Effects of natural killer cells on graft rejection, hematopoietic and immune reconstitution following allogeneic bone marrow transplantation*☆

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Abstract

BACKGROUND: The connection between Natural killer (NK)-cells and allogeneic bone marrow transplantation (allo-BMT) has aroused increasing attention.

OBJECTIVE: To explore the effect of NK cells on graft rejection, hematopoietic and immune reconstitution in mouse undergoing allo-BMT.

METHODS: Lethally and nonlethally irradiated BALB/c (H-2^d) mice were transplanted with C57BL/6(H-2^b) bone marrow plus donor peripheral T cells and/or NK cells.

RESULTS AND CONCLUSION: Compared with lethally irradiated and allo-BMT group without infusion of NK cells, the survival rate in lethally irradiated and allo-BMT group with infusion of NK cells significantly enhanced; leukocytes count, expression level of $CD19^+$ and $CD34^+$ cell count recovered rapidly; expression level of H-2^{b+} cell obviously increased. Expression level of $CD34^+$ cell in the group with infusion of NK cells was obviously lower than that of the group without infusion of NK cells at 28 days after transplantation, but there was no significant difference between the 2 groups at 60 days ($P > 0.05$). In nonlethally irradiated and allo-BMT group without NK cell infusion, expression level of H-2^{b+} cell significantly decreased at 30 days after transplantation, and reduced to before transplantation level at 60 days; while expression of H-2^{b+} cell yet could be detected with more than 80% at 60 days after transplantation in group infused with high and low concentration of NK cells. In allo-BMT mice, alloreactive NK cell inhibits graft rejection, enhances engraftment, promotes the reconstitution of hematopoiesis and immunity, and increases survival rates.

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INTRODUCTION

In 2003, Parham and McQueen [1] stated a review that natural killer (NK) cell can not only strengthen graft versus leukaemia, but also inhibit graft rejection and graft versus host disease, boost the implantation of hematopoietic stem cells. Therefore, NK cell plays an important role in allogenic hematopoietic stem cell transplantation (allo-HSCT). Taking the allo-BMT in mice as experimental model, our team has already examined the role of NK cells in allo-HSCT and proved that NK cell can prevent graft versus host disease[2]. This article will further study the effect of NK cell on graft rejection, hematopoietic and immune reconstitution.

MATERIALS AND METHODS

Materials

BALLB/c(H-2^d) female mice, aged 8−10 weeks, weighted 18-20 g, served as recipients; C57BL/6(H-2^b) male mice, aged 8−12 weeks, weighted 20 g, served as donors. All animals were purchased from Experimental Animal Center of Southern Medical University. Experimental protocols were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[3].

Methods

Preparation of recipient mice

BALB/c(H-2^d) female mice began to drink sterile water with Gentamicin (320 000 U/L)+Erythromycin (250 mg/L) at 5 days before transplantation, and put in γ ^{60}Co total body irradiation (TBI) at 4 hours before transplantation. Lethal-dose TBI: total dose at 8.5 Gy, dose rate at 0.5 Gy/min; non-lethal dose TBI: total dose at 6.5 Gy, dose rate at 0.5 Gy/min.

Preparation of bone marrow and peripheral T cells of donor mice

The C57BL/6(H-2^b) male mice were sacrificed by breaking off their spinal cord, separate femur, tibia and spleen under sterile condition, cut off the epiphysis of both femur and tibia, use injector to clear out the cells from the marrow cavity, spall red blood cells with lysis buffer, and washing-up with phosphatebuffer (PBS) for use. Grind and sift spleen to collect spleen cells by 200-mesh sift, obtain mononucleolus cell (MNC) through the Ficoll-Hypaque density separation method, and use nylon fiber to get rid of the major of the B cells and the peripheral T cells are obtained, among which $CD3^+CD19^-$ cells amounts to 80% according the flow cytometry (FCM) testing result. Then, washing-up the cells with PBS for use.

In vitro culture, expansion and separation of the NK cells from donor mice

Donor mice spleen MNC was obtained as above described at 15 days before the transplantation, place it into plastic culture flask and add concanavalin A (5 μg/mL). Cultivate the cells for 24 hours in the incubator with 5% CO_2 at 37 ℃ and only keep the suspension cells thereafter. Further add feeder cells and recombinant human interleukin-2 at 1 000 U/mL for culture for 2 weeks. Activate NK cells for proliferation and induce the adhesion. Then, NK cells were separated by two-step adherent methods[4], and adjusted the cell concentration to 5×10^6/mL for use through RPMI-1640 culture medium.

