Chapter 6

Donor Natural Killer Cells and Their Therapeutic Potential in Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract

Natural killer (NK) cells were first identified and named for their "natural" cytotoxicity to reject bone-marrow allografts in lethally irradiated mice. Different from T cells, NK cells require no prior sensitization or immunization to lyse transformed or virally infected target cells and are non-major histocompatibility complex (MHC)–restricted. However, recent progress in understanding of NK cells biology has proved that NK cells share some similar characteristics with T cells. During development, NK cells also undergo "education" according to "missing self" principle, thereby become mature and acquire effector function. The discovery that NK cells are able to "remember" prior certain stimulations indicates they may also contribute to adaptive immunity. After hematopoietic stem cell transplantation (HSCT), NK cells are the first donor-derived lymphogenous cells to reconstitute and alloreactivitiy of donor-derived NK cells have been shown to mediate graft-versus-leukemia (GvL) effect rather than to induce graft-versus-host disease (GvHD). These properties make donor-derived NK cells appealing for applications to benefit the outcome of HSCT. Here, we will review the improved understanding of NK cell biology, discuss characteristics of donor-derived NK cells which are associated with beneficial outcome of HSCT and explore novel methodologies that enhance the therapeutic effect of donor NK cells.

Keywords: hematopoietic stem cell transplantation, NK cells, immunotherapy, leukemia, cytomegalovirus, immune memory

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is one of the most effective therapy for leukemia and other malignant diseases [1]. Its major complications are

graft-versus-host disease (GvHD), infections, and leukemia relapse [2, 3]. Natural killer (NK) cells represent a key component of innate lymphoid cells and provide defense against microbial infection and malignant transformation by direct cytotoxicity and cytokine production [4, 5]. The role of NK cells in allo-HSCT was first demonstrated in haploidentical transplants [6]. During T-cell depleted haploidentical HSCT, the rapid recovery of donor-derived NK cells mediated potent graft-versus-leukemia (GvL) effect. Importantly, alloreactive NK cells mediated beneficial GvL effect without the occurrence of GvHD, which was consistently caused by donor T cells [7, 8].

Although the rapid reconstitution of donor NK cells plays a critical role in their GvL effect of the graft, they still take about 6 months or more to acquire maturation phenotype and full functionality [9]. This immaturity and insufficient education status may result in impaired function of donor NK cells in the early stage post transplantation. In addition, since the alloreactivity of donor NK cells was proved to account for the clinical benefit, genotyping the polymorphisms of killer cell immunoglobulin-like receptor (KIR), human leukocyte antigen (HLA) and Fcγ receptor (FcγR) are important for donor selection to maximize the GvL effect [10]. Sufficient numbers of allogeneic NK cells with high purity can also be generated and expanded through several sources including peripheral blood mononuclear cells, umbilical cord blood (UCB), and bone marrow–derived $CD34^+$ cells to be adoptively transferred after allo-HSCT [11, 12]. Many approaches involving the use of different feeder cells, engineered feeder cell, and cytokine stimulation were utilized to achieve sufficient numbers of donor NK cells with the most efficient GvL effect and clinical responses [13, 14]. Moreover, seven NK cell lines have been established to be used effectively during allo-HSCT, among which, NK-92 cell line has been shown to be safe and efficient in clinical trials [15–17]. Recently, genetic modification of NK cells has also been developed to enhance their function. Both gene transfer of chimeric antigen receptors (CARs) and expression of cytokine transgenes in NK cells are performed to improve the efficacy of NK cells therapy [18–20]. Furthermore, although traditionally considered as members of innate branch, increasing studies suggest that NK cells also "remember" prior certain stimulation like antigens, cytomegalovirus (CMV), or cytokines [21–26]. It draws particular interest to evaluate the role of adoptively transferred memory-like donor NK cells in allo-HSCT. Based on these research progresses of donor NK cell-mediated immunotherapy during allo-HSCT, in this chapter, we will describe the present state of donor NK cell therapy during allo-HSCT and its future direction aiming to improve therapeutic benefit of donor NK cells.

2. Overview of natural killer cell biology

NK cells represent a key component of innate lymphoid cells (ILC) and provide defense against microbial infection and malignant transformation. They belong to ILC1 group (according to their ability to produce type1 cytokine and transcription factors, they required for differentiation) and are so named for their capacity to mediate cytotoxicity toward cancer cells and virus-infected cells without prior sensitization [27–29]. NK cells constitute 10–15% of human

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peripheral blood lymphocytes and are defined by expression of CD56 without T cell marker CD3 [30]. Human NK cells can be further divided into two different subsets depending on the expression density of CD56 and CD16 [30]. The $CD56^{dim}$ $CD16^{bright}$ subsets account for majority (90%) of peripheral blood circulating NK cells and display cytotoxic function [31, 32], whereas $CD56^{bright}$ $CD16^{bright}$ subsets are predominantly found in lymph nodes and produce abundant amounts of inflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), tumor necrosis factor beta (TNF-β), IL-10, IL-13, granulocyte-macrophage colony stimulating factor (GM-CSF), etc., thereby promote adaptive immune responses [31, 33].

2.1. Natural killer cell receptors

NK cells immune function is mediated through an array of inhibitory and activating cell surface receptors. There are three main types of receptor families: KIR, C-type lectin receptors and natural cytotoxicity receptors (NCR) [34].

KIRs are proteins belonging to immunoglobulin superfamily that are encoded by a gene complex located on chromosome 19q13.4. Individuals may have up to 15 different KIR genes and two pseudogenes encoding for relevant receptors [35]. KIRs express stochastically on mature NK cells and recognize HLA molecules in a manner independent of antigen presentation. When bound to their ligands, these receptors transmit either inhibitory or activating signals. KIR genes are inherited as haplotypes due to the different number of KIR gene, and their high degree of diversity induced by various KIR gene content and allelic polymorphism [36]. Two major groups of KIR haplotypes have been distinguished based on gene content. Group A haplotypes contain a fix number of genes that are mainly inhibitory KIR, including KIR2DL1, KIR2DL3, KIR3DL1, KIR2DS4, and the pseudogene KIR2DP1. On the other hand, group B haplotypes are alterable both in numbers and content of KIR gene and have at least one of the following genes: KIR2DL2, KIR2DL5A, KIR2DL5B, KIR2DS2, KIR2DS5, and KIR3DS1 [37]. Group B haplotypes contain more activating KIR (up to 5) compared with group A haplotypes (only one).

