Role of Natural Killer Cells in Hematopoietic Stem Cell Transplantation: Myth or Reality?

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Key Words

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Abstract

Natural killer (NK) cells play a crucial role in the innate immune system and are responsible for the initial responses in the surveillance against malignant cells and virally infected cells. NK cells express their own repertoire of receptors, including activating and inhibitory receptors, which bind to major histocompatibility complex class I or class-I-related molecules. Binding of NK cell inhibitory receptors to their major histocompatibility complex class I ligands protects the target cells from NK cell-mediated cytotoxicity. NK cell alloreactivity has been put to use in allogeneic hematopoietic stem cell transplantation to reduce the rate of relapse and of graft-versus-host disease. A variety of findings have been observed in clinical studies, showing either beneficial or deleterious effects on clinical outcome. This article reviews the results of major clinical trials in relation to the model used to define NK cell alloreactivity.

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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for a variety of high-risk hematologic malignancies. Unfortunately, the

lack of suitable donors constitutes one of the major limitations to successful transplantation, because approximately only one third of candidates for allogeneic HSCT have human leukocyte antigen (HLA)-matched siblings. For those patients, alternative sources of stem cells for allogeneic HSCT include matched unrelated volunteers, umbilical cord blood and partially HLA mismatched or HLA haploidentical donors. Cure of malignancies by HSCT relies on the ability of the immune cells in the graft to recognize and eliminate the leukemia cells. Donor alloreactive T cells are the main effectors of the graft-versus-leukemia (GvL) effect and are directed against minor or major histocompatibility molecules. Unfortunately, these molecules are not only shared by leukemic cells, but also by normal host cells, resulting in graft-versus-host disease (GvHD), a lethal complication that limits the wider application of allogeneic HSCT.

In these settings, natural killer (NK) cells may play a crucial role in achieving successful transplantation. Indeed, contrary to T cells, NK cells recover very quickly after HSCT and may mediate a strong GvL effect without inducing acute GvHD (aGvHD), a dissociate effect that represents the ultimate goal for HSCT. This review will focus on various aspects of donor-derived NK cells in patients with leukemia receiving haploidentical HSCT.

Biology of NK Cells: A Polymorphic Repertoire to Maintain Self-Tolerance and Allow Alloreactivity

NK cells were originally defined by their ability to kill cells that had lost expression of one or more major histocompatibility complex (MHC) class I molecules on their surface without previous immunization and in an antigen-independent manner, unlike T or B cells. Although NK cells express a wide variety of inhibitory and activating receptors that recognize diverse self-molecules in response to cell stress, the ability to distinguish target cells that differ in their expression of MHC class I molecules is referred to as 'missing self-recognition'. It is now well-established that interaction between inhibitory receptors and their self-MHC class I ligands affect their education and provide NK cells with the capacity to recognize MHC class-I-negative target cells. Two major families of inhibitory NK receptors recognize MHC class I, i.e. killer cell immunoglobulin-like receptor (KIR) and the heterodimer formed by CD94/NKG2A receptors. Formally, four major inhibitory KIRs have been characterized and named KIR2DL2/KIR2DL3, KIR2DL1, KIR3DL1 and KIR3DL2. These receptors recognize HLA-C alleles with asparagine 80 (group C1), HLA-C alleles with lysine 80 (group C2), HLA-A and HLA-B alleles with Bw4 motifs at positions 77–83 and HLA-A3/11, respectively. The CD94/NKG2A heterodimer binds to the non-classical class Ib HLA-E molecule. It is likely that inhibitory KIR and CD94/NKG2A cooperate with each other to maintain tolerance to self-MHC class I [1, 2].

NK cell self-tolerance was first explained by the 'at least one' model in which each NK cell expressed at least one inhibitory KIR that recognized a self-MHC class I molecule to avoid autoreactivity [3]. Nevertheless, HLA and KIR genes segregate independently, and the KIR repertoire is predominantly determined by the KIR genotype and not by the HLA genotype. Of note, the HLA genotype might have a slight influence on the frequency of KIR-expressing cells, as KIR expression is increased in the presence of their cognate MHC class I ligands [4]. In addition, more recent data have demonstrated a significant frequency of NK cells that do not express receptors recognizing MHC class I molecules [5]. Importantly, these NK cells developing in the absence of MHC class I molecules became hyporesponsive and were unable to kill MHC class-I-deficient target cells; however, the mechanisms underlying NK cell education are still controversially debated. On the one hand, it has been proposed that engagement of self-MHC-specific inhibitory receptors directly confers functionality to NK cells, a pro-

cess referred to as 'licensing', but on the other hand, it has also been suggested that hyporesponsiveness of NK cells lacking self-specific inhibitory receptors is a consequence of persistent stimulation that is not counterbalanced by inhibitory signals. In this latter model, referred to as the 'disarming model', potentially autoreactive NK cells would be silenced by the lack of inhibition. In another model called 'rheostat', it was suggested that NK cell responsiveness is tuned along a continuum determined by the strength of the inhibitory input received by NK cells during education [6].

