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Role of HLA-G and NCR in protection of umbilical cord blood haematopoietic stem cells from NK cell mediated cytotoxicity

Ming Wang [a, b, #], Yuhua Qiu [c, #], Xuelei Wang [a, #], Fang Zhao [b, #], Min Jin [b], Ming Xu [a], Ruiming Rong [a], Hailiang Ge [b], Yanyun Zhang [b], Xiangdong Wang [d, *], Tongyu Zhu [a, d, *]

[a] *Department of Urology, Zhongshan Hospital, Fudan University, Shanghai, China*
[b] *Key Laboratory of Stem Cell Biology, Institute of Health Sciences and Shanghai Institute of Immunology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine, Shanghai, China*
[c] *Department of Immunology, Soochow University, Suzhou, China*
[d] *Shanghai Key Laboratory of Organ Transplantation, Fudan University, Shanghai, China*

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Abstract

Allogeneic umbilical cord blood haematopoietic stem cells (UCB–HSCs) can be transplanted into a host with the intact innate immunity with limited immuno-reaction, although the mechanisms remain unclear. The present studies aimed at investigating potential mechanisms of allogeneic UCB–HSCs escape from the cytolysis of natural killer (NK) cells. We compared UCB–HSCs ability to protect from NK-mediated cytotoxicity with peripheral blood or bone marrow haematopoietic stem cells (PB-HSCs and BM-HSCs). HSCs expressed lower levels of natural cytotoxicity receptor ligands including NKp30L, NKp44L and NKp46L than monocytes. Blocking these ligands respectively or in combination could increase the resistance of HSCs against NK cell mediated cytotoxicity. High expression of HLA-G was noticed on UCB–HSCs, rather than PB-HSCs or BM-HSCs, whereas blockade of HLA-G significantly elevated NK cell mediated cytolysis to UCB–HSCs. Thus, we conclude that natural cytotoxicity receptors and HLA-G on HSCs may contribute to the escape from NK cells, and activate and inhibitory NK cell receptors and their ligands can be novel therapeutic targets in cell transplantation.

Keywords: stem cell • HLA-G • NCRs • NK cell • umbilical cord blood • cytotoxicity

Introduction

Transplantation of haematopoietic stem cells (HSCs) is considered to be one of successful alternatives to treat malignant and non-malignant haematopoietic disease [1, 2], and a new therapy for non-haematologic diseases, including autoimmune diseases, multiple organ dysfunction and allogeneic transplantation rejection [3–7]. HSCs can be recovered from bone marrow (BM), peripheral blood (PB) and umbilical cord blood (UCB). HSCs from UCB have been suggested to be the major resource for clinical applications of HSC transplantation. UCB–HSC transplantation has a number of advantages, *e.g.* to reduce the incidence of acute graft-*versus*-host disease [8, 9], have faster availability of banked cryopreserved UCB units [10, 11], and process the tolerance of one to two human leucocyte antigen (HLA) locus mismatch [12]. However, it remains largely unknown that how can donor HSCs, particularly from UCB resource, engraft successfully *in vivo* in a host with intact innate immunity.

Natural killer (NK) cells play a fundamental role in the innate immune response through their ability to secrete cytokines and kill target cells without prior sensitization. The cytotoxic effect of NK cells is executed *via* natural cytotoxicity receptors (NCRs) expressed on NK cells and HLA-G expressed on target cells. NCRs is a main group of the killer cell activatory receptors, include NKp30, NKp44 and NKp46, through which NK cell activation-increased intracellular Ca^{2+} flux may trigger cytotoxicity and lymphokine release [13–16]. The recognition by NKp44 and NKp46 is critical to lyse cells expressing the corresponding viral glycoproteins [17, 18], whereas HLA-G can recognize and bind to killer cell inhibitory receptors on NK cells, protecting target cells from cytotoxicity of NK cells, $CD4^+$ and $CD8^+$ T cells and antigen-presenting

[#]These authors equally contributed to this study.
*Correspondence to: Dr. Tongyu ZHU, Xiangdong WANG MD, PhD, Department of Urology, Zhongshan Hospital, Fudan University, Shanghai, China.
Tel.: +86-21-6403-7287
Fax: +86-21-6403-7269
E-mail: tyzhu@fudan.edu.cn, xiangdong.wang@telia.com

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cells [19–21]. However, mechanisms by which UCB–HSCs can escape from the attack of NK cells remain unclear. This study aimed at investigating whether the NCRs/NCR ligands and HLA-G on the HSCs affect NK cell mediated cytotoxicity. We found that low levels of NKp30L, NKp44L and NKp46L on the surface of HSCs may contribute to HSCs' immune escaping from NK cells, whereas high level of HLA-G on UCB–HSCs may be responsible for the better ability of immune escaping from NK cells than PB-HSCs and BM-HSCs.

