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Delay expression of NKp30 on NK cells correlates with long-term mycophenolate mofetil treatment and higher EBV viremia post allogenic hematological stem cells transplantation

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ABSTRACT

Mycophenolate mofetil (MMF) is an immunosuppressive agent that is widely used in graft-versus-host disease prophylaxis because of its inhibitory function on T cells and B cells. However, the effect of MMF on natural killer cell reconstitution after allogenic hematological transplantation is largely unknown. The present study examined the effects of different MMF administration durations after haploidentical allo-HSCT on NK cell reconstitution. Ninety patients were enrolled in this study and defined into two groups in term of MMF duration. We found that MMF patients in the long-term MMF group were associated with a poor reconstitution of NK cells and a significantly lower cytotoxicity from day 30 to day 180 post-transplantation. Especially, the long-term MMF group inhibits reconstitution of NKp30 NK subsets, which correlated with higher risk of EBV viremia. Multivariate analysis showed that a better reconstitution of NKp30 cells was associated with lower EBV viremia (HR0.957, $p = .04$). In vitro experiments demonstrated that the active metabolite of MMF, mycophenolic acid (MPA), inhibited the proliferation and cytotoxicity of NK cells from healthy donors or patients at day 30 post-transplantation. In summary, our findings demonstrated that long-term MMF administration delayed the quality and quantity of NK cells, especially NKp30 subpopulations, which was associated with decreased EBV viremia post allogeneic HSCT.

1. Introduction

Graft-versus-disease (GVHD) and disease relapse are two major issues of allogeneic hematopoietic stem cells transplantation (allo-HSCT) for malignant hematological disease. Natural killer (NK) cells are the first lymphocytes to recover post-allo-HSCT, and these cells play important roles in graft versus leukemia (GVL) effects. Several reports demonstrated that NK cells killed alloreactive T cells to prevent GVHD occurrence post-allo-HSCT. Rapidly reconstituted NK cells are an indispensable factor against pathogen-induced infections following allo-HSCT. All of these consequences are attributed to the direct cytolysis of pathogens or indirect IFN-γ-induced inflammatory reactions by NK cells. Better reconstitution of NK cells predicts a decreased relapse of acute myeloid leukemia (AML) [1], improved engraftment [2] and reduced GVHD [3].

However, several factors influence the reconstitution of NK cells in different transplantation models [4–6], disease background [7] and treatment discrepancy [8]. MMF and other immunosuppressive agents are successfully used in the prevention of GVHD. Previous research demonstrated that the co-culture of MMF and NK cells decreased the cytotoxicity and proliferation capability of NK cells in vitro [9,10]. However, the effects of MMF on NK cell reconstitution are not clear and were studied in vivo in the background of allo-HSCT.

The effects of different durations of MMF administration post-transplantation under haploidentical allo-HSCT on NK cell reconstitution are not known. Antithymocyte globulin (ATG) followed by

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unmanipulated blood and marrow graft infusions without in vitro T cell depletion were successfully performed for haploidentical transplantation, and this procedure was termed the "Beijing Protocol." The Beijing Protocol includes two MMF treatment durations post-transplantation. The first protocol administers 0.5 g MMF orally every 12 h from day 9 pre-transplantation to day 30 post-transplantation, then 0.25 g MMF every 12 h for 1 to 2 months, which we termed "long-term MMF duration." The other regimen is the same protocol used for HLA-matched sibling allo-HSCT, in which MMF is administered orally at 0.5 g every 12 h from day 9 pre-transplantation to WBC engraftment post-transplantation, which we termed "short-term MMF duration". We designed a prospective observational study to compare the effects of short-term MMF treatment vs. case pair-matched long-term MMF treatment on NK quantity and quality reconstitution to investigate the hypothesis that short-term MMF treatment is superior to long-term MMF treatment for NK reconstitution in patients with standard-risk hematological malignances. Our comparisons demonstrated that short-term MMF treatment promoted the recovery of absolute numbers and function of NK cells and accelerated the maturation of NK cells post-haplo- HSCT. Meanwhile, the occurrence of EBV viremia had a trend to be lower in short-term MMF administration compared to long-term MMF administration. Long-term MMF administration delayed the expression of NCRs expression on NK cells, among them a rapid recovery of NKp30 + NK cells was associated with a lower risk of EBV viremia.

2. Materials and methods

2.1. Eligibility criteria and study design

We performed a prospective observational study to investigate NK cell reconstitution kinetics in patients with acute leukemia who underwent unmanipulated haploidentical allo-HSCT at the Peking University Institute of Hematology between Sep 2016 and Mar 2017. Patients with standard-risk acute leukemia in complete remission (CR) who received myeloablative allo-HSCT were eligible for inclusion in this trial. The specific transplantation procedure was reported previously [11,12]. Cyclosporin A (CsA) plus short-term MTX in combination with MMF were used as the GVHD prophylaxis regimen. We previously registered a clinical trial (ClinicalTrials.gov, #NCT02978274) to prospectively investigate the recovery characteristics of NK-cell subset reconstitution after haplo-HSCT from May 2016 to May 2017. Two durations of MMF treatment were used in the Peking University Institute of Hematology during the study. MMF was administered orally at 0.5 g every 12 h from day 9 pre-transplantation to day 30 post-transplantation followed by 0.25 g every 12 h for 1 to 2 months in Unit A (i.e., the long-term group). Unit B began in Dec 2016, and MMF was administered orally at 0.5 g every 12 h from day 9 pre-transplantation to WBC engraftment post-transplantation (i.e., the short-term group). Patients were enrolled into two groups based on MMF administration duration post-transplantation. Forty-five patients were selected from 135 patients for long-term MMF treatment using case-paired methods for age, donor age, patient-donor relationship, and conditioning regimen, and 45 patients accepted short-term MMF treatment to compare the effects of long-term and short-term MMF treatment on NK cell reconstitution. The NK reconstitution kinetics post-transplantation were compared between the 45 patients from the short-term group and 45 patients from the long-term group. The Institutional Review Board of Peking University approved the study, and written informed consent was obtained from all patients prior to study entry in accordance with the Declaration of Helsinki.