Transplantation groups with lethal-dose TBI

Prepared 3 normal BALB/c(H-2^d) female mice without TBI served as biologic comparative group. All mice with TBI were randomly divided into 5 groups with 19 mice on each one (among which nine mice will not be in survival observation. These mice were marked before the transplantation and they were killed respectively at 1, 2 and 3 weeks after transplantation for examination of peripheral white blood cells and CD34$^+$ cells in bone marrow). Each recipient mice were injected with grafts *via* tail vein 4 hours after TBI: Blank control group: 0.5 mL RPMI 1640; Allogenic bone marrow group: 4×10^6 bone marrow cells (BMC); Bone marrow+T cells group: 4×10^6 BMC+4×10^6 peripheral T cells; Bone marrow+NK cells group: 4×10^6 BMC+1×10^6 NK cells; Bone marrow+T+NK cells group: 4×10^6 BMC+4×10^6 peripheral T cells+1×10^6 NK cells.

Main outcome measures

Survival period: the survival rates of all lethal-dose TBI groups were counted at the 7, 15, 30, 60 and 120 days.

Hematopoietic reconstitution: the peripheral blood white blood cells (WBC) count of all transplanted mice in lethal-dose TBI were tested at the 1, 2 and 3 weeks after transplantation; and separated bilateral femoral bone and collected all BMC into the TruCont tube; add fluorescence antibody anti-mouse CD45-FITC and anti-mouse CD34-PE for staining; then use FCM to examine the CD34$^+$ cell absolute count.

Immune reconstitution: The peripheral blood of the mice in lethal-dose TBI were taken out respectively at 1, 2, 3, 4 weeks and 60 days after transplantation, the expression percentage of CD3$^+$ cell and CD19$^+$ cell in lymphocyte cells were measured by FCM after that anti-mouse CD3-PE and anti-mouse CD19-FITC fluorescence antibody double labeling.

Implantation of donor mice H-2K^{b+} cells: Peripheral blood were taken out from lethal-dose TBI mice surviving more than 120 days; measured the expression percentage of H-2K^{b+} cell in lymphocyte cells by FCM after labeling it with the fluorescence antibody Anti-mouse H-2Kb-PE and H-2K^d-FITC. On the other hand, apply the same way to the mice in non-lethal dose TBI respectively before the transplantation and in 15, 30, and 60 days after transplantation for the expression percentage of H-2K^{b+} cell.

Statistical analysis

Chi-square test was used for comparisons of the survival rate, and analysis of variance was used for comparisons of the mean; SPSS 10.0 was used to process relevant data. Average data were presented as Mean±SD.

RESULTS

Survival period

Recipient mice in the non-lethal dose TBI were all long survived. The lethal-dose results are shown in Table 1.

Hematopoietic reconstitution

The comparison of peripheral blood WBC and BM CD34$^+$ cell between all lethal-dose TBI groups and the biologic comparative group are shown in Table 2.

Table 1 Survival of mice in the lethal-dose total body irradiation groups at different time points after transplantation (n=10)

Group	Survival mice				
	+7 d	+15 d	+30 d	+60 d	+120 d
Blank control	6	0	0	0	0
Allo-BM	10	5	3	2	2
BM+T Cells	8	3	1	0	0
BM+NK Cells	10	10^a	8^a	7^a	6^a
BM+T+NK Cells	10	9^a	7^a	6^a	6^a

BM: bone marrow: NK: natural killer; ^aP < 0.01, *vs.* allo-BM and BM+T cells groups

Table 2 White blood count (WBC) in peripheral blood and BM CD34$^+$ cell in the lethal-dose total body irradiation groups at different time points (x±s)

Group	Count of peripheral blood WBC (1×10^9/L)		
	+7 d	+14 d	+21 d
Blank control		0.15±0.02	
Allo-BM	0.32±0.03	0.66±0.06	1.12±0.10
BM+T cells	0.72±0.07	1.40±0.11	1.20±0.10
BM+NK cells	0.24±0.02	0.69±0.06	5.50±0.25^a
BM+T+NK cells	0.41±0.04	1.36±0.11	5.44±0.26^a
Bio-comparative	6.85±0.65	6.80±0.60	6.80±0.60