Materials and methods

Fusion proteins, antibodies and cell lines

All recombinant human immunoglobulin (Ig) fusion proteins, including NKp30/Fc chimera, NKp44/Fc chimera and NKp46/Fc chimera, were purchased from R&D Systems (Minneapolis, MN, USA). Streptavidin-conjugated microbeads were purchased from Miltenyi Biotech (Auburn, CA, USA). Biotin-conjugated anti-CD34 antibody (Ab, 43A1) was obtained form Ancell Corporation (Bayport, MN, USA). Fluorescein isothiocyanate conjugated mouse anti-human IgG1 (4E3) and HLA-G (MEM-G/9) Abs were obtained from Southern Biotech (Birmingham, AL, USA) and Serotec (Oxford, UK), respectively. Neutralizing mouse anti-human HLA-G Ab (87G) were purchased from Exbio (Prague, Czech Republic). Other Abs used for immunofluorescence staining were obtained from BD Pharmingen (San Diego, CA, USA). NK-92 cells, provided by Dr. Zhigang Tian (University of Science and Technology of China, Anhui, China), were grown in α-MEM culture medium, which contains 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2- mercaptoethanol, 0.02 mM folic acid, 100U recombinant human IL-2, 12.5% horse serum and 12.5% foetal bovine serum, but lack of RNA and DNA.

Cell preparations and flow cytometry analysis

PB-HSCs, BM-HSCs or UCB–HSCs were obtained from the healthy adult or parturient and incubated with biotin-conjugated anti-CD34 Ab and the streptavidin-conjugated microbeads, followed by a magnetic selection, in order to produce HSC-depleted PB mononuclear cells. The study protocol was approved by the institutional review board of the Institute of Health Sciences (Shanghai, China). HSC-depleted PB mononuclear cells were prepared as PB-MNCs. NK cells were isolated from adult PB by fluorescence-activated cell sorting (FACS; FACSAria, BD Biosciences, San Diego, CA, USA) using anti-CD16 and anti-CD56 Abs. Informed consent was obtained from all study subjects before sample collection. Immunofluorescence analyses of cell surface phenotypes were performed using FACSAria (BD Biosciences). Expression of NCR ligands were detected by incubating cells with NKp/Fc fusion proteins and subsequently anti-IgG1 Ab as described [22].

Cytotoxic assays

The cytolytic activity of PB-NK cells and NK-92 cells against the HSCs was assessed in 4-hr lactate dehydrogenase (LDH) release assay using CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA). Effector and target cells were incubated together in 37°C, 5% CO_2 for three hrs and 15 min., and then 10 μl lytic solution was added into target cell maximal release group and corrected volume control group. After continue incubating for 45 min., the cytolytic activity was measured according to the assay procedure provided with the Assay kit. The blocking reagents were added to target cells to achieve the final protein concentration of 0.2–0.5 μg/ml. There were six groups, *i.e.* PB-MNCs depleting PB-HSCs as control, effect cells-spontaneous release, target cells-spontaneous release, target cells-maximal release, culture media background and volume corrected control. NK cytolysis rate (%) was calculated as follows = [(control well – spontaneous release)/(maximal release − spontaneous release)] $\times$ 100%. The spontaneous release was $<$25% of maximal release in all presented experiments.

Colony forming cell (CFC) assay

The numbers of HSCs with the ability to form colony *in vitro* assay of NK cells were measured using CFC Assay. Human Methylcellulose Complete Media Kit was obtained from R&D systems (Minneapolis, MN, USA). The methylcellulose media contained SCF, GM-CSF and EPO. Effector cells ($2 \times 104/100$ μl) and target cells ($2 \times 103/100$ μl) were added to each well of the round-bottom 96-well plates. Numbers of CFC in the cultures were numerated at the 14^{th} day after culture. The colony number was counted and the inhibition rate was calculated as follows: inhibition% = [target cell maximal proliferation well – (control well – effector cell control well)]/target cell maximal proliferation well $\times$100%.