C-type lectin receptors are heterodimers and comprise of two subunits including CD94 protein and a C-type lectin natural killer group 2 (NKG2) molecule [38]. The gene of this receptor family located on chromosome 12 encoding six different NKG2 proteins: NKG2A, NKG2C, NKG2E, NKG2F, NKG2B, NKG2H, as well as CD94 protein. Among these receptors, CD94/NKG2A and CD94/NKG2B are inhibitory receptors and bind HLA-E, whereas other receptors are activating. Activating receptor NKG2D also belongs to NKG2 family and is expressed on the surface of NK cells as a homodimer. NKG2D can recognize two different families of cognate ligands including nonclassical major histocompatibility complex (MHC) molecules (MIC-A and MIC-B) and UL16 binding protein family [39, 40]. The expression of NKG2D ligands is upregulated under the influence of cellular stress such as viral infection, inflammation, and tumor formation.

Natural cytotoxicity receptors including NKp30, NKp44, and NKp46 are activating receptors expressed on the surface of NK cells [41]. NKp30 can recognize ligands expressed on tumor cells such as the nuclear factor HLA-B-associated transcript-3 (BAT-3) and B7-H6 [42]. NKp30 also binds to viral ligands (CMV pp65 protein) [42]. Both NKp46 and NKp44 bind to viral-derived

proteins such as influenza hemagglutinin (HA) [43, 44]. Moreover, NKp44 has been shown to recognize the envelope glycorpoteins from West Nile and dengue viruses and NKp46 has been shown to recognize vimentin expressed on *Mycobacterium tuberculosis*–infected human monocytes [45]. However, the ligands of NCR have not been well identified yet.

2.2. Natural killer cell function

Upon education, the "licensed" NK cells acquire cytotoxic activity toward target cells lacking self MHC class-I molecules, meanwhile they are tolerant to normal cells expressing self MHC class-I molecules, namely "missing self" hypothesis. However, this hypothesis seems to oversimplify NK function regulation. Activating receptors have also been shown to play an important role to make the decision "to kill or not." For example, during murine allo-HSCT model, alloreactive donor NK cells do not respond to host's epithelial cells although donor NK cells lacking inhibitory receptors specific for host MHC class-I, mainly due to their low expression level of ligands specific for activating receptors of donor NK cells [6]. In some cases, the extremely high strength of activating signals may even overcome weaker inhibitory signals resulting in activation of NK cells [46, 47]. Therefore, triggering of NK cell activation is finally depended on the balance of activating and inhibitory receptors of NK cells.

Once NK cells are activated, they respond to target cells with function similar to $CD8^+$ T cells. Immune synapse forms and perforin and granzyme are released to induce apoptosis of target cells through activation of Caspase 3 [48]. NK cell activation also upregulates the expression of factor associated suicide (FAS) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and kill the target cells through Caspase 8 activation induced apoptosis [49, 50]. Furthermore, NK cells can also crosstalk with adaptive immune system through cytokine release and antibodies. Virus infected cells and tumor cells are recognized by B cells. These cells are then marked with antibodies covering their cell surface. NK cells recognize and bind to these cells through the interaction between activating receptor CD16 and the Fc end of antibodies [51]. This recognition mediates a potent activating signal in NK cells and results in target cells lysis. Moreover, NK cells are able to secret cytokines and chemokines upon activation, such as TNF-α, IFN-γ, regulated upon activation, norma, T-cell expressed and secreted (RANTES), GM-CSF, macrophage inflammatory protein-1-alpha and beta (MIP1-α and-β). NK cells can promote dentritic cells maturation through TNF-α and IFN-γ secretion [52]. IFN-γ production also affects helper T cell subset 1 (Th1) migration and effector function. Additionally, Th1 polarization in secondary lymphogenous organs is enhanced by IFN-γ secretion [53]. Thus, IFN-γ secretion of NK cells is critical for forming a bridge between the innate and adaptive immune responses.

3. Natural killer cell development and education

3.1. Natural killer cell development

NK cells develop from $CD34^+$ hematopoietic progenitor cells which can also give rise to T cells, B cells, and dendritic cells [54]. Particular transcription factors such as purine rich box-1 (PU.1), E26 transformation-specific (ETS-1), thymocyte selection-associated HMG box factor (TOX), the

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mammalian transcription factor E4 binding protein 4 (E4BP4), eomesodermin (Eomes), and T-box transcription factor (T-bet) are required for NK cell maturation stages [55–58]. Among these transcription factors, Eomes and T-bet are responsible for cytotoxicity and IFN-γ production in mature NK cells [59]. Furthermore, $CD56^{dim}$ NK cells express higher levels of T-bet and lower levels of Eomes than $CD56^{bright}$ NK cells, and T-bet/Eomes ratio increases with NK cells maturation [60]. A central cytokine required for NK cell development *in vivo* is IL-15, as mice lacking IL-15, CD122 (IL-15β-chain receptor), interleukin 15 receptor alpha (IL-15Rα), or signal transducer and activator of transcription 5 (STAT5) do not generate mature NK cells [61]. Stem cell factor (SCF), IL-7, and fms-like tyrosine kinase 3-ligand (Flt3-L) are also necessary for NK cell maturation since they induce pro-NK cell to express CD122 and CD132 (the common γ-chain receptor) so as to render cell to respond to IL-15 [62, 63]. Intriguingly, IL-15 is primarily accompanied by IL-15Rα and transpresented by accessory cells to support NK cell differentiation and proliferation *in vivo* rather than affecting NK cell in a soluble form [64].

After maturation in the bone marrow, NK cells migrate to peripheral circulation and take part in the defense against cancers and viral infections. Two major subsets in the peripheral blood are $CD56^{dim}$ and $CD56^{bright}$ NK cells [30]. These subsets differ in receptor expressions, functional capabilities, and tissue distribution. Although $CD56^{dim}$ NK cells have been considered to be cytotoxic population, it is now clearly proved that they also produce large amounts of cytokines upon receptor-induced NK cell triggering [65, 66]. These functional differences can be partly explained by different expression pattern of surface receptors. $CD56^{dim}$ NK cells express CD16, KIR and other activating or inhibitory receptors which are critical for their cytolytic functions, whereas $CD56^{birght}$ NK cells rarely express KIR and CD16 [67]. Interestingly, $CD56^{bright}$ NK cells could express KIR and CD16 after stimulation of IL-2 or IL-12 suggesting $CD56^{bright}$ NK cells are NK cell subsets in the stage of maturation [68, 69]. Thereafter, recent studies confirm that $CD56^{dim}$ NK cells indeed differentiate from $CD56^{bright}$ NK cells which display longer telomerase [70]. Due to different expression of chemokine receptors and adhesion molecules, $CD56^{dim}$ NK cells account for around 90% of peripheral blood NK cells, while $CD56^{bright}$ NK cells primarily exist in secondary lymphoid tissues or decidual tissues during early pregnancy [71].