Another crucial point is the extraordinary diversity of the NK cell repertoire, mainly orchestrated by KIRs. To date, 15 KIR genes and 2 pseudogenes have been described, encoded within a 100- to 200-kb region of the leukocyte receptor complex located on chromosome 19 (19q13.4); however, a physiologic function has only been attributed to a few of them, including inhibitory KIRs specific for HLA-C (KIR2DL1, KIR2DL2/KIR2DL3), HLA-Bw4 (KIR3DL1) and some alleles of HLA-A (KIR3DL2). KIR genes are organized into two basic haplotypes, which have been defined on the basis of gene content, and are termed 'haplotypes group A and B'. Group A haplotypes are uniform in terms of gene content and are composed of five inhibitory genes (KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2 and KIR3DL3), one activating gene (KIR2DS4) and KIR2DL4, which may have both inhibitory and activating capacity. In contrast, the group B haplotypes have variable combinations of activating and inhibitory receptors that are not part of the group A haplotypes. For Caucasians, group A and B haplotypes are present at an approximately equal frequency (58 and 42%, respectively). This gene content diversity represents the first level of genetic variation in the KIR system; thus, a longstanding hypothesis stated that KIRs are clonally distributed on NK cells [7]. In addition, only NK cells expressing inhibitory KIRs for self-HLA ligands are functionally active as they become educated, and therefore, are unable to exert alloreactivity against mismatched allogeneic targets. The second level of genetic variation is the high allelic polymorphism exhibited by some individual KIR genes. The polymorphism frequency varies across populations, with some being common while others are rare. Thus, the patterns of gene presence or absence and allelic polymorphism come together to generate a high degree of KIR heterogeneity between individuals; in fact, Shilling et al. [4] have defined 98 independent KIR haplotypes at the allelic level in 143 individuals from 34 families.

Table 1. Effects of the KIR ligand incompatibility model after HSCT

Reference	Pa- tients n	Malignant disease	KIR ligand MM, %	Graft	CDT	TCD/ TCR	ATG	IMS	Re- lapse	DFS	OS	TRM	GvHD	HvG
<i>Positive effect of KIR ligand incompatibility</i>														
Ruggeri et al. [9], 2007	112	AML	45	CD34 ⁺ PB	MA	TCD	yes	no	+++	+++	+++	d.n.p.	<>	<>
Willemze et al. [10], 2009	218	AML/ALL	32	CB	MA	TCR	yes	yes	+++	+++	+++	<>	<>	<>
Beelen et al. [11], 2005	374	AML/MDS/CML	58	BM ⁺ CSP	MA	TCR	no	yes	+++	d.n.p.	<>	<>	<>	-
Elmaagacli et al. [12], 2005	236	CML	33	BM/PB	MA	TCR	no	yes	+++	d.n.p.	<>	<>	<>	-
Giebel et al. [13], 2003	130	ALL/AML/CML	53	BM	MA	TCR	yes	yes	+++	d.n.p.	+++	+++	<>	<>
<i>Negative effect of KIR ligand incompatibility</i>														
Bishara et al. [14], 2004	62	ALL/AML/CML	52	CD34 ⁺ PB	MA	TCD	yes	no	<>	d.n.p.	--	d.n.p.	---	<>
De Santis et al. [15], 2005	104	various	27	BM/PB	MA	TCD	no	yes	<>	---	n.d.	---	---	---
Brunstein et al. [16], 2009	257	ALL/AML/CML	29	CB	MA/RIC	TCR	yes	yes	<>	d.n.p.	---	---	---	<>
Bornhäuser et al. [17], 2004	118	AML/CML	54	BM/PB	MA	TCD	yes	n.d.	---	<>	<>	<>	d.n.p.	-
Davies et al. [18], 2002	175	ALL/AML/CML	100	BM/PB	MA	TCR	n.d.	n.d.	<>	d.n.p.	--	d.n.p.	-	<>
Huang et al. [19], 2007	116	ALL/AML	26	PB	MA	TCR	yes	n.d.	---	d.n.p.	---	-	---	---
Kröger et al. [20], 2006	142	ALL/myeloid	15	BM/PB	MA	TCR	yes	yes	d.n.p.	---	---	---	d.n.p.	-
Morishima et al. [21], 2007	1,790	various	6	BM	MA	TCR	no	yes	---	d.n.p.	---	d.n.p.	---	---
Shaffer et al. [22], 2004	190	CML/other	12	BM/PB	MA/RIC	TCD	yes	yes	<>	d.n.p.	---	---	<>	d.n.p.
Sun et al. [23], 2005	378	various	31	BM/PB	MA/RIC	TCR	no	yes	---	---	---	--	<>	d.n.p.
Yabe et al. [24], 2008	1,489	various	32	BM	MA	TCR	no	yes	d.n.p.	d.n.p.	---	d.n.p.	---	d.n.p.
<i>No effect of KIR ligand incompatibility</i>														
Farag et al. [25], 2006	1,571	various	36	BM	MA	TCD	no	n.d.	<>	<>	<>	<>	<>	<>
Lowe et al. [26], 2003	105	CML	n.d.	BM	MA	TCD	n.d.	yes	<>/-	d.n.p.	<>/-	d.n.p.	<>/-	<>
Aversa et al. [27], 2005	104	ALL/AML	46	CD34 ⁺ PB	MA	TCD	yes	no	<>	d.n.p.	d.n.p.	d.n.p.	d.n.p.	d.n.p.