Statistical analysis

Significant differences were evaluated using the Student's *t*-test, except that multiple treatment groups were compared within individual experiments by ANOVA or Kruskal–Wallis test. Data are expressed as the mean $\pm$ S.D., and *P* values less than 0.05 were considered significant.

Results

UCB–HSCs resisted NK cell mediated cytolysis

Our results showed that all HSCs were resistive to the cytolysis mediated by freshly-isolated allogeneic NK cells, of which UCB–HSCs have the most powerful resistance (Fig. 1A). When the effector cell:target cell (E/T) ratio is 10, allogeneic NK cells killed 80.387 $\pm$ 4.499% of MNCs, whereas 30.187 $\pm$ 2.071% of PB-HSCs, 20.380 $\pm$ 1.156% of BM-HSCs and only 7.493 $\pm$ 0.737% of UCB–HSCs (Fig. 1A, $P < 0.05$ *versus* PB-MNCs). Both PB-HSCs and BM-HSCs were more sensitive to the cytolytic killing of NK cells, as compared to UCB–HSCs, respectively (Fig. 1A, $P < 0.05$). These data suggest that UCB–HSCs are able to escape from the cytolytic killing of NK cells, which is also evidenced by using control effector cell line NK-92 cells (Fig. 1B). We assayed CFC to test the role of effecter NK cells in the proliferation of HSCs and

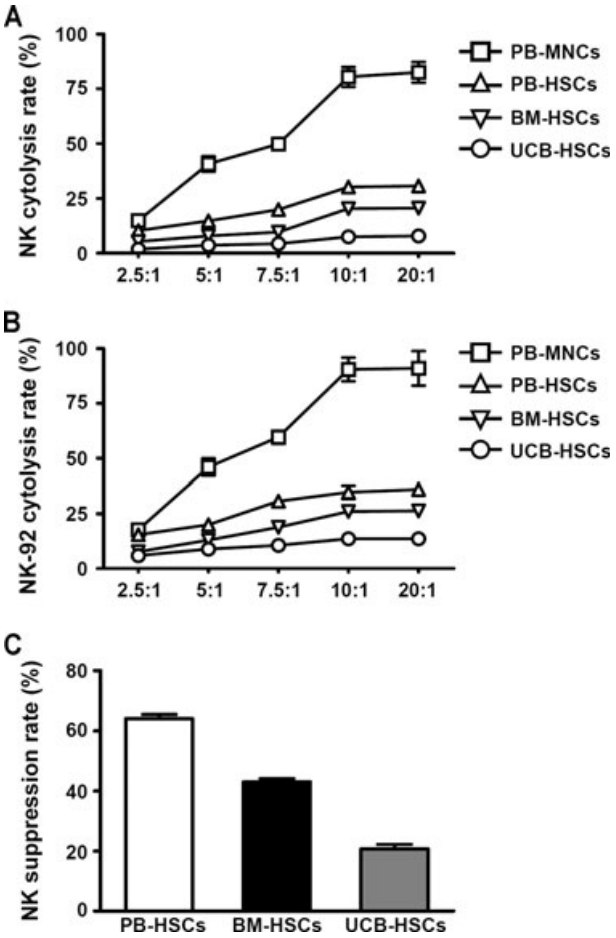

Fig. 1 NK cell cytolysis of peripheral blood NK cell (**A**) and NK-92 (**B**) in peripheral blood (PB), bone marrow (BM) and umbilical cord blood (UCB) haematopoietic stem cells (HSCs) by the 4-hr LDH release assay at five different effector cell:target cell (E:T) ratios. Peripheral blood monocytes depleting PB-HSCs are used as control cells. Inhibition of peripheral blood NK cell against the proliferation of HSCs (**C**) was measured by the Colony Forming Cell Assay after co-culture with effector cells for 4 hrs. E:T = 10:1. Data were expressed as means ± S.D. of six samples for each group and results represented three independent experiments.

we found NK cells significantly suppressed the proliferation of UCB–HSCs (20%), BM-HSCs (40%) and PB-HSCs (65%), respectively, as shown in Figure 1C ($P < 0.05$). Taken together, HSCs, in particular of UCB–HSCs, are resistive to NK cells.

HSCs expressed low levels of NCR ligands

Flow cytometry analysis showed that PB-MNCs expressed 47% NKp30L, 45% NKp44L and 46% NKp46L, respectively. As shown in Figure 2, all HSCs expressed significantly lower levels of these ligands, as compared to PB-MNCs ($P < 0.05$) and there was no significant difference among the three HSCs on the expressions of NCR ligands. This phenomenon suggests that low expression of NCR ligands may be contributable for the immune resistance of HSCs, but not the main factor that contribute to the UCB–HSCs' most powerful resistant ability.