2.2. Immunophenotyping and multiparameter flow cytometric analysis

NK cell surface phenotype was determined using 10-color flow cytometry (FACSCanto). Peripheral blood cells were collected from patients at 1 M, 3 M and 6 M routinely and labeled with CD3-V500 (BD Biosciences, clone UCHT1), CD56-PE (Biolegend, clone MEM-188), NKP46-PE-Cy7 (BD Biosciences, clone 9E2/NKP46), NKP30-V450 (BD Biosciences clone NK-1), and NKG2D-Percp (emBioscience REF46587842). Cytotoxicity was measured via the coculturing of peripheral blood mononuclear cells (PBMCs) with K562 at a ratio of 5:1 for 4 h, and anti-CD107a-BV421 antibody was added simultaneously. Samples were labeled with CD3-BV510 (BD Bioscience), CD56-APC-Cy7 (Biolegend clone HCD56), NKG2A-PE-Cy7 (Beckman clone B10246), NKG2C-APC-R700 (RD Systems clone FAB138N), CD57-BV605 (BD Bioscience clone NK-1), CD158a-APC (eBioscience clone HP-MA4), CD158e-FITC (BD Bioscience cat555966), and CD158b-PE (BD Bioscience cat 555,785). The data were analyzed using FlowJo V10

Table 1
Patient characteristics in the long-term group and short-term group.

Characteristics	Short-term group	Long-term group	P value
No. of patients	45	45	
Median age (range), years	35(14–58)	29(15–58)	0.541
Diagnosis (No)			0.484
AML	28(62.2%)	23(51.1%)	
ALL	13(28.9%)	15(33.3%)	
MDS	4(8.9%)	7(15.6%)	
HLA mismatch sites			1.000
1	2(4.4%)	2(4.4%)	
2	7(15.6%)	6(13.3%)	
3	36(80.0%)	37(82.2%)	
Donor relationship (%)			0.882
Parents to children	16(35.6%)	18(40.0%)	
Siblings	13(28.9%)	13(28.9%)	
Children to parents	16(35.6%)	14(31.1%)	
ABO-matched graft (%)			0.817
Matched	26(57.8%)	30(66.7%)	
Major mismatch	9(20.0%)	6(13.3%)	
Minor mismatch	6(13.3%)	6(13.3%)	
Bidirectional mismatch	4(8.9%)	3(6.7%)	
Donor age (range), years	30(15–62)	32(12–64)	0.568
Donor-recipient gender (%)			0.586
Male to-male	22(48.9%)	26(57.8%)	
Male to-female	11(24.4%)	8(17.8%)	
Female to male	5(11.1%)	7(15.6%)	
Female to female	7(15.6%)	4(8.9%)	
MMF(median, range), days	23(18–33)	32(20–80)	< 0.0001

Table 1 summarizes the characteristics of patients and donors in the long-term MMF group and short-term group. AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil. WBC: white blood cells; PLT: platelet ; aGVHD: acute graft versus healthy donors; CMV: cytomegalovirus; EBV: Epstein-Barr virus.

Table 2
Clinical outcomes between the long-term group and short-term group.

Characteristics	Short-term group	Long-term group	P value
WBC (range), days	13(9–23)	13(8–22)	0.613
Plt (range), days	18(10–76)	18.5(10–105)	0.364
aGVHD (%)			
I-IV	22(48.9%)	22(48.9%)	1.000
II-IV	8(17.8%)	16(35.6%)	0.057
III/IV	2(4.4%)	3(6.7%)	1.000
CMV anemia	38(84.4%)	42(93.3%)	0.180
Refractory CMV infection	20(44.4%)	16(64.4%)	0.389
EBV infection	8(17.8%)	12(26.7%)	0.310
Relapse			
Hematological relapse	3(6.7%)	1(2.2%)	0.616
Molecular relapse	10(22.2%)	5(11.1%)	0.258
Chronic GVHD			0.508
Mild	12(26.7%)	15(33.3%)	
Moderate	11(24.4%)	7(15.6%)	
Severe	1(2.2%)	0(0%)	

Table 2 summarizes the clinical outcomes between the long-term and short-term group. WBC: white blood cell; PLT: platelet; Agvhd: acute graft versus healthy donors; CMV: cytomegalovirus; EBV: Epstein-Barr virus.