MM = Mismatch, % of donor/recipient pairs having a KIR ligand incompatibility in the GvH direction; CDT = conditioning regimen; TCD/TCR = T-cell depleted/T-cell repleted; IMS = immunosuppression after stem cell transplant; DFS = disease-free survival; OS = overall survival; TRM = transplant-related mortality; HvG = host versus graft; PB = peripheral blood; MA = myeloablative; +++ = positive effect; d.n.p. = data not provided; <> =

no effect; CB = cord blood; MDS = myelodysplastic syndrome; CML = chronic myelogenous leukemia; BM = bone marrow; CSP = cyclosporine; - to --- = negative effect; various = malignant and non-malignant diseases; n.d. = not determined; myeloid = AML, myelodysplastic syndrome, chronic myelogenous leukemia, conditioning regimen; other = non-Hodgkin's lymphoma, myeloma, non-malignant diseases; RIC = reduced intensity conditioning.

Predictive Models of NK Cell Alloreactivity in HSCT

Most predictive models of NK cell alloreactivity are based on the 'missing self' hypothesis, i.e. the capacity of NK cells to attack self-cells that extinguish expression of MHC class I molecules. Clinical studies evaluating the impact of these predictive models are shown in tables 1–3. Four models of NK cell alloreactivity have been proposed.

The Ligand-Ligand Model (KIR Ligand Incompatibility between the Donor and the Recipient)

In this model, NK cell alloreactivity is stimulated when the recipient lacks one or more HLA class I allele specific for an inhibitory KIR, while this HLA class I allele is present in the donor. In this situation, there is a KIR ligand incompatibility between donor and recipient in the GvH direction. The group in Perugia (Italy) used this model to predict NK cell alloreactivity in their transplantation studies. Ruggeri et al. [8] reported that NK cell al-

loreactivity strikingly reduced the risk of relapse of high-risk acute myelogenous leukemia (AML) patients after HLA haploidentical transplants from NK cells with KIR ligand mismatched in the GvH direction while improving engraftment and protecting against aGvHD. Indeed, they noted a 5-year probability of relapse and survival of 0 and 60%, respectively, whereas in the absence of KIR ligand incompatibility in the GvH direction, the 5-year probability of relapse and survival was 75 and 5%, respectively. This GvL effect of predicted alloreactive NK cells was confirmed by the presence of cytolytic NK cell clones in the donor's and recipient's blood during the early post-transplant period. Interestingly, in another study in leukemic patients undergoing haploidentical HSCT, the same group reported that KIR ligand incompatibility was not a predictive factor of clinical outcome after haploidentical HSCT [27]. More recently, this group updated their previously published data and analyzed them according to the disease status at the time of transplant.

Table 2. Effects of the receptor ligand and the missing ligand models after HSCT

Reference	Patients n	Malignant disease	MM, %	Graft	CDT	TCD/ TCR	ATG	IMS	Re- lapse	DFS	OS	TRM	GvHD
<i>Receptor ligand model</i>													
Leung et al. [28], 2004	36	ALL, myeloid	64	PB	MA	TCD	yes	no	+++	d.n.p.	d.n.p.	d.n.p.	d.n.p.
Clausen et al. [29], 2007	43	myeloid, other	49	PB	MA	TCR	no	yes	+++	+++	+++	d.n.p.	<>
Hsu et al. [30], 2005	178	ALL, myeloid	63		MA	TCD		yes	+++	+++	+++	d.n.p.	d.n.p.
<i>Missing ligand model</i>													
Miller et al. [31], 2007	2,062	MDS	55	d.n.p.	d.n.p.	d.n.p.	d.n.p.	d.n.p.	+++	d.n.p.	d.n.p.	d.n.p.	---
Sobecks et al. [32], 2007	60	AML	d.n.p.	BM	MA	TCR	no	yes	+++	+++	+++	d.n.p.	<>
La Nasa et al. [33], 2007	45	β -thalassemia	d.n.p.	d.n.p.	MA	TCR	no	yes	d.n.p.	<>	<>	<>	+++
Hsu et al. [34], 2007	1,770	various	55	BM/PB	MA	TCR	d.n.p.	d.n.p.	+++/<>	d.n.p.	d.n.p.	<>	<>

MM = Mismatch, % of donor KIR/recipient ligand mismatch in the GvH direction; CDT = conditioning regimen; TCD/TCR = T-cell depleted/T-cell repleted; IMS = immunosuppression after stem cell transplant; DFS = disease-free survival; OS = overall survival; TRM = *transplant*-related mortality; myeloid = AML, myelodysplastic syndrome, chronic my-

eogenous leukemia, conditioning regimen; PB = peripheral blood; MA = myeloablative; +++ = positive effect; d.n.p. = data not provided; other = non-Hodgkin's lymphoma, myeloma, non-malignant diseases; <> = no effect; MDS = myelodysplastic syndrome; --- = negative effect; BM = bone marrow; various = malignant and non-malignant diseases.