Low level of NCR ligands partially protected UCB–HSCs against NK cytolysis

To further investigate whether the expression levels of NKp30L, NKp44L and NKp46L affect immune escaping ability, human Ig fusion proteins, including NKp30/Fc chimera, NKp44/Fc chimera and NKp46/Fc chimera, were used to block those NCR ligands, respectively or in combination. As shown in Figure 3A, addition of NKp30/Fc chimera, NKp44/Fc chimera or NKp46/Fc chimera significantly decreased the cytolytic killing activity of NK cells against PB-MNCs, as compared with controls ($P < 0.05$), whereas the combination of three NCR ligand blockers resulted in more significant inhibition of NK cytotoxicity in PB-MNCs than the correspondent each alone ($P < 0.05$). The similar inhibitory effects of blocking the expressions of NKp30L, NKp44L and NKp46L were noted on PB-HSCs, BM-HSCs and UCB–HSCs (Fig. 3B). All the results indicate that, lower expression of NCR ligands protects HSCs against NK cytolysis. However, it is just one possible mechanism in the protection of UCB–HSCs against NK cytolysis.

High levels of HLA-G protected UCB–HSCs against NK cytolysis

To further illuminate why UCB–HSCs have more potent ability than the other two HSCs to escape from NK cell mediated cytolysis, the expressions of immune tolerance molecule HLA-G were tested. As shown in Figure 4A, UCB–HSCs expressed high level of HLA-G (57.6%), whereas PB-HSCs and BM-HSCs expressed scarcely HLA-G (0.2% and 0.1%, respectively). When neutralized with anti-HLA-G monoclonal Ab, NK cell mediated cytolysis rate of UCB–HSCs significantly increased from 7.530 ± 0.429% to 25.467 ± 0.679% (Fig. 4B, $P < 0.05$), indicating that HLA-G contribute to the immune resistance of UCB–HSCs to the cytolysis of NK cells. Taken together, all the results demonstrate that, low-level cross-talking of NCRs with their ligands and high-level expression of HLA-G protect UCB–HSCs from NK-mediated cytolysis.

Discussion

Transplanted stem cells escape from the attacking by the host immune system as the first step of transplantation tolerance. Its mechanism remains elusive, although mixed haematopoietic chimerism was suggested to lead to tolerance [4]. The results

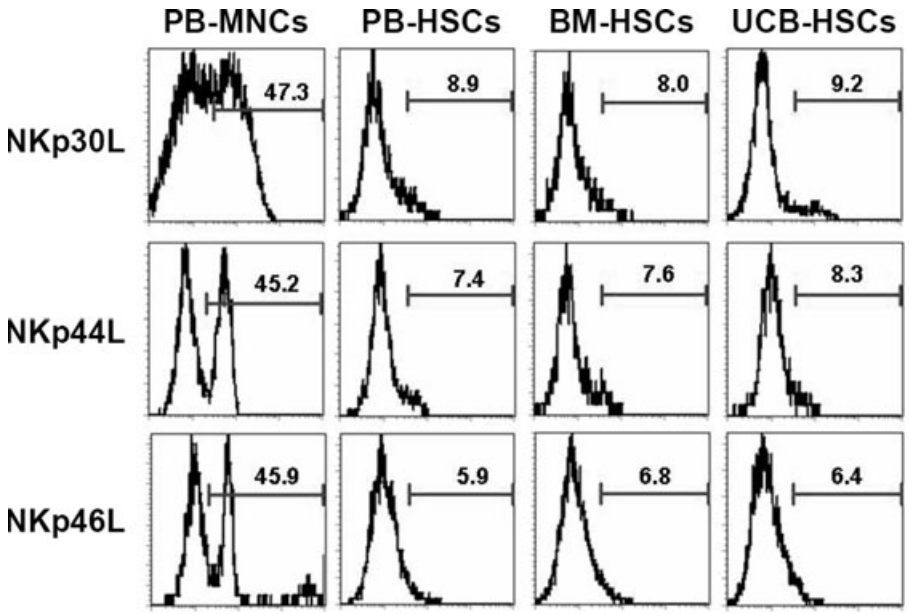

Fig. 2 Expression levels of NKp30L, NKp44L and NKp46L on peripheral blood (PB), bone marrow (BM) and umbilical cord blood (UCB) haematopoietic stem cells (HSCs) measured by the FACS analysis. NKp30/Fc, NKp44/Fc and NKp46/Fc fusion proteins were incubated with the cells respectively, and then stained with FITC-conjugated secondary Ab. Peripheral blood monocytes depleting PB-HSCs were used as control cells. Data represented three independent experiments.

