BL/6 mouse-derived NK cells.

DNMT1 silencing enhanced mouse survival, decreased GVHD score, and increased expression of KIR2DL1/2/3 in GVHD mouse models

The data in Fig. 3A showed that survival was enhanced in GVHD mouse models transfused with shDNMT1-transfected NK cells compared with that of GVHD mouse models transfused with shNC-containing NK cells. The GVHD score was lower in shDNMT1-containing GVHD mouse models than in shNC-containing GVHD mouse models (Fig. 3B,C, $p<0.001$). Furthermore, shDNMT1 increased the expression of KIR2DL1/2/3 on the surface of NK cells in GVHD mouse models (Fig. 3D-F, $p<0.01$).

DNMT1 silencing increased the secretion of Granzyme B, Perforin, and IFN-γ and the number of cytokine-producing NK cells in GVHD mouse models

Furthermore, in light of the results from flow cytometry, the levels of IFN-γ, Granzyme B, and Perforin, and the absolute values of IFN-γ, Granzyme B, and Perforin in spleen/liver tissues were all increased in shDNMT1-containing GVHD mouse models when compared with those in shNC-containing GVHD mouse

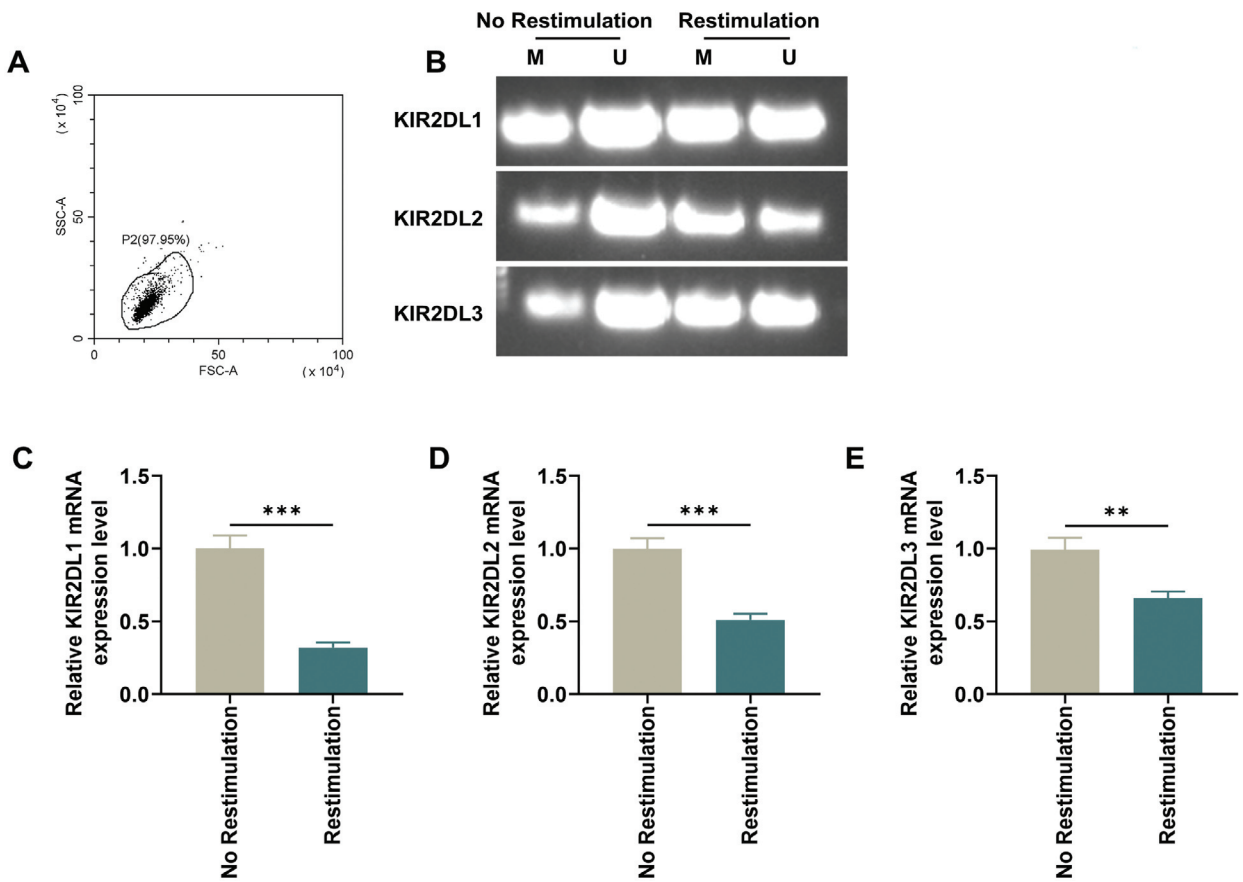

Fig. 1. Methylation of KIR2DL1/2/3 was increased and KIR2DL1/2/3 decreased in GVHD mouse model-derived NK cells. **A.** After BALB/c mice were transfused with NK cells (1×10^5) and bone marrow cells (1×10^7) via the tail vein to establish GVHD mouse models and the NK cells were isolated from the models and purified, we measured the NK cell purity via flow cytometry. **B-E.** After NK cells were isolated from the GVHD mouse models and purified, followed by *in vitro* expansion using IL-2. **B.** KIR2DL1/2/3 methylation level in GVHD mouse model-derived NK cells was evaluated via MSP. **C-E.** Expression of KIR2DL1/2/3 in these cells was tested via qRT-PCR, with GAPDH as the housekeeping control. $^{**}p<0.01$, $^{***}p<0.001$.GVHD, graft-*versus*-host disease; NK cells, natural killer cells; IL-2, interleukin 2; MSP, methylation-specific polymerase chain reaction; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KIR2DL1/2/3, killer-cell immunoglobulin-like receptor, two Ig domains and long cytoplasmic tail 1/2/3.