3.2. Generation of memory-like natural killer cells

Immune memory is previously considered as an exclusive property of T cells and B cells and defined as quicker and more robust responses to recurrent antigens [72]. However, accumulating evidence proved NK cell also have memory-like feature after certain modes of challenge including antigen-specific stimulation and antigen-independent activation. The hepatic NK cell memory was first observed by the Von Andrian's group [73]. They found NK cells can mediate delayed-type hypersensitivity (DTH) reactions in *Rag2*$^{-/-}$ mice model. These hapten-specific NK cells are exclusively recruited to the liver and identified as $CD49a^{+}$ $DX5^{-}$ NK cell population, expressing chemokine receptor CXCR6 [21, 73, 74]. The mechanism that generate this antigen-specific NK cells whose receptors cannot be somatically rearranged remain unknown. Moreover, similar hepatic memory response has not been proved to occur in humans.

Another differentiation pathway for antigen-specific NK cell memory response is CMV infection. CMV-triggered NK cell memory was first observed by the Lanier laboratory demonstrating

that Ly49H$^+$ NK cells can rapidly expand and response robustly after murine CMV (MCMV) re-infection [75]. NK cell co-activating receptor DNAX adhesion molecule-1 (DNAM-1) is required for the initial expansion of MCMV-induced memory NK cells [76]. Furthermore, transcription factor Zbtb32 and pro-inflammatory cytokine pathway also play important role in MCMV-induced expansion [77, 78]. In human, natural killer Group 2, member C positive (NKG2C$^+$) NK cells also expand during acute human CMV (HCMV) infection and display a CD56^{dim}CD57$^+$NKG2A$^-$ phenotype [79, 80]. These HCMV-induced memory NK cells have increased expression of IFN-γ, which may be induced by epigenetic remodeling of *IFNG* locus [81, 82]. The role of HCMV-induced NK memory during HSCT will be described in section 6 of this chapter.

Antigen-independent NK cell memory response was first observed by the Yokoyama laboratory [24]. They showed mouse NK cells which were pre-activated with the combination of IL-12/15/18 overnight produced increased level of IFN-γ upon re-stimulation. Additionally, these cytokine-induced memory-like NK cells displayed enhanced proliferation and upregulation of CD25 expression in response to re-stimulation with cytokines [24, 25]. Intriguingly, human NK cells also can give rise to cytokine-induced memory-like NK cells with similar key properties [26]. Series of studies explored the antitumor responses of cytokine-induced memory-like NK cells, and we will discuss their role during HSCT in section 7 and 9 of this chapter.

3.3. Natural killer cell education

3.3.1. Inhibitory receptors are critical for natural killer cell education

The genes encoding for KIR are located on chromosome 19, while HLA genes are on chromosome 6 [83]. Therefore, KIR genes and HLA genes are inherited independently, and this randomness may result in the expression of KIR on NK cells in the absence of their corresponding HLA ligands, thereby NK cells may have chance to attack normal cells. In fact, NK cells are tolerant to our body most of the time. It is interesting to understand how NK cells are educated to become tolerant? Inhibitory receptors have been shown to play an important role in this education process. During NK cell maturation, when the inhibitory receptors recognize self-MHC class I, the stochastic expression of KIR genes is switched off, and signals are transmitted to promote NK cell maturation and generate effector function. Oppositely, when the inhibitory receptors of NK cells fail to recognize self-MHC class I, they remain in a state of hyporesponsiveness and are not able to attack normal autologous cells [35, 84]. Collectively, only NK cells expressing "at least one" self-recognizing inhibitory receptor are "licensed" and acquire effector function, whereas "unlicensed" NK cells that recognize non-self-MHC are hyporesponsive and tolerant to normal cells. This process of "licensing" was proved in "peptide transporter-associated antigen processing" (TAP)-deficient patients [85]. Cells from these patients hardly express HLA class I molecules on their surface, thereby potentially render self-cells to be targets of NK cells. However, most of these patients do not have autoimmune disorders because of the hyporesponsiveness of NK cells. Additionally, NK cells isolated from TAP-deficient patients are also unresponsive to autologous HLA class I-negative B lymphoblastoid cell lines [86].

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3.3.2. The state of "unlicensed" natural killer cells is reversible

It is important to note that the hyporesponsive state of "unlicensed" NK cells is reversible. During viral infection, "unlicensed" NK cells regain cytotoxic activity and are even more effectively in controlling murine cytomegalovirus (MCMV) infection compared with "licensed" NK cells owing to the lack of inhibitory effect of self MHC class-I expression on target cells [87]. The activity of "unlicensed" NK cells can also be restored after *in vitro* culture in the presence of IL-2 or IL-12+IL-18 or through activating receptors mediated strong signals [88].

The education process is more complex during allo-HSCT as donor-derived progenitors of NK cells posses both possibilities to undergo either donor type or host type education. Besides this, adoptively transferred mature NK cells can undergo "re-education" following MHC mismatched HSCT. It is now believed that donor HSC-derived NK cells generate a KIR repertoire of donor type, thanks to the high number of infused donor cells during haplo-HSCT and both nonhematopoietic as well as hematopoietic cells may present self MHC class-I molecules to precursors of NK cells thereby are involved in NK cell education [34, 89].

4. Donor natural killer cell reconstitution after hematopoietic stem cell transplantation

NK cells need to develop *in vivo* to acquire effector function following HSCT. Accordingly, they are the first donor-derived lymphogenous cells to reconstitute and can be detected as early as the first month post-transplant [90]. The reconstitution kinetics and functional properties of NK cells are variable due to the primary activity of NK cells in the graft and different graft source including bone marrow, G-CSF-mobilized peripheral blood stem cell, and UCB. Series studies demonstrated that different grant source did not affect the rate of NK cell reconstitution and during the early stage post-HSCT, the primary NK cell subsets were $CD56^{bright}$ donor NK cells [91, 92]. These results indicated that the early reconstituting NK cells after HSCT were mainly generated from the differentiation and maturation of NK precursors but not the expansion of mature NK cells in the graft. Nevertheless, the expansion of adoptive transferred NK cells also influences the reconstitution of donor NK cells since Eissens et al. find that in HLA-matched allogeneic peripheral blood stem cell transplantation, $CD3^+/CD19^+$-depleted grafts which contain more mature NK cells than $CD34^+$-selected grafts lead to more rapid NK cell reconstitution [93].

Although donor-derived NK cells are observed very early post-HSCT, they have an immature $CD56^{bright}$ KIR^- $NKG2A^+$ phenotype for at least 3-6 months [94]. Interestingly, the ability of these immature $CD56^{bright}$ NK cells to produce IFN-γ is significantly impaired in response to tumor cell line or primary leukemia cells [95, 96]. The expression of T-bet and mucin-containing domain-3 (Tim-3) is much lower in the patients post-HSCT compared with the healthy individuals [97]. Both of these two molecules are critical for induction of IFN-γ production, thus their reduced expression may partly account for the decreased recovery of cytokine production.