Table 3. Effects of the donor KIR genes after HSCT

Reference	Pa- tients n	Malignant disease	Relevant KIR	Graft	CDT	TCD/TCR	ATG	IMS	Re- lapse	DFS	OS	TRM	GvHD
<i>Positive effect of donor KIR genotype</i>													
Cook et al. [37], 2006	234	various	D haplotype B	d.n.p.	MA/RIC	TCR		d.n.p.	yes	d.n.p.	d.n.p.	d.n.p.	+++
Cooley et al. [38], 2009	448	AML	D haplotype B/x	BM	MA	TCR		d.n.p.	d.n.p.	+	+++	+++	---
Cooley et al. [39], 2010	1,049	ALL, AML	D haplotype B/x	d.n.p.	MA	TCR		d.n.p.	d.n.p.	+++	d.n.p.	+++	d.n.p.
Kim et al. [40], 2007	53	AML	D KIR2DS1	BM	MA	TCR	no	yes	d.n.p.	+++	+++	<>	<>
Ludajic et al. [41], 2009	124	various	D KIR2DS2	BM/PB	MA	TCR	+/-	yes	d.n.p.	d.n.p.	d.n.p.	d.n.p.	+++
Verheyden et al. [42], 2005	65	ALL, myeloid	D KIR2DS1/DS2	BM	MA	TCD/TCR	no	yes	+++	d.n.p.	+++	<>	<>
<i>Negative effect of donor KIR genotype</i>													
Gagne et al. [44], 2002	75	various	R genotype<D	d.n.p.	d.n.p.	d.n.p.		d.n.p.	d.n.p.	d.n.p.	d.n.p.	d.n.p.	---
Giebel et al. [45], 2006	25	ALL, myeloid	D KIR2DS2	BM	MA	TCR	yes	yes	<>	---	---	<>	-
McQueen et al. [46], 2007	202	various	D haplotype B	PB	MA	d.n.p.		d.n.p.	d.n.p.	---	---	d.n.p.	---
Schellekens et al. [47], 2008	83	various	D and R KIR2DS5/S3	d.n.p.	MA/RIC	TCR		d.n.p.	d.n.p.	---	d.n.p.	d.n.p.	d.n.p.

CDT = Conditioning regimen; TCD/TCR = T-cell depleted/T-cell repleted; IMS = immunosuppression after stem cell transplant; DFS = disease-free survival; OS = overall survival; TRM = transplant-related mortality; various = malignant and non-malignant diseases; D = donor; d.n.p. = data not provided; MA = myeloablative; RIC = reduced intensity conditioning; + and +++ = posi-

tive effect; BM = bone marrow; - and --- = negative effect; <> = no effect; myeloid = AML, myelodysplastic syndrome, chronic myelogenous leukemia, conditioning regimen; R genotype<D = recipient KIR genotype is included in donor KIR genotype; PB = peripheral blood; R = recipient.

They confirmed a favorable impact of NK alloreactivity, and concluded that disease status was the main predictive factor of relapse for AML and acute lymphoblastic leukemia (ALL) patients after haploidentical HSCT [9].

Many studies further evaluated the impact of ligand incompatibility (HLA-C or HLA-B) in the GvH direction in unrelated donor transplants, as compared to ligand-

compatible transplants (including HLA-matched transplants and HLA-C- or HLA-B-mismatched but KIR ligand-compatible transplants). As shown in table 1, the impact of KIR ligand incompatibility has remained controversial; both favorable and adverse effects have been reported in recent large studies, which merit further examination and discussion [9–27]. Morishima et al. [21]