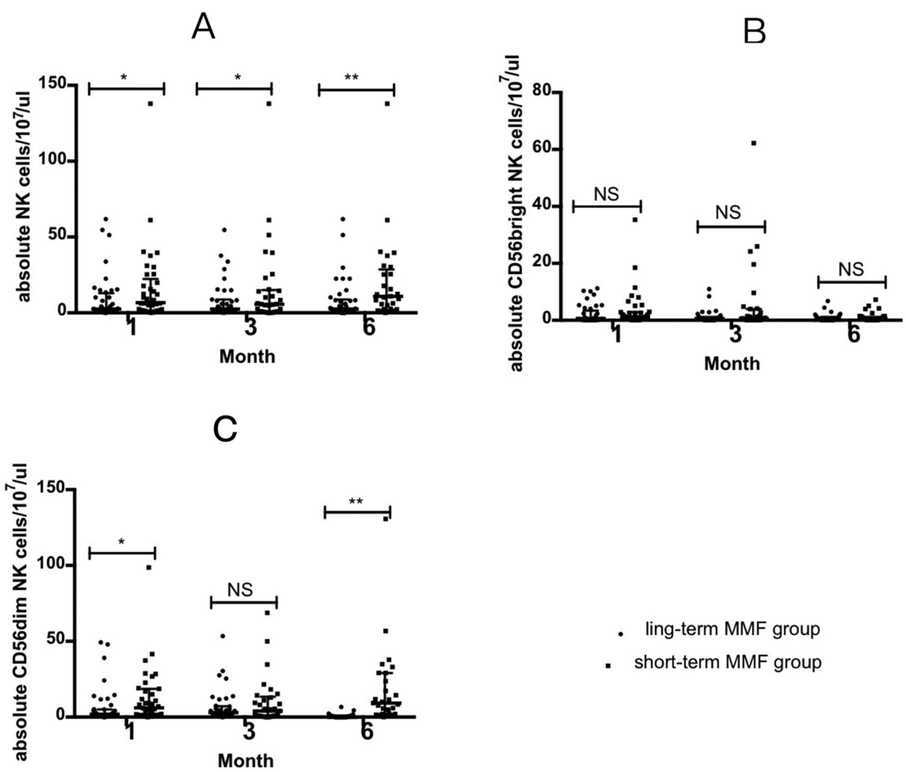

Fig. 1. Effects of MMF on the absolute numbers of NK cell reconstitution (A), CD56bright (B) and CD56dim (C) in vivo. Data are shown as the means ± SE. *, $p < .05$; **, $p < .01$ between short-term MMF group (1 M, $n = 40$; 3 M, $n = 31$; 6 M, $n = 28$) and long-term MMF group (1 M, $n = 39$; 3 M, $n = 38$; 6 M, $n = 37$).

software.

2.3. Ex vivo proliferation and cytotoxicity assays of NK cells

PBMCs were obtained from healthy donors ($n = 6$) and patients 30 days post-allo-HSCT ($n = 5$). NK cells were sorted using an NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's recommendations. Enriched NK cells contained > 90%–95% CD56 + CD3- cells (data not shown). Enriched NK cells were cultured in IL-2 (100 μg/ml) and IL-15 (10 ng/ml) with or without the active metabolite of MMF mycophenolic acid (MPA) (10 μg/ml) [10]. Purified NK cells were stained with carboxy fluoroscein succinimidyl ester (CFSE) using the Cell Trace Proliferation Kit (Invitrogen, Carlsbad, CA) to examine the proliferation ability under MPA (Sangon Biotech, Shanghai) for 7 days. Purified NK cells were cultured with MPA for 5 days to measure NK cell cytotoxicity and cocultured with K562 for 4 h to examine the effect of MPA on NK cell cytotoxicity. Briefly, purified NK cells, treated with or without MPA, were obtained and labeled with CD107a and cocultured with K562 (target:effector = 5:1) in RPMI 1640 supplemented with 10% bovine serum [13].

2.4. Statistical considerations

Patient characteristics and clinical outcomes between two groups were analyzed using the χ2 test or Fisher exact probability test for categorical variables and the Mann-Whitney *U* test for continuous variables (Table 1). A two-sided Mann-Whitney U test or Student's *t*-test was used to compare the reconstitution of NK cells from different subgroups within the first 180 days using repeated measures analysis [14]. Associations between levels of NK subsets and incidences of EBV were calculated using logistics, multivariate Cox models to assess the proportional hazard assumption, and interaction terms with covariates were tested. Factors with univariate analysis *P*-values of < 0.1 were included in the Cox model. The final multivariate models were constructed using a forward stepwise selection approach. A *p* value < .05 indicated statistical significance and indicated using stars ($0.01 < p < .05$ marked as *, $p \leq .01$ marked as **). Numerical data are expressed as the means ± standard of error (SEM). All analyses were performed using the SPSS 23.0 and Graphpad Prism 7.0 packages.

3. Results

3.1. Characteristics and clinical outcomes

Tables 1 and 2 show patient characteristics and clinical outcomes. Ninety patients diagnosed with standard-risk AML, ALL or MDS received stem cell grafts from related family members. The two groups were comparable in donor and recipient genders, the ages of donors and recipients, disease type, disease risk category, HLA-disparity sites, and donor-recipient relationships (all ps > 0.05). Overall survival at the end of follow-up was 92.2%. Clinical outcomes, including acute GVHD, CMV and EBV infection, hematological relapse and molecular relapse, were all comparable between the short-term and long-term groups (Table 2).

3.2. Effect of MMF on NK expansion in vivo and in vitro

The absolute numbers of NK cells were significantly different between the long-term and short-term MMF group at 1 M ($p = .021$), 3 M ($p = .035$) and 6 M ($P = .001$) post-transplantation (Fig. 1A). However, no significant difference was found in the absolute numbers of T cells or the percentages of T cells in lymphocyte subsets between the two groups post-transplantation (data not shown).