It is difficult to clearly define the effect of T cell content in the graft on NK cell reconstitution as it is also depended on immunosuppressive therapy. By far, graft T cell content is thought to

significantly influence functional reconstitution of NK cells rather than numerical reconstitution. One study observed donor-derived NK cells reconstitution following HSCT with three different kinds of donor source including T-cell-replete adult grafts, T-cell-deplete adult grafts, and UCB [95]. Both of the T-cell-replete group and UCB group were treated with immunosuppression. They found KIR expression in T-cell-replete group was the highest among these three groups indicated donor-derived NK cells in this group may have better effector function. Accordingly, in an earlier study by the same group, they found IFN-γ production was more potent following HSCT with T-cell-replete grafts compared to T-cell-deplete transplantation [98]. These results were consistent with the observations by Nguyen et al. which demonstrated that without immunosuppression, recovery NK cells in the partial T-cell-depleted group had lower cytotoxicity than NK cells in the full T-cell-depleted group [92]. In summary, graft T cell content trends to enhance functional reconstitution of NK cells irrespective of immunosuppression. This promotion effect may be due to activation from T-cell-derived IL-2, or stimulation from other inflammatory cytokine such as IL-12 and IL-18, which are increased following donor T cell engraftment. Finally, CMV infection and GvHD may also have potent effect on NK cell reconstitution, and we will discuss this in the following sections.

5. Association between natural killer cell alloreactivity and graft-versus-leukemia effect

5.1. The complexity of hematopoietic stem cell transplantation may affect the graft-versus-leukemia effects of alloreactive natural killer cells

HSCT is well-established curative treatment for hematologic malignances. The major limitation of HSCT is the absence of HLA-matched donor. Therefore, the HLA-haploidentical relatives are increasingly used as donor sources of HSCs. During the haplo-HSCT, donor and recipient share one identical HLA haplotype, while other haplotype is fully mismatched [99]. The GvL effect of NK cells was first observed in a landmark study which demonstrated that NK cells rapidly reconstituted and displayed potent anti-leukemia activity after extensive T-cell depleted haplo-HSCT [6]. After inhibitory receptor KIR was identified as a mediator of missing-self response, the GvL effect of NK cells was assumed to be mainly due to mismatches between donor KIR and recipient HLA class I which increase NK cell function thereby effectively lyse residual leukemia cells in the recipient. This hypothesis was first verified by Ruggeri L et al. [100]. They isolated donor-derived NK cells from haplotype-mismatched HCT recipients and tested their cytotoxicity toward recipient lymphocytes. Their results demonstrated donor alloreactive NK cells indeed effectively killed recipient myeloid leukemia cells. The follow-up study of this group demonstrated therapeutic utility of NK cell alloreactivity. They found transplants with KIR ligand mismatch were associated with decreased probability of relapse and increases in disease-free survival in the absence of GvHD [6].

Series studies subsequently tested NK cell alloreactivity-mediated GvL effect in the KIR ligand incompatibility model. However, not all studies supported the benefit role of alloreactive NK cells. During haplotype-HCT with less T cell depletion, KIR ligand mismatch patients

even developed more acute GvHD (aGvHD), and the overall survival was poor. In a cohort using unrelated donors, no effect of predicted NK cell alloreactivity was observed, whereas in another similar cohort with T-cell depletion, KIR ligand mismatch prolonged overall survival [101, 102]. Frag et al. examined the clinical impact of NK cell alloreactivity in over 1500 unrelated transplants and found KIR ligand mismatch was not associated with relapse prevention [103]. In a retrospective study, data from 2062 unrelated transplant recipients were analyzed for acute myelocytic leukemia (AML), chronic myeloid leukemia (CML), and myelodysplastic syndrome (MDS) [104]. Missing one or more KIR ligands in the recipient were associated with less relapse in patients with early myeloid leukemia and in CML patients during first chronic phase. Nevertheless, KIR ligand mismatch was also associated with an increased risk of chronic GvHD (cGvHD) in CML patients.

The effect of NK alloreactivity in recipients of UCB grafts has also been tested. Willemze et al. evaluated the impact of KIR ligand incompatibility in recipients of UCB grafts with T-cell depletion and found KIR ligand mismatch was associated with relapse prevention and improved overall survival [105]. However, Brunstein et al. reported different results [106]. They investigated the impact of KIR ligand mismatch in patients of UCB grafts after different intensity conditioning regimens. After myeloablative conditioning, KIR ligand mismatch did not exert protective effect on GvHD, relapse, transplantation-related mortality, or survival. Following reduced intensity conditioning, KIR ligand mismatch patients developed more aGvHD and poorer overall survival.

The different results among these studies using either adult grafts or UCB grafts may be explained by the complexity of HCT, such as variable preparative regimens, donor sources, and degree of HLA mismatch with the donor. For instance, the degree of T-cell depletion was different among these studies, the grafts were extensively T-cell depleted in some studies, whereas only were partially depleted in others. T cell content may outcompete NK cells for cytokines and thereby influence the beneficial effect of alloreactive NK cells. Additionally, different intensity of preparative regimens also affects numeral and functional reconstitution of alloreactive NK cells after HSCT thus influences HSCT outcomes. Finally, the origin of leukemic blasts also affects HSCT outcomes. No beneficial effects of alloreactive NK cells in adult acute lymphoblastic leukemia (ALL) patients were observed probably due to the lack of expression of activating ligands [100]. However, allorecative NK cells have been proved to positively affect the outcome of HSCT in children with ALL [107]. Accordingly, the intensity of HLA expression on the leukemic lymphoblasts was shown to be critical for NK-mediated cytotoxicity in an experimental study [108].

5.2. Donor killer immunoglobulin-like receptors affect beneficial effects of alloreactive natural killer cells and outcome after hematopoietic stem cell transplantation

As we have mentioned above, KIR genes can be simplified into two main haplotypes: haplotype A and haplotype B. A hapltotype consists of main inhibitory KIR and single-activating KIR, KIR2DS4. B haplotype contains variable KIR gene encoding both inhibitory and activating KIRs which can be further divided into either centromeric (Cen) or telomeric (Tel) regions. The role of these haplotypes in HSCT is also evaluated. Mc Queen et al. first demonstrated

grafts from KIR A/A genotype donors into KIR B recipients resulted in poorer survival in HLA-matched T-cell-replete sibling transplants [109]. In contrast, Stringaris et al. reported transplants from KIR B haplotype donors including genes for KIR2DS1, KIR3DS1, and KIR2DL5 improved overall survival and prevented relapse specific for AML patients with T-cell depletion during HLA identical siblings HSCT [110]. A larger cohort was performed to further evaluate the effect of KIR B haplotype in AML patients. The results indicated that grafts from donors with KIR B haplotype significantly reduced the risk of relapse and improved overall survival [111]. A follow-up study expanded numbers of AML patients with T-cell-depleted unrelated donor transplants and found that donor KIR B haplotype-mediated relapse prevention was enhanced in recipients who have HLA-C1 alleles rather than C2 homozygous recipients [36]. Michaelis SU et al. reported transplants from KIR B haplotype donors resulted in better HSCT outcome in ALL patients [112].