determined the impact of KIR ligand mismatch in the GvH direction after T-cell-repleted marrow transplants from 1,790 patients with either myeloid diseases (n = 1173) or ALL (n = 617). This study revealed that KIR ligand incompatibility was associated with a significant increase in aGvHD and with poor clinical outcome in both myeloid and lymphoid patients. Interestingly, HLA-C mismatch (regardless of KIR ligand mismatch) significantly reduced the risk of relapse in ALL patients, while KIR ligand incompatibility increased it. This suggests that NK cell alloreactivity may inhibit the alloreactive T-cell-mediated GvL effect. This cohort was reanalyzed by Yabe et al. [24] according to the administration of anti-thymocyte globulin (ATG) in the conditioning regimen to deplete donor T cells in vivo. Consistent with the data from Morishima et al. [21], in 1,489 T-cell-repleted HSCT, the KIR ligand incompatibility is associated with both a robust incidence of aGvHD and a lower overall survival, but only when patients did not receive ATG. In agreement with these observations, shown in table 1, most studies on unrelated donor transplants were T-cell repleted or minimally T-cell depleted and reported adverse effects of ligand incompatibility in the GvH direction on overall survival that correlated with an increased risk of relapse or aGvHD. Nevertheless, these studies are difficult to reconcile due to the large heterogeneity in the number of patients, the type and disease status at transplant, the donor/recipient HLA matching, the intensity of the conditioning regimen, the use of ATG, the graft source, the manipulation of the graft, or the immunosuppressive drugs after transplant. The use of ATG and/or the extensive T-cell depletion of the graft might be important factors that enhance NK cell alloreactivity by eliminating the putative effects of alloreactive T cells, but their impact on clinical outcome remains to be proven, as only one study reported an improved overall survival after unrelated donor HSCT [13].

Interestingly, Willemze et al. [10] reported a favorable impact of KIR ligand incompatibility after unrelated cord blood transplantation (UCBT) after myeloablative regimen or reduced-intensity conditioning. HLA-C1, HLA-C2, HLA-Bw4 and HLA-A3/A11 incompatibility in the GvH direction had a beneficial effect on relapse, overall survival and GvHD in AML patients (n = 94), but not in ALL patients (n = 124). On the contrary, Brunstein et al. [16] recently reported an adverse effect of KIR ligand incompatibility in the reduced intensity conditioning group patients after UCBT, as shown by an increased risk of transplantation-related mortality, GvHD and worse overall survival.

The Receptor Ligand Model (Donor KIR Recipient Ligand Incompatibility)

In this model, NK cell alloreactivity is predicted when at least one of the donor KIRs does not recognize any HLA allele in the recipient ligand repertoire. Analysis of the donor KIRs by genotyping and phenotyping is required in this model. Studies examining the receptor ligand model are shown in table 2 [28–30]. Leung et al. [28] demonstrated that phenotypic assessment of donor KIR expression is more predictive of NK cell alloreactivity than donor HLA typing in the haploidentical HSCT setting. They compared three predictive models in 36 pediatric patients who received haploidentical HSCT for leukemic disease: (1) NK cell cytotoxicity assessment by chromium release analysis; (2) ligand-ligand model as defined by Ruggeri et al. [9], and (3) the receptor ligand model. The first model (cytotoxicity) was not relevant. The ligand-ligand model was able to predict a low risk of relapse, but the receptor ligand model was more accurate at predicting both low and high risks of relapse. In addition, phenotypic rather than genotypic analysis of the KIR repertoire was more accurate. Indeed, allelic polymorphism and epigenetic silencing have previously been demonstrated to affect the levels of KIR expression. These factors alone can lead to disparities in 25% of cases between expected KIR expression from DNA-based typing and phenotyping results. Hsu and colleagues [30] evaluated the impact of the lack of recipient KIR ligand for donor-inhibitory KIRs in 178 T-cell-depleted, related HLA-matched HSCT for various leukemic diseases. Analysis of donor KIR genotype with recipient HLA genotype demonstrated that about two thirds of the patients lacked an HLA ligand (usually HLA-C1 or HLA-C2 and/or HLA-Bw4) for an identified donor inhibitory KIR (KIR2DL1, KIR2DL2/KIR2DL3 or KIR3DL1 gene in donor); this had a significant effect on overall survival and relapse in patients receiving transplants for AML and myelodysplastic syndromes, but not in patients with chronic myelogenous leukemia or ALL. The authors suggested that the absence of an HLA class I ligand in the recipient for a donor inhibitory KIR could be a favorable prognostic factor of outcome in HLA-identical sibling transplantation for AML and myelodysplastic syndromes.

The Missing Ligand Model (Missing KIR Ligand in the Recipient)

This model is an extension and simplification of the receptor ligand model. Regardless of HLA expression, most individuals express the full complement of inhibitory KIRs. Thus, an individual who is homozygous for

KIR ligand epitopes would be predicted to have a subset of NK cells expressing an inhibitory KIR for the absent ligand. Studies examining the missing ligand model are shown in table 2 [31–34]. Hsu et al. [34] reanalyzed the impact of the missing KIR ligand in a larger retrospective study that included 1,770 patients who received myeloablative, T-cell-repleted transplants from HLA-matched (n = 581) or -mismatched (n = 622) unrelated donors for myeloid (n = 1,022) or lymphoid (n = 180) leukemia. They observed that patients homozygous for the HLA-C1-C1 or Bw6-Bw6 epitopes (but not for HLA-C2-C2) who received transplants from HLA-mismatched donors had a lower rate of relapse than heterozygotes, although there was no effect on overall survival. Similar effects were observed in ALL and myeloid patients, but no effect was observed after HLA-matched transplants. Of note, no effect of KIR ligand incompatibility in the GvH direction was reported in this large study. Similarly, Miller et al. [31] analyzed 2,062 unrelated donor transplants with myeloid malignancies. Of these patients, 70% were missing one or more KIR ligands. When the patients were stratified according to disease stage, significant differences emerged such as the protective effect against relapse provided by the absence of one or more KIR ligands in patients with early disease. This finding was independent of HLA matching and of T-cell depletion, consistent with the observed beneficial NK cell-mediated effects in transplantation with HLA-matched sibling donors. Unlike the data from Hsu et al. [34], this effect was seen in both HLA-matched and -mismatched transplants. Similarly, Clausen et al. [29] have analyzed outcomes following related HLA-matched, T-cell-repleted transplants in 43 patients with myeloid disease. They concluded that missing HLA-B and/or HLA-C ligand combined with missing HLA-A3/11 and a high number of NK cells in the graft could predict a lower risk of relapse.