NK cells are classified into two major subsets in peripheral blood (PB) based on the levels of CD56 surface marker. The major proportion is $CD56^{dim}$ NK subsets, which constitute approximately 90% in PB and are characterized by cytotoxic capacity. In contrast, the $CD56^{bright}$ NK subset performs regulatory functions. Several investigators

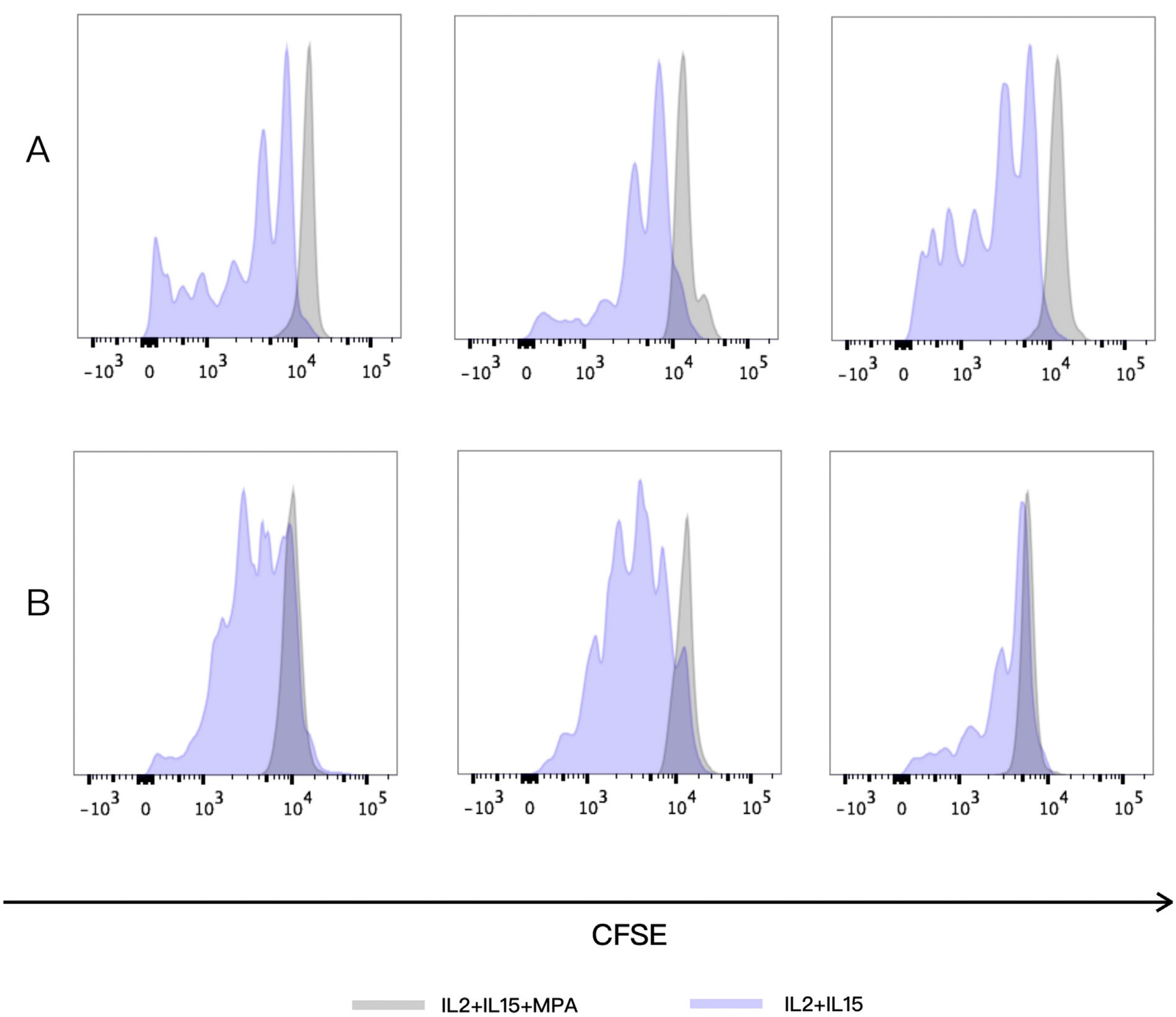

Fig. 2. Effects of MPA on NK cell proliferation in vitro (A) in healthy donors (n = 3). (B) Thirty days post-haplo-HSCT in patients (n = 3). NK cells isolated from heathy individuals and patients 30 days post-transplantation were cultured in the presence of IL-2 (100 U/ml) and IL-15 (10 ng/ml) with or without MPA (10 μg/ml). CFSE contents in CD3-CD56 + cells were determined using flow cytometry after 7 days of culture. The figure shows a representative set of histograms from 6 healthy individuals and 5 patients 30-days post-transplantation. The black background means the culture medium with IL-2, IL-15 and MPA, and the purple background indicates the culture medium with IL-2 and IL-15. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