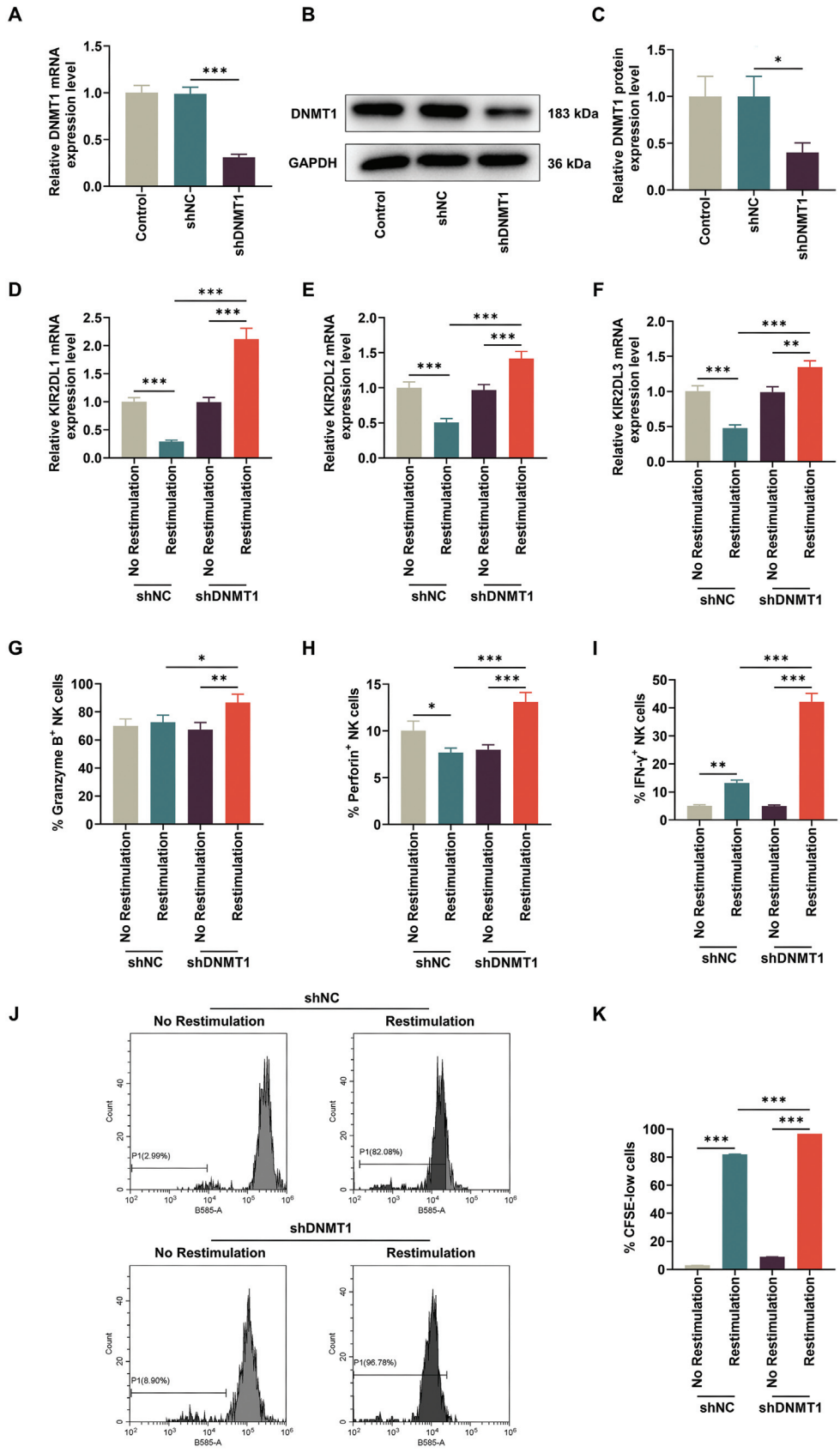

Fig. 2. DNMT1 silencing increased KIR2DL1/2/3 expression, and enhanced cytokine secretion and proliferation of C57BL/6 mouse-derived NK cells. **A-C.** We successfully transfected shDNMT1 into C57BL/6 mouse-derived NK cells, and transfection efficiency was measured via qRT-PCR and western blot, with GAPDH as the housekeeping control. **D-I.** Following transfection and IL-2 stimulation in C57BL/6 mouse-derived NK cells, expression of KIR2DL1/2/3 **(D-F)** in these cells was measured by qRT-PCR (GAPDH as the housekeeping control), cytokine levels **(G-I)** and NK cell proliferation **(J-K)** were assessed via flow cytometry. *p<0.05, **p<0.01, ***p<0.001. shDNMT1, short hairpin RNA targeting DNA methyltransferase-1; IFN-γ, Interferon-γ; CFSE, carboxyfluorescein succinimidyl ester; shNC, short hairpin RNA of negative control.

models (Fig. 4A-F, $p<0.01$). Also, shDNMT1 increased IFN-γ levels, decreased Granzyme B and Perforin levels, and elevated the absolute value of IFN-γ, Granzyme B, and Perforin in lung tissues of GVHD mouse models (Fig. 4M-R, $p<0.01$).

Discussion

GVHD is considered a vital cause of transplant failure (Xu et al., 2018). NK cells act as promoters in the progression of GVHD, and demethylation has a GVHD-promoting effect by enhancing the activation of NK cells (Shi et al., 2016). The research on NK-cell receptors revealed that demethylation can activate receptor genes, thereby promoting the expression of NK-cell receptors (Costello et al., 2015). Demethylation therapy can induce changes in cellular immune function (Tsirogianni et al., 2019), and high methylation of histones can inhibit NKG2D activity, thus repressing the activation of NK cells (Shi et al., 2016). More importantly, a prior study