Collectively, it is logical to hypothesize that the presence of activating KIR in donor KIR B haplotype is favorably associated with better transplant outcome. However, it is difficult to evaluate the contribution of each activating KIR to relapse protection and better overall survival due to their unknown ligands. KIR2DS1 has been proved to interact with HLA-C group 2 alleles and involved in the killing of leukemic blasts from HLA-C2 patients. The interaction between KIR2DS1 and HLA-C2 was found to overcome NKG2A mediated inhibitory signals in vitro [113, 114]. These results suggested KIR2DS1-mediated activating signal may break the barrier of NKG2A and exert positive effect during HSCT. Venstrom et al. investigated the role of KIR2DS1 from donor in unrelated HSCT in 1277 patients with AML and found that KIR2DS1 expression in donor mediated a significant GvL effect [114]. Notably, no beneficial effect was observed when donors were HLA C2 homozygous as in those donors, NK cells expressing KIR2DS1 cannot be educated and acquire effector function. Therefore, at least one copy of HLA-C1 in donor is needed for KLR2DS1-dependent GvL effect. In addition, KIR3DS1 was also found to associate with lower rate of relapse and infection. Mancusi A et al. reported KIR3DS1 was associated with reduced infection mortality [115]. Further studies are still needed to identify ligands for activating KIR so as to confirm the effect of each activating KIR gene on allo-HSCT outcome.

The intensity of interaction between KIR and HLA may also influence the alloreactivity of NK cells during allo-HSCT. HLA alleles have been shown to bind NK cells with different affinities. The HLA class I molecules are highly polymorphic, and HLA-B alleles can be divided into HLA-B alleles with an isoleucine at position 80 (80I) and with a theonine (80T). The affinity between certain HLA-B alleles with an 80I and KIR3DL1 was found to be much stronger than those with an 80T [116, 117]. The impact of this different affinity was tested in HIV patients [118]. Presence of 80I but not 80T in HIV patients associated with slower AIDS progression. These results indicated strong interaction with KIR3DL1 may promote NK cell education and enhance their effector function. HLA-C1 alleles can also bind to NK cells with different affinity [119]. Donors who were homozygous for HLA-C*07 produce higher level of IFN-γ compared to donors expressing either HLA-C*01, 03*,*08, *1402, or 1403*. Since the strength of the interaction between KIR and HLA is critical for NK cell education and function, it is tempting to speculate that during allo-HSCT certain HLA combination may enhance NK cell activity and contribute to better outcome of HSCT. The differing KIR/HLA affinity also can be

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generated by KIR allelic differences. KIR3DL1 is one of the most polymorphic KIR genes with more than 60 alleles [120]. KIR3DL1*01052 binds to Bw4 more firmly than KIR3DL*007 due to their high-expression level [119]. Although the surface expression of KIR3DL1*002 is similar to KIR3DL*007, the former generates stronger interaction with Bw4 than the latter [121]. KIR-recognizing HLA-C alleles also have varying interactions. KIR2DL1 has been shown to be the strongest KIR for HLA-C2 followed by KIR2DL2 then KIR2DL3 [122]. These differing affinities may explain the beneficial effect of haplotype B from donors as these donors are homozygous for KIR2DL2 which may account for the generation of NK cells with enhanced function.

Besides using "missing self" model to predict alloreactivites of NK cells as described above, "missing ligand" model has also been used to speculate alloreactivites. According to this model, NK cell alloreactivity may also be observed when the KIR on the surface of donor NK cells cannot recognize the ligands either from donor or recipient [123, 124]. As we have mentioned, these "unlicensed" NK cells are hyporesponsive in situ but can have potential to acquire effector function in recipients. Thus, NK cell alloreactivity can occur even after autologous HSCT. This model is supported by the observation that most individuals have 3 inhibitory KIR, but only 1 or 2 corresponding HLA KIR ligands are expressed on the surface of their own cells [125, 126]. Several comparative analyses have been performed to test the prediction rate of this model. Some groups found the selection of donors based on this model indeed was associated with better outcome, while others reported opposite results [127–129]. These variable results may be due to the complexity and variables of HSCT.

6. The impact of cytomegalovirus infection on natural killer cell-mediated graft-versus-leukemia effect: reconstitution promotion and memory induction

6.1. The impact of cytomegalovirus infection on natural killer cell reconstitution

Although in healthy individuals, CMV infection is potentially controlled by T cells and NK cells, it can cause life-threatening complication in immunodeficient transplant recipients. Lethal CMV-caused illness is now uncommon thanks to the use of drugs such as ganciclovir and foscarnet. In view of the strong immune response caused by CMV, it is attractive to investigate whether CMV reactivation can reduce the risk of relapse in patients after HSCT. As early as 30 years ago, Lonnqvist et al. reported that CMV infection was associated with lower relapse in BMT recipients with various hematological malignancies [130]. Subsequently, a number of reports confirmed that CMV reactivation following HSCT resulted in reduced relapse in AML patients. Among these reports, Elmaagacli et al. found CMV reactivation occurred within the first 100 days after HSCT significantly reduce the rate of AML relapse from 42 to 9% [131]. Remarkably, this CMV reactivation-mediated relapse prevention effect was independent of aGvHD. Furthermore, in another study of large cohort of 2566 patients with variable hematological malignancies, Green et al. comprehensively analyzed the results among all kinds of disease with adjustment for potential variables and reported that CMV reactivation was significantly associated with a decreased risk of relapse [132].

Upon the association between CMV reactivation and decreased rate of relapse after HSCT was confirmed, several studies next tried to test whether CMV-specific donor T cells accounted for this beneficial effect. However, CMV-specific donor T cells appeared not to impact relapse rates after HSCT [133]. On the other hand, NK cells have been shown to play an important role in CMV reactivation-mediated relapse prevention. Foley et al. demonstrated that the beneficial effect of CMV reactivation was specifically associated with the clonal-like expansion of NK cells characterized by the NKG2C$^+$ NKG2A$^-$ self-KIR$^+$ CD57$^+$ CD56^{dim} signature [80]. These NK cells have been shown to exhibit enhanced cytolytic activity and cytokine production. During CMV reactivation, both NKG2C$^+$ NK cell percentages and numbers are significantly increased with enhanced capability of producing IFN-γ upon the stimulation of myeloid leukemia cell line K562. Nevertheless, the mechanisms underlie the increased expansion and functions of NKG2C$^+$ NK cells are not entirely clear. Guma et al. reported that after *ex vivo* stimulation with CMV-infected fibroblasts, NKG2C$^+$ NK cells from seropositive healthy donors rapidly proliferated [134]. These results indicated that NKG2C may play a unique role in NKG2C$^+$ NK cell expansion. However, the exact ligands of NKG2C on CMV infected cells are not completely confirmed. Although HLA-E has been shown to be a ligand for NKG2C [135], other undefined ligands may also take part in NKG2C-mediated effect. Leukemic cells can retain HLE expression while downregulate the level of classical class I HLA molecule [136]. Therefore, it is now believed that the change in receptor repertoire from inhibitory receptor NKG2A to activating receptor NKG2C may play a crucial role in this NK cell subset-mediated GvL effect [137].