Group	Count of BM CD34$^+$ cell (1×10^6 cells)		
	+7 d	+14 d	+21 d
Blank control		0.999±0.029	
Allo-BM	1.289±0.038	3.312±0.096	6.338±0.183
BM+T cells	1.489±0.054	6.997±0.287	6.745±0.261
BM+NK cells	1.297±0.039	5.129±0.151	8.931±0.282^{ab}
BM+T+NK cells	1.329±0.051	5.117±0.205	8.866±0.354^{ab}
Bio-comparative	9.116±0.183	9.116±0.186	9.116±0.182

BM: bone marrow: NK: natural killer; ^aP < 0.01, *vs.* allo-BM and BM+T cells groups; ^bP > 0.05, *vs.* biologic comparative group

Within 2 weeks after transplantation, the count of peripheral blood WBCs on the BM+T cell group increases mostly; count of WBCs at 14 day in the BM+T cells and BM+T+NK cells groups shows an obvious increase in comparison with the ones without injection of T cells; on the other hand, the mice with higher WBC in BM+T cell group all die at 3 weeks after transplantation; the WBC count at 21 days in BM+NK cells and BM+T+NK cells groups was higher than that without injection of donor NK cells.

The CD34$^+$ cells on each group make little difference at 7 days after transplantation; CD34$^+$ cells count is higher in BM+T cell group than others at 14 days (yet the mice with higher CD34$^+$ cell count in the group all die during 3 weeks after transplantation); The CD34$^+$ cells already come back to the normal level in BM+NK cells and BM+T+NK cells groups at 21 days after transplantation and higher than the other groups without the injection of NK cells.

Immune reconstitution

The immune reconstitution of mice in lethal-dose TBI is

described in Table 3.

Table 3 Expression percentage of CD3+ and CD19+cells in lymphocyte cells of all groups at different time points after transplantation ($\bar{x}\pm s$, %)

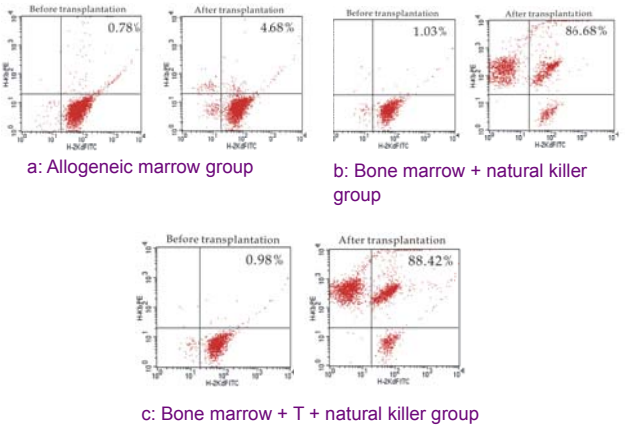
Group	CD3+ cell expression		
	+14 d	+28 d	+60 d
Allo-BM	45.07±4.01(6)	50.40±5.06(4)	56.06±5.08(2)
BM+T cells	47.33±5.03(4)	56.95±5.95(2)	
BM+NK cells	44.12±4.03(10)	33.69±3.36(9)a	57.50±5.65(7)
BM+T+NK cells	46.54±5.66(10)	35.28±4.42(8)b	56.68±5.86(6)
Bio-comparativ	58.43±5.75(3)	58.68±5.70(3)	58.30±5.65(3)

Group	CD19+ cell expression		
	+14 d	+28 d	+60 d
Allo-BM	3.13±0.32	10.60±1.13	20.03±1.98
BM+T cells	1.66±0.20	4.92±0.61	
BM+NK cells	5.88±0.68[a]	20.62±1.83[a]	24.91±2.03[a]
BM+T+NK cells	3.88±0.42b	17.80±1.65b	23.32±2.01
Bio-comparative	29.36±2.54	29.25±2.65	29.63±2.60

BM: bone marrow: NK: natural killer; [a]$P < 0.01$, *vs.* allo-BM groups; [b]$P < 0.01$, *vs.* BM+T cells group; number in parentheses indicates the number of mice at different time points