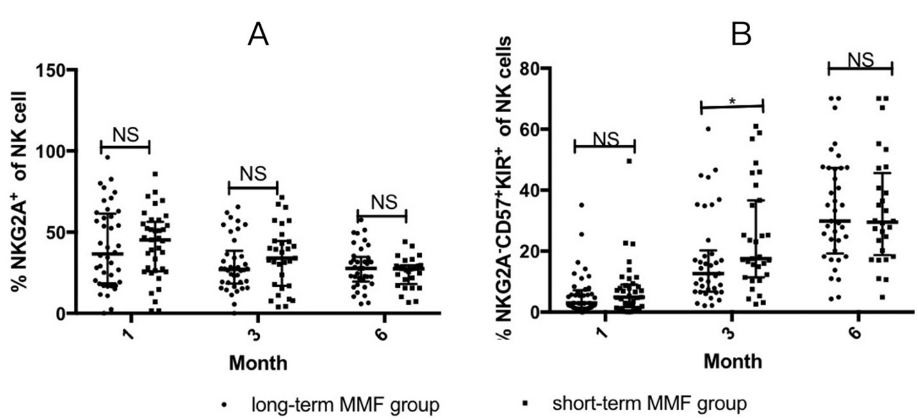

Fig. 3. Effects of MMF on NK cell reconstitution in vivo. Changes in the expression levels of NKG2C, NKG2A, and NKG2A-CD57 + KIR + in NK cells are shown as means ± SE. *, p < .05; **, p < .01 between the short-term MMF group (1 M, n = 40; 3 M, n = 31; 6 M, n = 28) and long-term MMF group (1 M, n = 39; 3 M, n = 38; 6 M, n = 37). The vertical axis represents the percentages of subsets in total NK cells.

demonstrated that CD56^{bright} subsets were immature stages of CD56^{dim} subsets [2]. We analyzed the absolute numbers of CD56bright and CD56^{dim} NK cells at three different time points in both groups to evaluate effects of MMF on NK differentiation states. The absolute numbers of CD56^{dim} at 1 M and 6 M were significantly higher in the short-term MMF group compared to the long-term MMF group (p = .030 and p = .001, respectively). A trend for increased numbers in the CD56^{dim} NK subset was noted 3 M posttransplantation in the short-term group compared to the long-term group (p = .093) (Fig. 1B). There were no significant differences in the absolute numbers of CD56^{bright}

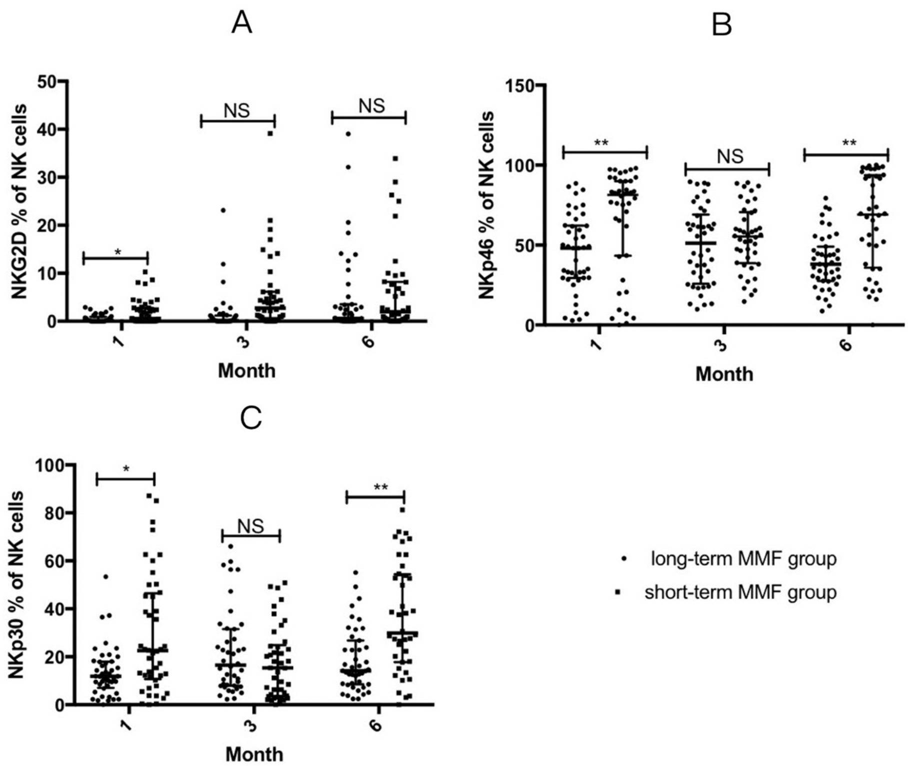

Fig. 4. Changes in the activation receptor with different courses of MMF treatment in vivo. Samples were collected at 1 M, 3 M and 6 M from the short-term MMF group and long-term MMF group; data are shown as means ± SE. *, $p < .05$; **, $p < .01$ between the short-term MMF group (1 M, $n = 44$; 3 M, n = 40; 6 M, n = 37) and long-term MMF group (1 M, n = 44; 3 M, $n = 42$; 6 M, n = 37).

NK subsets post-transplantation (Fig. 1C) at 1 M, 3 M, or 6 M. Repeated analysis revealed a significant difference between the two groups ($p = .029$).

MPA was added to the purified NK cells to further identify the inhibitory effect of MMF on proliferation. MPA inhibited NK cell proliferation in healthy donors and recipients at day 30 posttransplantation after a 7-day coculture (Fig. 2).

3.3. Effects of MMF on the maturation status of NK cells post-transplantation

NK cells differentiate and mature in bone marrow, and dynamic phenotype changes occur during development [7]. Immature NK cells exhibit a high expression of NKG2A/CD94 [8] and are termed CD56bri cells. These cells acquire KIR, NKG2C and CD57 [9] and become mature NK cells. We classified the NK cells into different development stages based on NKG2A, NKG2C, CD57 and KIR expression. The percentages of NKG2A subsets were not significantly different between the two groups at 1 M, 3 M or 6 M post-transplantation (Fig. 3), and no significance in NKG2C was observed until 6 M ($p = .000$).