It has been shown that NK cells from UCB transplants will undergo slower reconstitution compared with NK cells from adult grafts. In contrast, in CMV-infected UCB transplantation patients, NK cells are found to reconstitute rapidly with decreased NKG2A expression and increased KIR expression [138]. Their functional reconstitution is also promoted as they can produce high level of IFN-γ in response to K562 cells. Intriguingly, during CMV-infected UCB transplantation, Della Chiesa et al. detected a unique subset of hyporesponsive NK cells which shared similar surface markers with the expanded NKG2C$^+$ NK cells except for CD56 expression [139]. Briefly, this NK cell subset does not express CD56. IL-2 stimulation can reverse the hyporesponsive state of CD56$^-$ NK cells thereby this NK cells subset may represent a stage of NK cell differentiation in the case of cytokine deficiencies.

The effect of CMV reactivation was also investigated in recipients received grafts either from peripheral blood or bone marrow [140]. Strikingly, the expansion of NKG2C$^+$ NK cells seemed to be dependent on the serostatus of recipient. The percentage of NKG2C$^+$ NK cells was significantly increased when CMV seropositive recipients received grafts from CMV seronegative donors. Oppositely, when grafts from CMV seropositive donors were infused into CMV seronegative recipients, NKG2C$^+$ NK cells failed to expand, and their percentage was declined to the levels similar to CMV seronegative donor/recipient pairs.

Besides NKG2C, activating KIR may also contribute to CMV-induced NK cell expansion. Della Chiesa et al. proved that when donor grafts were lack of NKG2C, NKG2C$^-$ NK cells can rapidly expanded expressing activating KIR in the recipients following CMV infection [141]. These data indicated that activating KIR can recognize CMV and promote NK cell reconstitution

independent of NKG2C. Correspondingly, the increased presence of donor activating KIR has been shown to result in reduced risk of CMV infection [142, 143]. However, the ligands expressed on the CMV infected cells specific for activating KIR are still elusive, which become an obstacle to explore the actual role of activating KIR in CMV-infected HSCT.

Additionally, CMV seropositivity has been shown to be associated with presence of a population of NK cells which does not express FcγRIγ [144]. FcγRIγ is an adapter molecule that is used by CD16, NKP30, and NKp46 for signal transmitting. CD3ζ is also required for CD16 associated signal transduction. Lack of both FcγRIγ and CD3ζ in HIV-infected patients was proved to diminish CD16 signaling [145], while single down-regulation of FcγRIγ in CMV seropositive healthy donors resulted in enhanced CD16 signaling [146]. The deficiency of FcγRIγ induced NK cells hyporesponsive to CMV infected fibroblasts, whereas CMV specific antibody can reverse NK cells to degranulate and produce IFN-γ and TNF-α against CMV infected fibroblasts.

6.2. Cytomegalovirus infection induces natural killer cell memory

Although traditionally viewed as a member of innate immune cells, NK cells have been demonstrated to mount memory response to hapten and mediate hapten-induced contact hypersensitivity responses [21]. In MCMV model, NK cells were also observed to develop memory-like properties including viral ligand m157 specific expansion, persisting over time and enhanced effector function after rechallenge with MCMV [22, 23]. This MCMV-induced memory-like population of NK cells is characterized by the expression Ly49H. Intriguingly, during acute human CMV, the expanded $NKG2C^+$ $NKG2A^-$ self-KIR^+ $CD57^+$ $CD56^{dim}$ NK cells which we have described above was proved to be human analog of $Ly49H^+$ memory-like NK cells [139, 147]. After HSCT, these CMV-specific memory-like NK cells can be detected in peripheral blood of CMV-infected HSCT recipients. As we have mentioned, when CMV-seropositive recipients received grafts from CMV-seropositive donors, these $NKG2C^+$ NK cells underwent expansion even in the absence of detectable viremia, which meant a nonclassical recall response was developed [140]. However, the mechanisms underlined the generation of memory NK cells have not been exactly defined. Recently, two studies demonstrated that CMV infection resulted in modified effector function through driving epigenetic alterations in these "adaptive" NK cells [148]. These memory-like NK cells were proved to lack expression of signaling proteins such as Fc epsilon receptor I (FcεRIγ), spleen tyrosine kinase (Syk), ewing's sarcoma-associated transcript 2 (EAT2) and exhibit increased capacity to mediate ADCC as well as produce IFN-γ in response to tumor targets. In contrast, memory-like NK cells produced extremely less level of IFN-γ in response to cytokines such as IL-12 and IL-18 mainly due to the downregulation of cytokine receptors.

In summary, $NKG2C^+$ NK cells expanded after CMV-infected HSCT display memory-like properties and associated with reduced risk of relapse. Nevertheless, it is still unclear whether the GvL effect observed in CMV-infected HSCT is mainly due to $NKG2^+C$ NK cells cytotoxicity toward leukemic blasts or primarily due to the cytolytic effect of CMV. This potent effect of CMV reactivation in shaping immune response after HSCT may provide new choice for therapeutic strategies, such as utilizing CMV vaccine to mimic this beneficial effect, isolating $NKG2C^+$ NK cells for adoptive transfer setting and optimizing the criterion for donor selection.

7. The effects of natural killer cells on graft-versus-host disease

Allo-HSCT has curative potential through donor immune effectors-mediated GvL effects. However, the donor versus host direction effect of alloreactive T cells also causes GvHD which is a major complication of allo-HSCT [2]. Depletion of donor T cells in grafts and application of immunosuppressive pretreatment are often used to eliminate GvHD; however, these treatments also hampers GvL effects at the same time. NK cells have also been shown to play an important role in GvHD. In murine models, alloreactive NK cells are shown to kill antigen presenting cells (APCs) thereby prevent the initiation of aGvHD [100]. Correspondingly, another study demonstrated that alloreactive NK cells can be recruited into the lymph nodes and suppress T cells activation through cytotoxicity toward allogeneic dentritic cells (DCs) [149]. Donor NK cells may also mediate suppression of aGvHD through the direct killing of activated donor alloreactive T cells [150]. The possible mechanism involves the interactions between activating receptor NKG2D and its ligand highly expressed on the surface of activated T cells, which help NK cells to discriminate activated T cells from normal T cells and initiate killing [151, 152]. NK cells were also found to inhibit aGvHD through cytokine production such as TGF-β [153]. Furthermore, NK cells were able to inhibit the proliferation of donor $CD4^+$T cells and reduce the risk of cGvHD [154]. Remarkably, donor NK cells are believed not to attack normal host tissues mainly due to the absence of activating receptor ligands on normal nonhematopoietic cells [155].