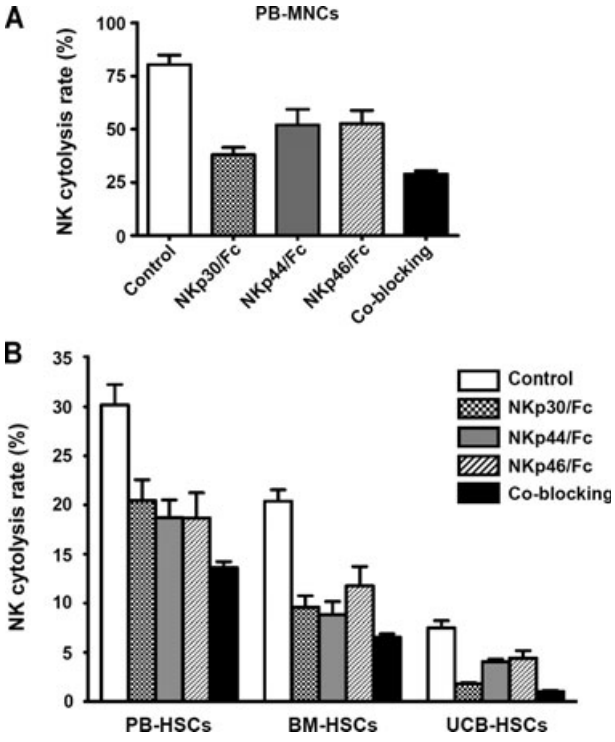

Fig. 3 The NK cell cytolysis rate in peripheral blood monocytes depleting haematopoietic stem cells (HSCs, **A**), peripheral blood (PB, **B**), bone marrow (BM, **C**) and umbilical cord blood (UCB) HSCs (**D**) after blocking NK30pL, NKp44L and NKp46L using Ig fusion proteins. The cytolysis was tested by the 4-hr LDH release assay at the effector cell:target cell ratios = 10:1. Data were expressed as means ± S.D. of six samples for each group and results represented three independent experiments.

from this study demonstrate that UCB–HSCs are the most powerful to resist NK cell mediated cytolysis when comparing with PB-HSCs and BM-HSCs. It seems that low expression of NKp30L, NKp44L and NKp46L may play the critical role in the occurrence of NK cell-resistance in all HSCs. However, it is still not enough to explain the mechanism by which UCB–HSCs had better ability of the resistance than other HSCs. From the present results, we demonstrate that HLA-G expressed on UCB, but not on other HSCs, contribute to the best immune resistance.

It was hypothesized that donor allogeneic HSCs are eliminated by host NK cells because of the lack of expression of recipient MHC proteins on the transplanted cells [23], where NK cells need the activating signal to initiate such effects. It was found that NCRs functioned as the main activating receptors to trigger human NK cell mediated cytolysis to allogeneic cells [24, 25], probably through different transmembrane-anchored polypeptides associated with immune tyrosine-based activating motifs bearing adapter molecule DAP12 [26]. Although the ligands recognized by NCRs are still unknown, there was suggestive evidence that the pattern of ligand expression might vary between cell types. The findings from the previous and present studies confirm that NCRs play a major role in the process of the NK-mediated lysis in tumour cells and HSCs. Our data indicate that the low-level cross-talking of NKp30, NKp44, NKp46 with their corresponding ligands may contribute to the property of resistance of HSCs, as evidenced by the fact that the blocking of them increased the level of immune resistance. This was supported by the findings that human NK cytotoxicity against porcine cells was partially triggered by NKp44 after the xenotransplantation and reduced using anti-NKp44 Ab [27]. It was also supported by other findings that low cell-surface density of ligands for NCRs resulted in tumour cell insensitive to recognition and killing by NK cells,