Implantation of donor H-2K^{b+} cells

Lethal-dose TBI group: Expression percentage of H-2K^{b+} in peripheral blood lymphocyte cells was (86.68±4.45) and (88.42±4.65) in the BM+NK and BM+T+NK cells groups, both of which were significant higher than the allo-BM group (4.68±0.32, $P < 0.01$). The expression percentage of H-2K^{b+} among three groups of mice that survive 120 days and the comparison with before transplantation respectively are shown in Figure 1.



a: Allogeneic marrow group

b: Bone marrow + natural killer group

c: Bone marrow + T + natural killer group

Figure 1 Comparison of flow cytometry assay for H-2K^{b+} cells in the transplantation groups (8.5 Gy total body irradiation)

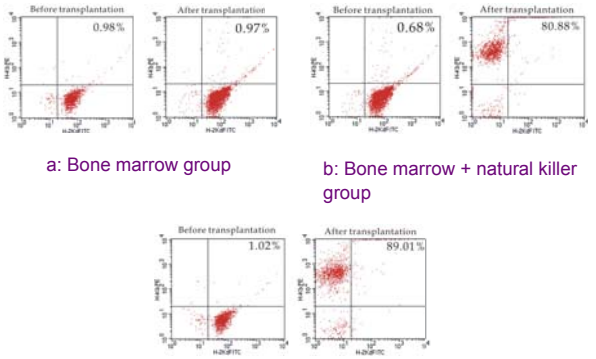
Non-lethal dose TBI groups: At 60 days after transplantation, the expression percentage of H-2K^{b+} cells on both inject with 1×10^6 and 2×10^6 NK cells groups were over 80%, which decreased to the level before transplantation in the allo-BM group. The expression percentage of H-2K^{b+} cells of each group at 15, 30 and 60 days after transplantation are shown in Table 4.

The comparison of H-2K^{b+} cells in both with and without NK cells injection groups between before transplantation and at 60 days after transplantation (Figure 2).

Table 4 Expression percentage of H-2K^{b+} cells in non-lethal dose total body irradiation groups at 15, 30 and 60 d after transplantation (n=10, %)

Group	Survival mice		
	+15 d	+30 d	+60 d
Blank control	0.98±0.24	1.04±0.27	0.97±0.28
Allo-BM	6.94±0.57	1.27±0.30	0.97±0.29[a]
BM+ NK cells	66.38±4.27[a]	73.65±4.69[a]	80.88±4.78[a]
BM+NK cells (high-conentration)	77.54±4.95[a]	82.75±4.91[a]	89.01±4.32[a]

BM: bone marrow: NK: natural killer; [a]$P < 0.01$, *vs.* allo-BM group



a: Bone marrow group

b: Bone marrow + natural killer group

c: Bone marrow + natural killer (high concentration) group

Figure 2 Comparison of flow cytometry assay for H-2K^{b+} cells in the transplantation groups (6.5 Gy total body irradiation)

DISCUSSION

Recent research demonstrates that in haplotype-mismatched allo-HSCT, if recipients are short of one killer-cell immunoglobulin receptor (KIR) epitope on donor NK, that is, the KIR ligand incompatibility in the graft-versus-host direction, the MHC class Ⅰ specific inhibitory KIR cannot recognize the ligand and will hence intercept the inhibitory signal. On the other hand, other non-MHC class Ⅰ specific activating NKRs (such as NKG2D) can recognize ligand, and activate alloreaction to aim directly at recipients allogenic cells mediated by donor NK cells. Alloreaction may probably greatly affect the allo-HSCT outcome, such as graft versus leukaemia, graft versus host disease, graft rejection, implantation of stem cells, hematopoietic and immune reconstitution[1, 5-6].

The alloreaction of mice NK cell are regulated by the recognition between lectin-like receptors (Ly49 family) and its correspondent MHC class I molecule ligand. Similar to human KIR, the combination of the mice Ly49 receptor with the MHC class Ⅰ molecule ligand can inhibit the activity of NK cell[7-9]. The NK cell in H-2^b mice mainly expresses Ly49C receptors, and the NK cell in H-2^d mice expresses Ly49A receptor epitope. The Ly49A can recognize specifically the ligand of H-2d and cannot be combined with H-2^b[10]. Our research is to experiment allo-BMT on H-2^b and H-2^d mice with mismatched Ly49 receptor epitope, study the effect of alloreactive NK cell on graft rejection and hematopoietic and immune reconstitution. The result proves that the allo-reactive NK cell promotes the engraftment

of hematopoietic stem cells, and the reconstitution of hematopoiesis and immunity. Among long survival recipient mice in the lethal-dose TBI groups, the expression of H-2K^{b+} cells over 80% on both the BM+NK cells and BM+T+NK cells groups; in contrast, the H-2K^{b+} cells expression of the two long survival mice on Allo-BM group only comes to (4.68±0.32)%. Such result suggests that NK cells inhibit graft rejection and help to steady high level donor chimerism.