Although these previous experimental studies suggested that donor NK cells may have potential role in modulating GvHD, the clinical results are variable. Ruggeri L et al. reported NK cell alloreactivity was favorably associated with the lower risk of aGvHD [6]. However, this benefit effect cannot be observed in most groups, some groups even found KIR mismatch may worsen GvHD [156, 157]. Notably, in a recent clinical trial, IL-15/4-1BBL-activated donor NK cell infusion was found to contribute to aGvHD in T-cell depleted HSCT [158]. Collectively, these contradictory results indicated the role of alloreactive NK cells in GvHD may be variable. They could either aggravate or attenuate GvHD probably depended on different immune microenvironment in HSCT. Correspondingly, in our study of murine acute GvHD model, IL-12/15/18-preactivated donor NK cells significantly inhibited severe aGvHD, whereas they accelerated the development of aGvHD in a mild aGvHD model (unpublished data).

8. Donor selection based on killer cell immunoglobulin-like receptor typing

Since alloreactive NK cells play a critical role in the successful treatment of leukemia patients receiving alloreactive grafts, it is crucial to ensure sufficient number of NK cells in the best possible donor. As we have discussed above, the polymorphisms of KIR and its ligand HLA obviously affect NK cell alloreactivity. Therefore, genotyping of these two families of genes provides the probability to identify the size of alloreactive NK cell populations in donor. Up to now, there are three levels of KIR typing. The first level is genotyping to test the gene content of KIR families [159]. Based on these results, the KIR haplotype of donor is confirmed and scored.

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The preferable donors defined in this level are those possess inhibitory KIR specific for HLA-I alleles absent in the recipients with highest haplotype B score [128, 160]. The second level of KIR typing is using flow cytometry and quantitative PCR to assess the number of KIR mismatched NK cells and the expression of KIR gene [125]. Based on the results of this level, the donors possess the largest number of KIR mismatched NK cells with high frequency of KIR gene expression are preferable. The third level is KIR typing of alleles [161]. As we have discussed above, different alleles of KIR gene may result in different intensity of NK cell alloreactivity. Therefore, donors with stronger KIR alleles should be selected. Finally, FcγR polymorphisms are also needed to analyze when the treatment is associated with ADCC effect of NK cells [162]. Additionally, the assess of NK cell cytotoxicity toward leukemic blasts from patients could also help to predict the outcome of HSCT.

9. Adoptive transfer of donor natural killer cells and natural killer cell expansion

Despite their potential beneficial role in allo-HSCT, donor NK cells derived from the hematopoietic stem cells may be functionally impaired due to their immaturity and insufficient education. Thus, adoptively transfer of mature donor NK cells has been employed to provide the immunotherapeutic benefits for allo-HSCT. The first trial of utilizing allogeneic NK cells as immunotherapy in humans was published in 2005 focused on the impact of different chemotherapy preparative regimens on infused donor NK cells [163]. Three different chemotherapy preparative regimens were used in their study: high cyclophosphamide and fludarabine (Hi-Cy/Flu), low cylcophosphamide and methylprednisone, or fludarabine alone. Their results demonstrated that the infusion of NK cells was well tolerated by patients, donor-derived NK cells expansion can only be detected in patients receiving Hi-Cy/Flu. Furthermore, long-term remission was observed in 5 of 19 patients. An obvious increase in endogenous IL-15 concentration was detected in patients receiving high intensity of the conditioning regimen and was associated with the expansion of adoptive transferred donor NK cells. These results indicated the Hi-Cy/Flu treatment may eliminate recipient's lymphocytes thereby provide "space" and adequate cytokines for expansion of transferred donor NK cells. These results also suggested besides IL-2, IL-15 might be a good choice to promote NK cell expansion *in vivo*. In the follow-up study, the criteria for successful donor NK cell expansion were defined as a measurement of more than 100 donor NK cells/μl of peripheral blood 12–16 days after infusion [113].

Although remission of patients was observed in the above studies, this benefit effect was not permanent as patients ultimately relapsed. To test whether host-mediated rejection affected the expansion of donor NK cells thereby resulted in remission, total body irradiation was added to Hi-Cy/Flu to deplete recipient cells [138]. The results demonstrated that measurable NK cell expansion was obviously increased in patients received total body irradiation, which was associated with better leukemia clearance effect. These data again indicated the importance of enough "space" for successful expansion of donor NK cells during HSCT. In a pediatric study, Rubnitz et al. reported after high intensity of lymphodepeleting

chemotherapy, infused donor NK cells could successfully expand in the patients of AML [164]. Remarkably, among 10 patients, three patients were observed to have detectable NK cells up to 1 month after infusion and have a disease-free survival rate of 100% at a median of 2 years. This promising outcome may due to the higher cell dose of transferred donor NK cells used in this study; thus, higher doses of infused NK cells may be important for better HSCT outcome.

To produce sufficient number of donor NK cells and achieve the optimal expansion of NK cells *ex vivo*, numbers of studies were performed with variable approaches, such as cytokine stimulation and utilizing engineered feeder cells. Up to now, clinical NK cell doses can be reached even up to 10^8 NK cells/kg [165]. However, there are numerous factors that may affect the effector function of expanded NK cells. A crucial factor among these complicated production protocol is how to activate NK cells *ex vivo*. Addition of cytokines such as IL-2 or IL-15 was used to promote survival and expansion of NK cells. Although IL-2 stimulation may result in activation-induced cell death (AICD) of NK cells *in vivo*, IL-15 stimulation does not have this side effect [166, 167]. Subsequently, human-derived feeder cells were used to develop approaches in expanding NK cells. Fujisaki et al. first reported the use of genetically modified APCs that could expand NK cells from peripheral blood 500- to 1000-fold [13]. These artificial APCs can be further modified with costimulatory molecules and membrane-bound cytokines [168, 169]. The expanded NK cells express high level of activating receptors and CD16 molecule and are proved to be potent mediators of cytotoxicity. However, these activated NK cells often become "exhausted" and cannot maintain their effector function and expansion *in vivo* [170]. Denman CJ et al. used APCs modified with membrane-bound form of IL-21 to break this barrier [168]. However, after cytokine withdrawal, these *ex vivo* expanded NK cells have been shown to have shorter survival *in vivo* compared with freshly activated NK cells [171].