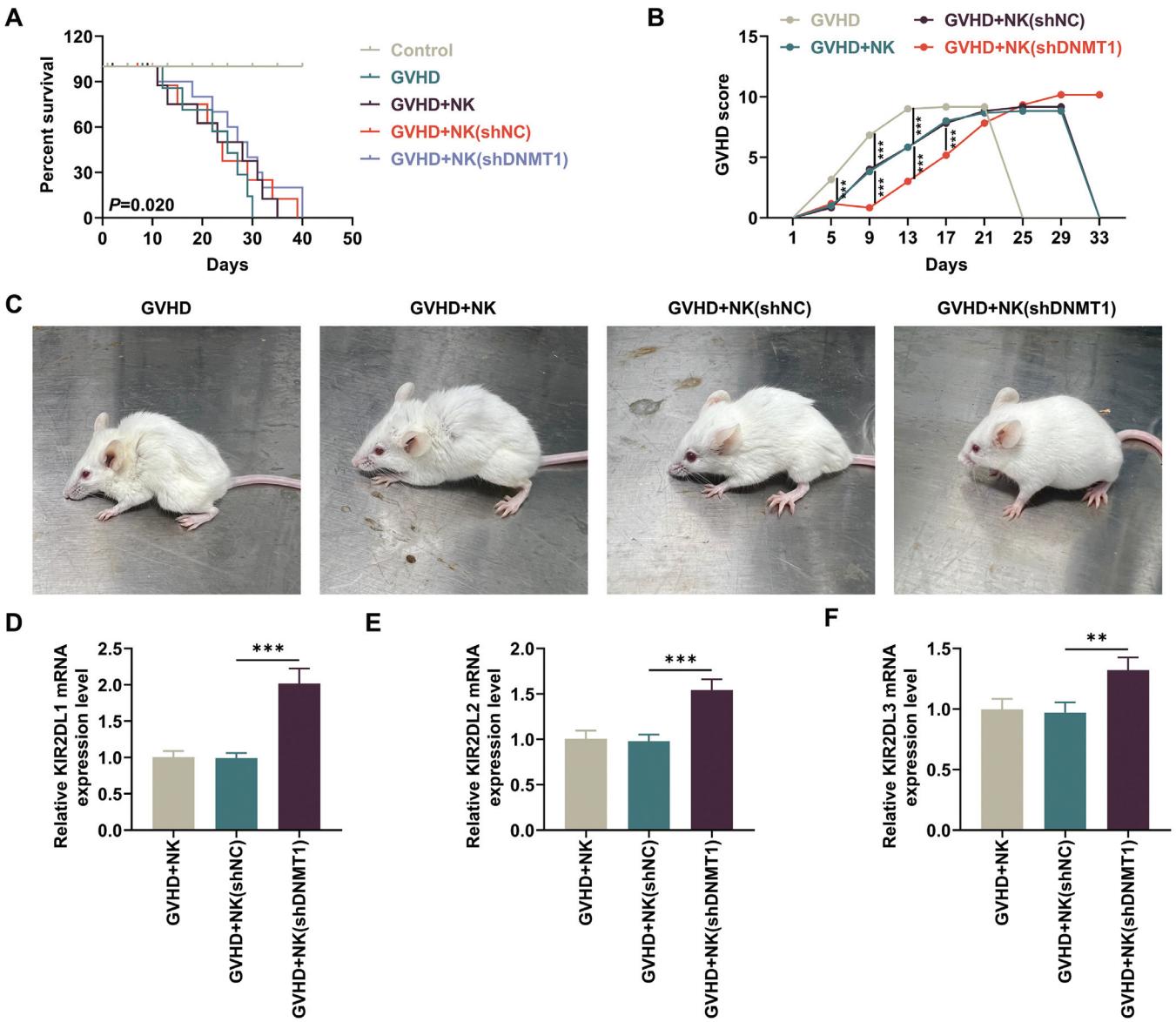

Fig. 3. DNMT1 silencing enhanced mouse survival, decreased GVHD score, and increased expression of KIR2DL1/2/3 in GVHD mouse models. **A.** After BALB/c recipient mice were transfused with C57BL/6 donor mouse-derived NK cells (1×10^5), with/without shDNMT1, and bone marrow cells (1×10^7) via the tail vein to establish GVHD mouse models, these mice were observed daily for survival **(A)** and clinical signs for GVHD scores **(B-C)**, along with the detection of **(D-F)** KIR2DL1/2/3 expression on the surface of GVHD mouse model-derived NK cells via qRT-PCR (GAPDH as the housekeeping control). $^{**}p<0.01$, $^{***}p<0.001$.