The receptor ligand and the missing ligand models are quite close, as KIR2DL1 and KIR2DL2/KIR2DL3 are expressed by most of the donors. The receptor ligand model might be more accurate than the missing ligand model for the HLA-Bw4 epitope, as KIR3DL1 is expressed by the majority of donors, but not by all (about 85% of individuals express this receptor). The advantage of these two models is the absence of relevance of the donor HLA typing, making it possible to analyze the impact of these predictive models of NK cell alloreactivity after HLA-matched HSCT. The observation that NK cell alloreactivity might exist in HLA-matched settings is intriguing. Indeed, as NK cells are educated to become tolerant to self-HLA class I molecules, they should also become tol-

erant to the recipient after HSCT when the donor and recipient are HLA matched. Yu et al. [35] showed that the favorable impact on clinical outcome of the missing ligand model observed in HLA-matched HSCT might be correlated to a delay in the education process during the early period after transplant. In this situation, NK cells generated from HLA-matched donor CD34⁺ stem cells express inhibitory KIRs that do not recognize any ligand in the recipient and are not ‘disarmed’ during the first few months after transplant, thereby mediating a transient GvL effect. Another hypothesis is that quiescent autoreactive NK cell clones in the donor are able to reactivate in the recipient after transplantation due to inflammatory signals or other cytokine changes, thereby becoming alloreactive. Nevertheless, another recent study reported no delay in the licensing process after transplantation, making the mechanisms of NK cell alloreactivity after HLA-matched HSCT still unresolved [36].

The Donor KIR Genotype Model

There is mounting evidence suggesting that the variability in the number and type of KIR genes in the donor could have an impact on clinical outcome after HSCT. The influence of the KIR gene number has been reported by Cooley et al. [38] in a study of 448 AML patients receiving myeloablative conditioning, followed by T-cell-repleted unrelated donor transplantation (47% HLA matched, 53% HLA mismatched). They found that unrelated donors with one or two KIRs of the group B haplotypes confer significant improvements in overall and relapse-free survival in HLA-matched and -mismatched transplants, with the exception of the subgroup with HLA mismatch and KIR ligand (HLA-C) mismatch transplants. The same group recently analyzed donor KIR genotyping in a larger cohort including 1,409 unrelated transplants and confirmed the favorable impact of donor KIR B haplotypes on relapse and survival in AML patients, but not in individuals with ALL [39]. Importantly, these benefits were increased when donors’ B genes were located in the centromeric region of both KIR haplotypes. De Santis et al. [15] reported increased overall survival and decreased aGvHD when the donor’s KIR gene content was higher than that of the recipient. In addition, Sun et al. [23] demonstrated a correlation between increased aGvHD and higher inhibitory KIR in the recipient than in the donor and/or higher activating KIR in the donor than in the recipient in HLA-matched unrelated transplantation for AML. Interestingly, several other studies have observed a favorable impact of a higher number of activating KIRs on the risk of cytomegalovirus reactivation, or bacterial infections

[37]. On the contrary, other studies have reported an adverse effect on the outcome of a high number of donor activating KIR genes after HSCT. Gagne et al. [44] observed that when the recipient KIR genotype was included in the donor KIR genotype (i.e. when the donor has more KIR genes than the recipient, especially activating KIR genes), the risk of GvHD was strongly increased after unrelated HSCT, but not after related HSCT. Consistent with these data, McQueen et al. [46] showed an increase in aGvHD in AML patients when the donor had a greater number of activating KIR genes than the recipient, but only if the donor and the recipient missed the HLA-C2 ligand. Similarly, Kröger et al. [20] showed in 142 patients with leukemia that the risk of relapse was reduced, resulting in a significantly better disease-free survival after in vivo T-cell-depleted (ATG), unrelated HSCT with donors carrying a low number of activating KIR genes (group A KIR haplotype).