Another alternative approach is to generate "memory-like" NK cells *ex vivo*. Cooper et al. reported that IL-12/15/18-preactivated NK cells obtained the ability to produce increased IFN-γ upon restimulation for up to 4 months after adoptive transfer [24]. Therefore, cytokine preactivation before infusion may amplify and sustain the beneficial effects of NK cells during allo-HSCT. The enhanced antitumor activity of murine IL-12/15/18-preactivated NK cells was first demonstrated in a murine tumor model [172]. Similar to murine NK cells, human IL-12/15/18-preactivated NK cells have been recently shown to have memory-like properties, including increased IFN-γ production, enhanced proliferation, and high expression of IL-2 receptor [26]. Recently, a phase I study of adoptively transferred cytokine-induced memory-like NK cells has demonstrated sustained anti-leukemia responses in patients with relapsed or refractory AML [173]. Therefore, cytokine-induced memory-like NK cells may possess long-lasting effector function *in vivo*, which can be harnessed in the treatment of leukemia patients. However, the safety of such therapy could be complicated by the induction of aGvHD after allo-HSCT. Adoptively transfer of donor-derived IL-15/4-1BBL-activated donor NK cells contributed to aGvHD [158], likely through upregulation of activating receptor expression and inflammatory cytokine production. Therefore, the effect of IL-12/15/18-preactivated NK cell infusion on GvL and GvHD after allo-HSCT needs further investigation.

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10. Methods to enhance natural killer cell function and future perspectives

Monoclonal antibodies are now increasingly being studied for their effect on enhancement of NK cell activity. The isotype IgG1 subclass is often used to induce ADCC through stimulating activating Fc receptors on NK cells. The most effective monoclonal antibody used in hematological malignancy is Rituximab, which targets antigen CD20 on B cells [174]. Notably, it was proved that such antibodies should be administered at appropriate time point as NK cells reconstituted early after HSCT are immature with lower expression of CD16 [95]. Interactions between inhibitory KIRs and HLA molecules transmit a potent inhibitory signal to the NK cell, thus monoclonal antibodies blocking inhibitory KIR receptors are used to enhance NK cell activity. The preclinical results demonstrated these antibodies appear safe and do not induce autoimmunity [175]. Additionally, a disintegrin and metalloprotease-17 (ADAM17) was demonstrated to induce activated NK cells to lose expression of CD16 and CD62L [176]. Therefore, pharmacologic inhibition of ADAM17 is used to enhance the cytotoxicity of NK cells toward Rituximab bound lymphoma cells. Following the improvement of antibody production, bispecific killer engagers (BiKE) and trispecific killer engagers (TriKE) are used to crosslink specific tumor antigens such as CD19, CD20 with a potent stimulator on NK cell such as CD16 [177–179]. CD16 × 19 BiKEs and CD16 × 19 × 22 TriKEs have been constructed and shown to significantly activate CD16 signaling of NK cells [180, 181]. Furthermore, a CD16 × 33 BiKEs was constructed recently and proved to result in potent cytotoxicity of NK cells in patients of refractory AML [182]. However, the very short half-life of bi- and trispecific antibodies might limit their therapeutic effect [179].

Another important focus is to enhance the immune response of NK cells through sensitizing target cells. Bortezomib is a drug classically used to treat multiple myeloma and mantle cell lymphoma. As a proteosome inhibitor, Bortezomib can sensitize tumor cells to TRAIL-mediated apoptosis [14]. Moreover, drugs such as doxorubicin and depsipeptide have been proved to upregulate the expression of death receptors including Fas, TRAIL-R1, TNFR1, thereby sensitize tumor cells to the cytotoxicity of NK cells [183, 184].

Recently, NK cells are also genetic engineered to express CARs which represent an effective therapy. The preclinical studies demonstrated that primary NK cells expressed CAR constructs can result in potent killing of B cell tumors [185, 186]. However, it is difficult to express exogenous genes in primary NK cells and to reach enough number for immunotherapy. Therefore, human NK-like cell line, NK-92 becomes another candidate for genetic engineering. Based on the results of phase I clinical trials of NK-92 cells [187], CAR-expressing NK-92 cells may be easily expanded *ex vivo* and well tolerated in patients.

11. Conclusions

A number of recent studies demonstrated that NK cells played a critical role in disease-relapse prevention. Based on the progress made in the field of NK cell therapy, the criteria

for choosing HSCT donor have been significantly changed and shown to be associated with better outcomes. However, the role of NK cells in HSCT is not fully understood. The GvL effect of NK alloreactivity may be affected by the complexity of HSCT including the degree of T-cell depletion, donor sources, degree of HLA mismatch, different intensity of preparative regimens, and the origin of leukemic blasts. Furthermore, the haplotype of donor KIR genes and the intensity of interaction between KIR and HLA may also influence the GvL effect of NK cells. Donor KIR B haplotype may be favorably associated with better overall survival. The education of NK cells is also critical for their beneficial effect. The stronger KIR/HLA affinity may contribute to the generation of NK cells with enhanced GvL effect. Intriguingly, "unlicensed" NK cells may also have chance to undergo "re-education" and acquire potent effector function after HSCT. Several studies reported the favorable association between CMV reactivation and better HSCT outcome. This beneficial effect of CMV reactivation was mainly due to the expansion of $NKG2C^+$ NK cells. Moreover, these $NKG2C^+$ NK cells display memory-like properties and enhanced cytotoxicity toward leukemic blasts. NK alloreactivity has been demonstrated to play potential role in suppression of aGvHD. However, some groups found KIR mismatch or alloreactive donor NK cell infusion also worsen aGvHD. Therefore, the role of NK alloreactivity in GVHD still needs to be carefully studied.

Since the GvL effect of NK alloreactiviy is affected in various aspects. Some critical questions are still remained to answer so as to ensure NK cells play the most desirable role during HSCT. The fate of NK cells is still unpredictable after transfusion. The optimal approach to expand most powerful NK cells which can maintain their proliferative potential and effector function *in vivo* is still not confirmed. The risk of the clinical usage of these activated NK cells is still needed for evaluation. The ligands of several activating receptors are undefined and the interactions between NK cells and other immune cells need further investigation. Further studies are also required to explore the mechanism underlying the process of NK cell "education" and "memory". Recently, monoclonal antibodies and immunomodulatory drugs are utilized to modulate NK cell function and proved to have benefit effect on the outcome of HSCT, whereas it is still needed to confirm the suitable hematopoietic malignancies for these treatments. The answers to these questions and continuous progress in understanding NK cells biology will optimize donor NK cells-based therapy and benefit the outcomes of HSCT.

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