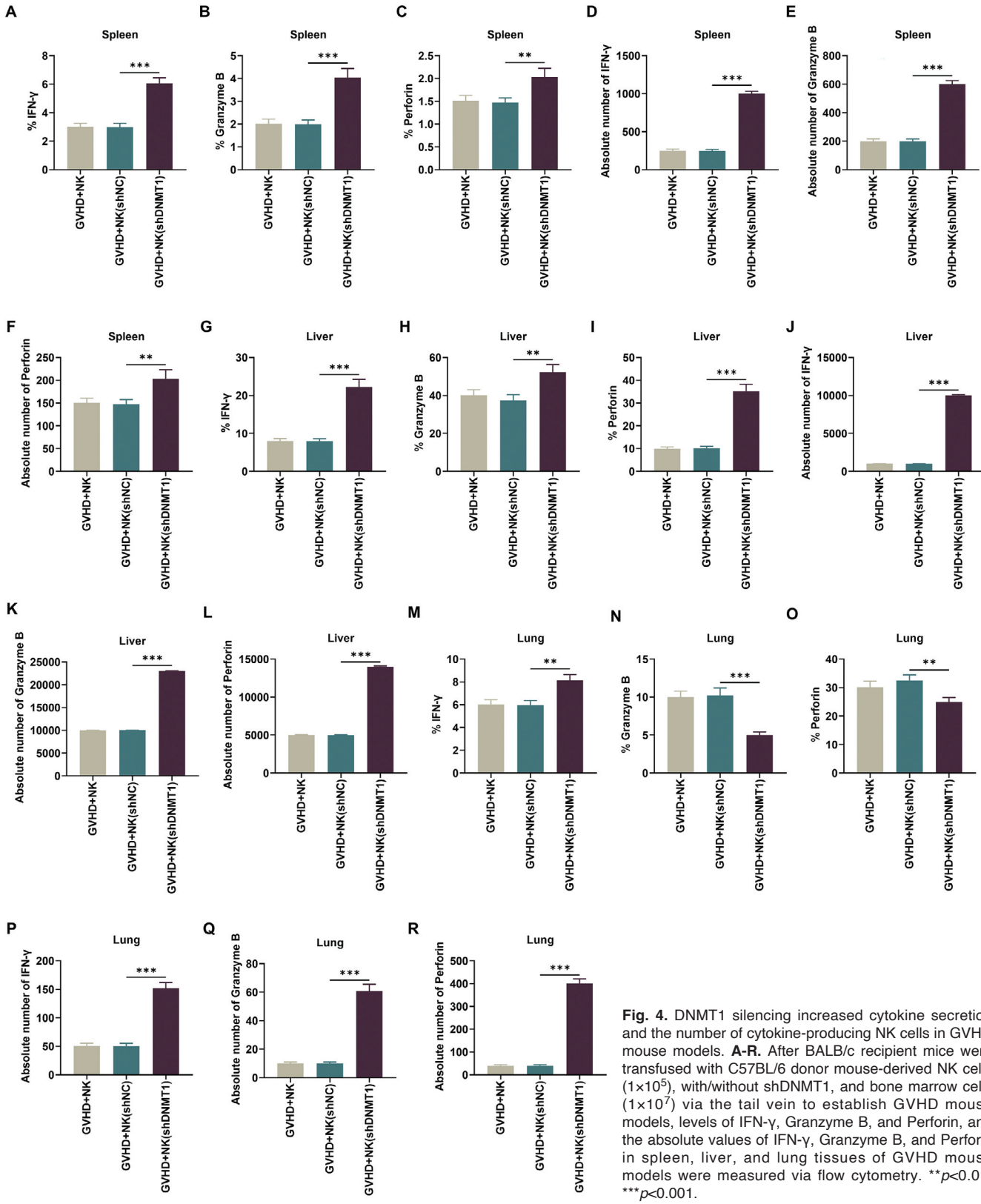

Fig. 4. DNMT1 silencing increased cytokine secretion and the number of cytokine-producing NK cells in GVHD mouse models. **A-R.** After BALB/c recipient mice were transfused with C57BL/6 donor mouse-derived NK cells (1×10^5), with/without shDNMT1, and bone marrow cells (1×10^7) via the tail vein to establish GVHD mouse models, levels of IFN-γ, Granzyme B, and Perforin, and the absolute values of IFN-γ, Granzyme B, and Perforin in spleen, liver, and lung tissues of GVHD mouse models were measured via flow cytometry. $^{**}p<0.01$, $^{***}p<0.001$.

demonstrated that demethylation causes an increase in KIR expression in NK cells (Wu et al., 2020). KIR is involved in regulating NK cell function, and KIR discordance between donors and recipients improves transplantation success and reduces transplantation-related mortality by reducing the incidence of recurrence, implantation failure, and GVHD (Ruggeri et al., 2002; Lanier, 2005). In this study, we further revealed that DNMT1 silencing induces KIR expression in NK cells through reducing methylation to alleviate GVHD after allo-HSCT.