$NKG2A^-CD57^+KIR^+$ subsets are important cells that play important roles in the GVL effect. Therefore, we evaluated the reconstitution of $NKG2A^-CD57^+KIR^+$ NK subsets (% of NK cells) after allo-HSCT and found that the short-term MMF group exhibited a tendency of a higher percentage of $NKG2A^-CD57^+KIR^+$ subsets of NK cells over time ($p = .355$, $p = .016$, and $p = .507$ at 1 M, 3 M and 6 M, respectively). The repeated measures analysis supported a significant difference between the two groups ($p = .039$, Fig. 3C).

3.4. Effects of MMF on the expression of activation receptors on NK cells

We compared the reconstitution dynamics of activation receptors in both groups to evaluate the effects of MMF on the killing capacity of NK cells. The results demonstrated that the expression of activation receptors gradually increased on NK cells over time. The frequency of NKP30 and NKP46 on NK cells was higher at 1 M and 6 M in the short-term MMF group than the long-term MMF group ($p = .012$ and $P < .01$ at 1 M and 6 M, respectively, for NKP30; $p < .0001$ and $p < .01$ at 1 M and 6 M, respectively, for NKP46; Fig. 4 A-B). Repeated measure analysis demonstrated that the percentage of NKP46 and NKP30 on NK cells were significantly different between two groups ($p = .05$ and $p = .026$, respectively). The expression of NKG2D on NK cells at 1 M was higher in the short-term group compared to the long-term group ($P = .047$, Fig. 4C).

3.5. Effects of MMF duration on NK cell cytotoxicity

Repeated measure analysis demonstrated that the frequency of CD107a in total NK cells against K562 cells was comparable between the short-term and long-term MMF group ($p = .564$) (Fig. 5A). We analyzed CD107a expression on different NK cell subpopulations. The results demonstrated that CD107a expression on $CD57^+$NK subsets was significantly higher in the short-term MMF group than the long-term MMF group at all three time points (1 M $p = .005$; 3 M $p < .00$; and 6 M $p = .002$, respectively). Analysis of $NKG2A^-CD57^+KIR^+CD107a^+$ subsets (% of NK cells) revealed an increasing tendency in both groups. There was no difference between the two groups at 1 M ($p = .868$) or 3 M ($p = .420$), but a difference was found at 6 M ($p = .032$). Repeated measures revealed that the overall reconstitution in the short-term group was significantly higher than the long-term group ($p = .015$, Fig. 5C). In vitro experiments confirmed that MPA treatment significantly decreased the cytotoxicity of NK cells against K562 in healthy donors and recipients 30-days post-transplantation (Fig. 5D and E respectively).

3.6. Association of NK recovery and EBV reactivation

There was a tendency of decreased EBV reactivation between short-term and long-term MMF group. We therefore analyzed the correlation of subsets above showed statistical differences between the long-term MMF group and the short-term MMF group with EBV anemia. The results showed that rapid recovery of NKG2A-CD57 + KIR+ correlate