In the allo-BMT under the condition of non-lethal dose TBI, a further research into the effect of NK cell on graft rejection tells that the recipient mice reject the transplanted allogenic cells and survive long due to restoration of self hematopoiesis. The results further reveal that over 80% expression of H-2K^{b+} cells are still with two groups of recipient mice with the infusion of both highly-condensed and lowly-condensed NK cells at 60 days after transplantation. Ruggeri *et al*[5] also discovered in their haplotype-mismatched BMT experiment with H-2d/b donors and H-2^{b}/ H-2^{d} recipients that, if injecting 2×10^{5} alloreactive NK cells, even the recipients only receiving non-lethal dose TBI (≤7 Gy), the possibility of donor chimerism goes to 80%. In this research and Ruggeri L's transplantation model, incompatible NKR epitope in graft-versus-host direction exists and can activate allo-reaction to aim directly at recipients' allogenic cells mediated by donor NK cells. Furthermore, the similar result from 2 different transplantation models attests that alloreactive NK cell can inhibit graft rejection and improve the implantation of stem cells. These function of NK cell in allo-BMT is probably caused by the NK cell alloreaction which can attacks the recipients T cells, that is immune functionary cells of graft rejection, and suggests that alloreactive NK cell can partially substitute for function to restrict the recipient immunity in place of TBI.

It is noticeable that, alloreactive NK cells would benefit to restore the immune function of B cell. The expression of CD3^{+} cell in both allo-BM and BM+T cell groups increased gradually after transplantation, while it decreased before the increase on the two groups with the infusion of NK cells. Whether the phenomenon is related to the clearance of recipients T cells due to the effect of NK cell's alloreaction, it is yet to be answered because of the deficiency of the evaluation index in absolute count. In any case, the experimental result suggests that the alloreactive NK cell can inhibit graft rejection, improve the implantation of hematopoietic stem cells and advance hematopoietic and immune reconstitution.

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自然杀伤细胞对异基因骨髓移植中移植排斥和造血及免疫重建的影响*☆

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摘要

背景：近几年，自然杀伤细胞与异基因骨髓移植的关系备受关注。

目的：探讨自然杀伤细胞在小鼠异基因骨髓移植中对移植排斥、造血及免疫重建的影响。

方法：以 C57BL/6(H-2b) 小鼠为供鼠、BALB/c(H-2d)小鼠为受鼠，分别在致死剂量和非致死剂量(≤7.0 Gy)全身照射的预处理条件下进行异基因骨髓移植，移植同时输注供鼠外周 T 细胞和(或)自然杀伤细胞。

结果与结论：在致死剂量全身照射的移植中，输注自然杀伤细胞与不输注自然杀伤细胞的移植组比较，存活率显著增高；白细胞计数、CD19^{+}细胞表达及骨髓CD34^{+}细胞计数恢复快；H-2K^{b+}细胞的表达水平高。移植后 28 d，输注自然杀伤细胞组 CD3^{+}细胞表达水平明显低于不输注自然杀伤细胞组，60 d 两组比较差异无显著性意义($P > 0.05$)。在非致死剂量全身照射的移植中，移植 30 d，异基因骨髓组 H-2Kb^{+}细胞表达百分率明显下降，60 d 已下降至移植前水平；而输注高浓度和低浓度自然杀伤细胞的两组在移植后 60 d 仍能检测到 80%以上的 H-2K^{b+}细胞表达。提示，在小鼠异基因骨髓移植中，同种异基因反应性自然杀伤细胞可以抑制移植排斥，提高造血干细胞的植入水平，促进造血及免疫重建并增加移植受鼠的生存率。

关键词：自然杀伤细胞；天然；骨髓移植；全身照射；同种异基因反应性

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