NK cells are not B/T lymphocytes; however, they have been proven to share some phenotypic features with T lymphocytes (Kim et al., 2022). Following the isolation of NK cells from GVHD mouse models, a 97.95% NK cell purity was detected in this study. In light of the published research, short-term *in vitro* culture with cell growth-promoting cytokine media may enhance the killing activity and the numbers of NK cells. IL-2 is frequently used to induce the expansion of NK cells purified from allogeneic donors (Becker et al., 2016). In this study, we also adopted IL-2 to achieve the *in vitro* expansion of NK cells. Considering the effects of demethylation and KIR expression in NK cells on GVHD, we investigated the mechanism of de-methylation on NK cells after transplantation by analyzing the change in KIR expression on the surface of NK cells and its roles in GVHD in C57BL/6-BALB/c mice. As expected, the results revealed that methylation levels of KIR2DL1, KIR2DL2, and KIR2DL3 in GVHD mouse model-derived NK cells were elevated after IL-2stimulation, along with the downregulated expressions of KIR2DL1, KIR2DL2, and KIR2DL3 on the surface of these NK cells. Therefore, we had reason to infer that GVHD induced methylation of KIR2DL1/2/3 in NK cells.

Methylation is one of the modes of epigenetic modification of genes (Skvortsova et al., 2019). Demethylation therapy has received much attention. Recently, it was found that the post-transplant administration of demethylation drugs in transplanted patients with relapsed myelodysplastic syndromes (MDS)/acute myeloid leukemia (AML), or high-risk AML, reduces the occurrence and severity of GVHD (Cao et al., 2020), prolongs survival, and decreases the recurrence rate (Koyama et al., 2022). NK cells play an important role in the development of GVHD in post-transplantation immune regulation, and KIR expression in NK cells is increased after demethylation (Li et al., 2019). Also, particular attention has been paid to the mechanism of demethylation in the KIR promoter (Wu et al., 2020). The present work explored the relationship between NK cells and GVHD by fathoming the mechanism of demethylation affecting NK cells and thus attenuating GVHD after allo-HSCT. Given this, we took an interest in DNMT1, considered the key enzyme responsible for intracellular DNA methylation (Ren, 2022). Accordingly, whether DNMT1 affects NK cell functional activity by regulating KIR methylation was investigated in the present study. By employing shDNMT1, we verified that DNMT1 silencing promoted the expression of KIR2DL1/2/3 on the surface of NK cells. Besides, existing findings unveiled that NK cells have cytotoxic effects on virally infected cells and cancers, and can kill target cells by secreting cytokines, such as granulysin, perforin, IFN-γ, and Granzymes (Feehan et al., 2022; Wu et al., 2022). We thus measured the secretion of cytokines in NK cells. As expected, shDNMT1 promoted the secretion of Granzyme B, Perforin, and IFN-γ in NK cells, as evidenced by their elevated levels. These findings provided strong evidence that DNMT1 silencing affected NK cell function by regulating the methylation of KIR2DL1/2/3.

Furthermore, the effects of shDNMT1 on GVDH mouse models were explored in this study, and we demonstrated that DNMT1 silencing enhanced mouse survival, decreased the GVHD score, upregulated KIR2DL1/2/3 expression, and increased corresponding cytokine levels and cytokine-producing NK cells in the models. Hence, we conjectured that DNMT1 silencing can induce KIR expression in NK cells by reducing methylation to alleviate GVHD after allo-HSCT. Nevertheless, the correlation between changes in KIR expression on the surface of NK cells and the occurrence of GVHD needs further research.

In conclusion, we corroborate that DNMT1 silencing induces KIR expression in NK cells by reducing methylation to alleviate GVHD after allo-HSCT. Considering the findings in this study, the development of GVHD can be predicted according to KIR expression on the surface of NK cells, and the early intervention of GVHD can be achieved based on changes in cytokines. All findings in the present study, we hope, can lay the foundation for the application of NK cells in immunotherapy towards GVHD after allo-HSCT.

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