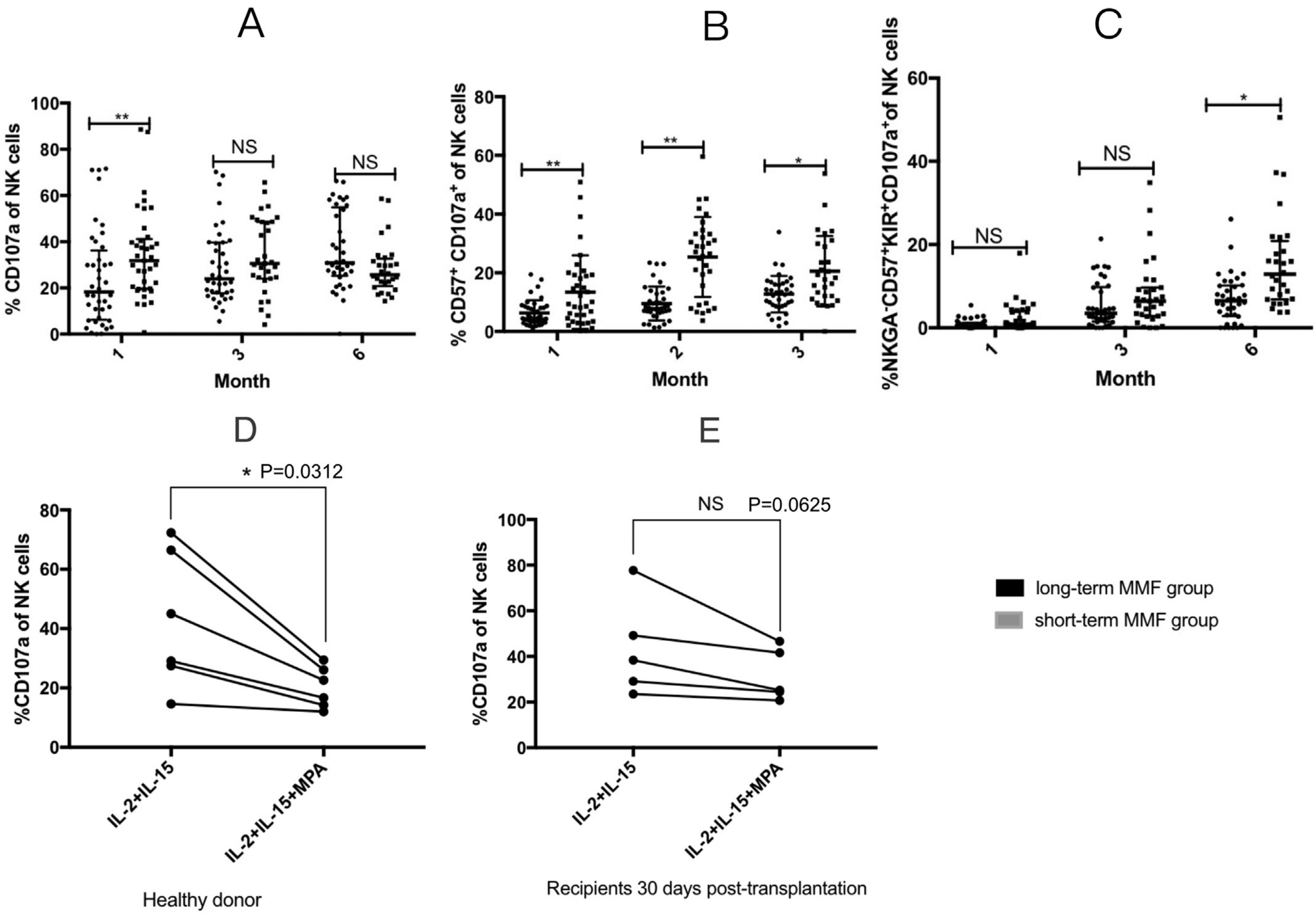

Fig. 5. Cytotoxicity of NK cell changes with MMF treatment in vivo and in vitro. (A,B,C) The percentages of CD107a, CD57 + CD107a + and NKG2A-CD57 + KIR +CD107a + subsets of NK cells are shown as means ± SE. *, p < .05; **, p < .01 between the short-term MMF group (1 M, n = 40; 3 M, n = 31; 6 M, n = 28) and long-term MMF group (1 M, n = 39; 3 M, n = 38; 6 M, n = 37) in vivo. (D,E)The percentages of CD107a in NK cells among healthy individuals (n = 6) and patients 30 days post-transplantation (n = 5) are shown.

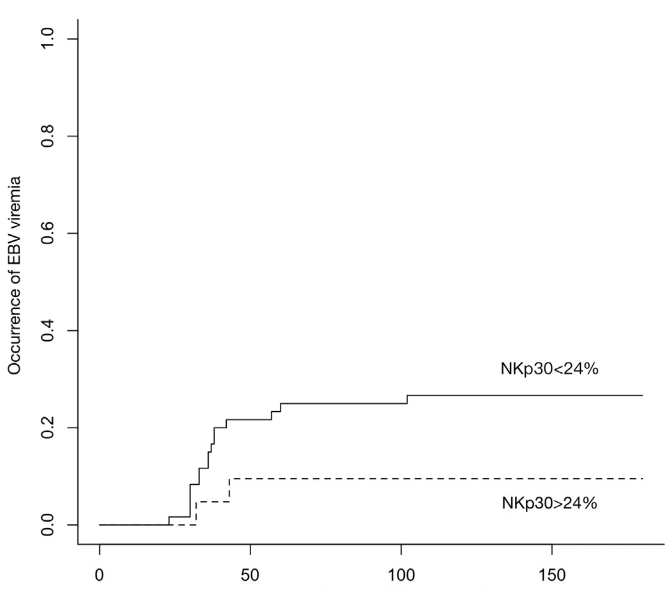

Fig. 6. High levels of NKp30 reconstitution in 1 M were collated with lower risks of EBV viremia. Cumulative occurrence of EBV viremia for all patients who underwent haploidentical transplantation.

with a lower rate of EBV in 1 M (coef = −0.1519; p = .004). The same tendency had been found in the association between higher expression of NKp30+, NKp46+ or NKG2D+ on NK cells at 1 M (p = .000,0.008,0.004 respectively) with a lower rate of EBV. Indicating that patients without EBV reactivation showed rapid recovery of several subsets of NK cells early post-transplantation compared to those patients with EBV reactivation. However, no statistical differences were seen in the NKG2A-CD57 + KIR +CD107a + subpopulations in terms of correlation with EBV (data not shown).

Based on the ROC curve of NKp30(% of total NK cells) expression on day 30 to predict the occurrence of EBV, the cut-off value was selected as 24%, with the sensitivity 80.5% and specificity 60.7%. Patients with high NKp30 expression (> 24%, N = 20) showed decreased EBV reactivation (5.0% versus 27.9%; p = .035, Fig. 6) compared to those with low NKp30 expression (< 24%, N = 68). While no significant difference was seen in the occurrence of EBV viremia when the cut-off point were made based on the ROC of NKp46 + and NKG2D+ NK subsets(data not shown).

Multivariate analysis further confirmed the expression of NKp30(% of NK cells) on day 30 predicted lower EBV reactivation (Table 3, p = .04).

4. Discussion

The present prospective observational study found that long-term

Table 3
Multivariable analysis of factors related with Epstein-Barr virus (EBV) infection post allo-genic stem cell transplantation.

Covariate	Univariate analysis			Multivariate analysis		
	coef	95% CI	P	HR	95% CI	P
NKp30 in1M(%of total NK cells)	−0.0783	−0.119,-0.037	0.000	0.957	0.918，0.998	0.04
NKp46 in1M(%of total NK cells)	−0.233	−0.033,-0.013	0.000			
NKG2D in1M(%of total NK cells)	−1.7675	−3.068,-0.467	0.008			
$NKG2A^-CD57^+KIR^+$% in 1 M	−0.1519	−0.256,-0.048	0.004			
$NKG2A^-CD57^+KIR^+CD107^+$% in 1 M	0.077	0.897,1.300	0.419			

Univariate factors that shows $P < .1$ were included in the multivariable analysis.