Impact of the Donor Activating KIR Genotype

Activating KIRs have gained credence as new important 'players' in mediating NK cell alloreactivity, and a more specific analysis of KIR gene content is needed to better understand the discrepancy between all the studies reporting conflicting results on the impact of the number of activating KIR genes in the donor. Activating forms of KIRs were identified and cloned, but the specificity for HLA class I molecules has only been unequivocally documented for KIR2DS1 and KIR2DS4. Indeed, KIR2DS1 binds to HLA-C2 ligands (with a lower affinity than its inhibitory counterpart KIR2DL1), and this interaction induces interferon (IFN)- γ production. On the contrary, KIR2DS2 does not seem to bind to HLA-C1 ligands. Analysis of KIR genotypes in HLA-matched transplantation for AML, chronic myelogenous leukemia and ALL showed that the joint effect of two donor activating KIRs, i.e. KIR2DS1 and KIR2DS2, correlated with decreased relapse [42]. Similar effects were observed in 124 HLA-matched unrelated HSCT for malignant diseases where a missing HLA-C2 ligand for donor inhibitory KIR2DL1 was significantly associated with an increased risk of aGvHD (II–IV), whereas transplantation of HLA-C1-C2 patients with KIR2DS2-positive grafts was associated with a decreased risk of aGvHD (II–IV) [41]. Kim et al. [40] showed that donor KIR2S1⁺ gene improved overall survival, whereas donor KIR2DS2 and KIR2DS4 were associated with increased GvHD. As recently shown, KIR2DS1 can overcome CD94/NKG2A-mediated inhibition, resulting in the killing of C2-C2 blast cells. Thus, the expression of KIR2DS1 may reveal NK cells capable

of alloreactivity and allow a more precise understanding of the size of the alloreactive NK cell subset. Nevertheless, other groups reported adverse effects correlating with one particular activating KIR gene in the donor. Donor KIR2DS2 was associated with higher GvHD and transplantation-related mortality, and decreased overall survival, especially when the recipient was homozygous for C1-C1 [40, 24, 43]. Some studies also reported an adverse effect of donor KIR2DS1⁺ on GvHD or survival, especially when the recipient was homozygous for C2-C2 [45, 47]. All these studies suggest that individual activating KIRs may play distinct biologic roles in HSCT, such as having a different impact on GvL effect, GvHD and control of infections. The mechanisms that could explain such an effect are not completely understood, as the ligands for all the activating KIRs are not well defined, and the effector cells responsible of such effect can be NK cells, but also activating KIR-expressing T cells. One can speculate that when a donor has a specific activating KIR2DS, and the recipient is homozygous for this receptor ligand, the activating signal will be strong (and the inhibiting signal will be weak, as there is a missing ligand for an inhibitory KIR), resulting in a global state of activation of the KIR-expressing cells. These effector cells (T or NK cells) can have various effects depending on the activated signal pathways, such as production of IFN- γ by NK cells and/or stimulation of alloreactive T cells that might increase GvHD, or activation of NK cell cytotoxicity against tumor cells or infected cells that might control relapse or infections.

Reconstitution of NK Cells after HSCT

Biological studies of NK cells, and in particular of their reconstitution after HSCT, is of major importance to better understand the factors that influence NK cell alloreactivity. Even though NK cells are the first lymphoid cells to repopulate the marrow after HSCT and reach normal numbers within 1 month after transplant, regardless of donor type or patient age, the quality of the reconstitution might be impaired or delayed. Few studies have questioned the importance of NK cell reconstitution in preventing leukemia relapse after T-cell-depleted haploidentical HSCT, where NK cells were reported to be the major effectors of the GvL effect. We showed that NK cells generated after haploidentical HSCT exhibited an immature phenotype, characterized by a strong diminution of the cytotoxic CD3⁺CD56^{dim} NK cell subset, a lower expression of KIR and NKp30 and an overexpression

of CD94/NKG2A [48]. As CD94/NKG2A binds to HLA-E molecules, which are present in all HLA class I cells, including primary myeloid leukemic blasts, this results in a striking reduction in the size of the putative alloreactive NK cells, as defined by the expression of one inhibitory KIR lacking its ligand in the recipient, and by the absence of the inhibitory CD94/NKG2A receptor. These distinctive phenotypic features were associated with a defective *ex vivo* cytotoxicity against primary mismatch AML blasts, correlating with CD94/NKG2A expression. These characteristics fit the model of Freud and Caligiuri [49] based on the different maturation stages of NK cells. According to this model, NK cells go through four maturation stages before they become mature, circulating NK cells (stage V). In stage IV, before reaching maturity, NK cells express CD94/NKG2A and high levels of CD56. We and others have observed that most of the NK cells circulating in the peripheral blood in the first 6 months after matched or haploidentical HSCT have the characteristics of stage IV NK cells, i.e. CD56^{bright}, KIR^{low}, NKG2A⁺. A sustained high number of NK cells resembling stage IV cells could be associated with poor clinical outcome, particularly in patients with a high leukemia burden or an unstable state of remission at the time of the allograft transplantation.