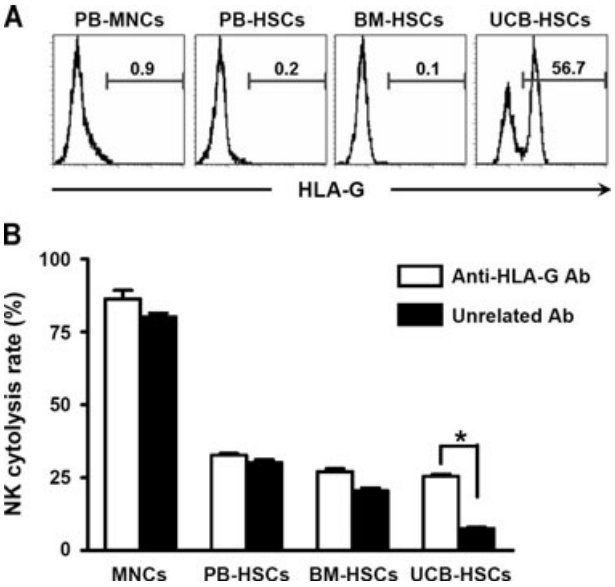

Fig. 4 The expression of HLA-G (**A**) on peripheral blood (PB), bone marrow (BM) and umbilical cord blood (UCB) haematopoietic stem cells (HSCs) measured by the FACS analysis. The cytolysis rate (**B**) of controls cells, PB-HSCs, BM-HSCs and UCB–HSCs after the treatment with HLA-G mAb or unrelated mAb. Peripheral blood monocytes depleting HSCs were used as control cells. Data were calculated as means ± S.D. of six samples for each group and results represented three independent experiments; $*P < 0.05$.

whereas up-regulation of such ligands increased the susceptibility of cells to NK-mediated cytolysis [28].

However, the results from this study indicate that the ligand of killer cell inhibitory receptors, *e.g.* HLA-G, may play even very important role in the occurrence of the resistance among HSCs. The human non-classical MHC class I molecule HLA-G has long been known as a molecule selectively expressed by cytotrophoblastic cells. By inhibiting the cytolytic function of decidual NK cells, HLA-G protects the HLA-A and HLA-B negative semi-allogeneic embryonic tissue against the mother's immune system [29]. In patients with heart transplantation, HLA-G was found to be correlated with better graft acceptance [20]. The expression of HLA-G by transplanted tissue might contribute to the graft protection and soluble HLA-G could induce apoptosis of activated $CD8^+$ T cells and modulate NK response, inhibiting both NK cell and T cell mediated cytolysis [30, 31]. The protective effect of HLA-G on xenogeneic NK cell cytolytic and migratory functions was also noticed in transfection systems [32]. Our results demonstrate that UCB–HSCs expressed much higher levels of HLA-G than the control cells, PB-HSCs and BM-HSCs and anti-HLA-G Ab disserved the protection of UCB–HSCs against the cytolysis of NK cells, more than other HSCs. It confirms that HLA-G is involved in the protective process of transplanted cells from NK-mediated cytotoxicity and could play the unique role in the ability UCB–HSCs to antagonize NK killing.

NK cells are involved in the immune rejection after allogeneic transplantation. Previous studies showed that NK cells could mediate allograft damage and rejection in the absence of $CD8^+$ T cells [33], and host NK cells have been known to be capable of mediating bone marrow cell allograft rejection in irradiated mice [34, 35]. Nevertheless, HSCs seems be able to escape from the control of host immune cells, but the mechanism remains elusive and controversial. Our research works raise an important question about how HSCs induce NK-tolerance. In normal physiological status, NK cells present in immune-privileged sites, but exhibit NK cell-tolerance, for example, in placenta. In pregnant woman, presence of HLA-G molecular expressed on foetal trophoblast cells play important role in maternal–foetal tolerance through recognized by MHC-I specific inhibitory receptors on NK cells [36]. High level of HLA-G is also detected in cornea [37]. These results suggest possible mechanisms by which immune tolerance can be established and maintained in individual development, and our researches also show that engraft UCB–HSCs acquire powerful NK-resistant capability by similar way. All these findings suggest possible strategy to establish and maintain NK cell tolerance in allogeneic organ or cell transplantation by regulating the NCR ligands and HLA-G in graft.

In conclusion, our data showed that UCB–HSCs had better ability to protect from NK-mediated cytotoxicity, as compared with PB-HSCs and BM-HSCs. The low expressions of NKp30L, Nkp44L and NKp46L on HSCs contribute to the mechanisms by which they could escape from NK cells. HLA-G was found to be the responsible factor to explain the better ability of UCB–HSCs to escape from NK cells. Our findings provide a better understanding into the role of activating and inhibitory NK cell receptors and their ligands in UCB–HSCs transplantation, suggesting a novel strategy and alternative to improve cell therapies.

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