MMF treatment delayed the early period of NK cell reconstitution and a delayed reconstitution of NKp30 subsets was associated with a decreased risk of EBV viremia. To the best of our knowledge, this study is the first investigation of the effect of different MMF administration durations on NK cell reconstitution in vivo post-allo-HCST.

Previous in vitro research demonstrated that the active metabolite of MMF, MPA, inhibited the proliferation and cytotoxicity and altered the receptor expression of NK cells from healthy donors [9]. These results are consistent with our studies. The absolute numbers of NK cells were reduced significantly following long-term MMF stimulation in vivo, which suggests inhibitory effects of MMF on NK proliferation. The inhibition of DNA synthesis or increasing apoptosis of NK cells may underlie these effects, which are identical to the effects on T and B cells [15,16]. Our in vitro experiment confirmed the inhibitory effect of MPA on the proliferation capacity of NK cells from donors and patients on day 30 post-transplantation. However, the effects of MPA on different NK subsets were also different. NK cells are a heterogenic cell population and divided into two primary subsets, $CD56^{bri}$ and $CD56^{dim}$. These NK subsets are characterized by CD56 expression in peripheral blood. Eissens et al. [10] recently described that MPA in vitro inhibited the $CD56^{bright}CD16^{+/-}$, and most of the NK population treated with MMF maintained a stable $CD56^{dim}$ phenotype. Interestingly , not only our study demonstrated that MMF primarily influenced the subpopulations of $CD56^{bright}$ cells , but also the $CD56^{dim}$ subpopulations. There were significant differences in the absolute numbers of $CD56^{bright}$ NK subsets and $CD56^{dim}$ NK subsets in NK cells post-transplantation between the long-term and short-term groups. However, the percentage of NKG2A of NK cells shows no significant differences while the percentage of $NKG2A^-CD57^+KIR^+$ NK subsets of NK cells post-transplantation were significantly lower in the long-term MMF group, which indicates MMF may involve in the maturation of NK cells. The underlying mechanism of MMF in vivo administration on NK cell maturation must be examined further.

The effects of MMF on NK cell repertoire were primarily investigated in vitro, and only one in vivo study in a mouse model was found. Dong S et al. analyzed mice treated with 30 mg/kg /d MMF for 1 month and demonstrated lower NKG2D expression, which accounts for decreased cytotoxicity [17]. Treatment with long-time MMF therapy decreased the expression of activated receptors, including NKP46, NKP30 and NKG2D, on NK cells compared to the long-term MMF group. However, no differences were observed in inhibitory receptors (single KIR and NKG2A). The functionality of NK cells depends on the balance of activation and inhibitory receptors [18]. Phenotype differences were reflected in the cytolytic activity of NK cells against K562 cells. Nguyen et al. investigated which haplo-HSCT with T cell depletion and demonstrated that the decreased fraction of CD56dim NK cells during the first months following transplant was associated with reduced NK cell cytotoxicity [19], which is consistent with the results of our study. Our results demonstrated that the percentages of CD107a subsets were apparently higher in the short-term MMF group in 1 M. Further subgroup analysis of NK cells revealed that long-MMF treatment primarily inhibited the cytotoxicity of the mature $NKG2A^-CD57^+KIR^+$ NK subset, which is a major subpopulation involved in the GVL effects. Theoretically, a better and early reconstitution of $NKG2A^-CD57^+KIR^+$ indicates reduced relapse or infection without GVHD. However, we did not further analyze the contribution of recovery of $NKG2A^-CD57^+KIR^+$ NK cells to relapse because of the limited number of patients who relapsed.

In this study, rapid NK reconstitutions correlate with lower EBV infection, indicating a defense of NK cells against EBV virus. Studying mice with a humanized model revealed that depletion of NK cells increase primary EBV infection and promote EBV-associated tumorigenesis [20].However, the exact ways of NK cells defending against EBV infection are unclear. In this study, we firstly found that higher expression of NCRs, especially NKP30 on NK cells correlated with decreased EBV reactivation further support the anti-EBV function of NK cells. A higher expression of activating NKG2D, NKp30 were also seen when culturing with EBV indicated that NKG2D and NKP30 expression was affected by EBV infection. However,the effect of NKP30 expression on NK cells function against EBV virus need be further explore.

The role of NK alloreactivity, as determined by the KIR and HLA from the donor and recipient, in the clinical outcome post-haploidentical transplantations for malignant hematological disease remains controversial [21–24].In these results may be attributed to heterogeneity in transplantation protocols at different centers. Our study first demonstrated that long MMF administration delayed the maturation status and cytotoxicity of NK cells, which would partially account for the controversial roles of NK cell alloreactivity under different transplant settings.

There were several limitations in this study. This study was a prospective observational study rather than randomized clinical trial; lack of monitoring in IFN- γ secretion by NK cells. Whatever, short-term MMF treatment did not increase the occurrence of acute GVHD, had a trend to decrease EBV reactivation and promoted NK cells quality and quantity recovery would support the performance of randomized clinical trials in the future to confirm the results of this cohort.

In conclusion, our study demonstrated that MMF played a role in the regulation of NK cell reconstitution, particularly the early stage after haplo-HSCT. Further research is warranted to evaluate the effect of immunosuppressive agents on NK cells.

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Conflicts of interest disclosures

The authors made no disclosures.

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