Recently, Vago et al. [50] confirmed these data and showed that reconstitution of KIR⁺ alloreactive NK cells from hematopoietic stem cells is hampered by several features intrinsic to NK cell physiological maturation, even when alloreactive NK cells arise. They showed that in haploidentical HSCT, differentiation of KIR⁺ alloreactive NK cells from hematopoietic stem cell precursors may require at least 6–8 weeks. Thus, the anti-leukemia effect of NK cells may occur only after this time period. In the case of high residual tumor burden and/or rapidly proliferating leukemia blasts, this delay (or lack) of appearance of KIR⁺ alloreactive NK cells or their hypofunctional status during the first months after HSCT may represent a major limitation and may result in leukemic relapses. In view of these data, one may ask how donor NK cell precursors undergoing maturation in the mismatched recipient can give rise to alloreactive NK cells capable of killing leukemia cells. We cannot exclude that in haploidentical HSCT, the megadoses of transfused CD34⁺ cells may provide a particular microenvironment that drives NK cells to undergo maturation, predominantly of donor type. Under these conditions, the process of NK cell education would be similar to that occurring in the donor and would allow the generation of ‘licensed’ alloreactive NK cells, capable of destroying HLA class I (and KIR li-

gand) incompatible tumor cells in the haploidentical host. Another puzzling question is the role of T-cell depletion from the graft on the reconstitution of NK cells, as T-cell depletion and ATG have been shown to be important factors in clinical studies (table 1). T cells play a major role in the development of GvHD; however, their elimination from the graft by ATG leads to profound immune deficiency and loss of T-cell-mediated GvL effect for at least 6–12 months after haploidentical T-cell-depleted HSCT. This peculiar effect has been studied by comparing haploidentical HSCT recipients who underwent either partial or extensive T-cell depletion [51]. Our study showed that less extensive T-cell depletion of the haploidentical graft was associated with better maturation of NK cells and restoration of cytolytic capacities against primary haplo-mismatched blasts, as compared to the effects of extensive T-cell depletion. These effects might have an impact on clinical outcomes as patients who received a less extensive T-cell-depleted graft developed strong GvHD, but had also less relapse than patients in the highly purified CD34⁺ group. Of note, Cooley et al. [52] found different results, showing that T cells in the graft affect *in vivo* KIR reconstitution and NK cell function and correlate with increased GvHD and poor clinical outcomes after transplantation.

All together, these findings suggest that mature donor T cells and/or other cells (mature NK cells, monocytes, facilitating cells) in the graft may play a key role in NK cell differentiation *in vivo* after HSCT. An interesting approach, consisting in CD3/CD19 depletion of the haploidentical graft rather than in a positive CD34⁺ selection, resulted in the infusion of a large dose of mature NK cells, monocytes and facilitating cells. Recent studies reported that reconstitution of NK cells was better after CD3/CD19-depleted HSCT, as compared with CD34⁺-selected HSCT, potentially providing beneficial effects on immune reconstitution and NK cell-mediated GvL effect [53, 54]. In the study by Eissens et al. [53], functional NK cells were already observed 14 days after HSCT, with KIR-expressing NK cell numbers similar to those found in the donor before HSCT. After 14 days, the cytolytic activity and number of KIR-expressing cells increased even further and remained high during follow-up. In pediatric patients, Pfeiffer et al. [54] also found a fast recovery of functional CD56^{dim}CD16⁺ cells with high cytolytic activity against K562 and strong antibody-dependent cellular cytotoxicity activity against neuroblastoma and leukemic blasts as early as day 14 after haploidentical transplantation with T- and B-cell-depleted grafts. Of interest, they found better overall and event-free survival and lower re-

lapse probability in patients homozygous for C2-C2 or heterozygous for C1-C2 than in patients homozygous for C1-C1. This effect could be caused by the faster and higher reconstitution of KIR2DL2/DL3-positive NK cells, which should be efficiently blocked in patients homozygous for C1-C1. This particular kinetic of KIR reconstitution after HSCT (KIR2DL2/DL3-expressing NK cells appearing before KIR2DL1) has been observed in many studies independently from the type of transplant, suggesting that the acquisition of inhibitory KIRs is sequential and genetically programmed *in vivo* in humans [51, 55, 56]. Nevertheless, the impact of such sequential KIR acquisition remains controversial as other studies reported a worse outcome in patients homozygous for C2-C2 [55]. Indeed, as KIR2DL2-expressing NK cells reconstitute first, they can interact with C1 ligands in the recipients, and thus, become licensed and capable of killing infected cells or tumors. On the contrary, in C2-C2 patients, KIR2DL2-expressing cells are not able to become licensed and are thus hyporesponsive, having an unfavorable impact on clinical outcome. All together, these studies suggest that the beneficial effect of NK cell activity after HSCT is dependent on the fast reconstitution of a cytolytic NK cell repertoire that is regulated by KIR and that a slower reconstitution toward a KIR-dependent repertoire is associated with poor clinical outcome. However, more extensive studies are needed to reveal whether these findings have an effect on clinical outcome.

Fast reconstitution of NK cells was also achieved after UCBT. We have shown that after UCBT, NK cells appeared very rapidly and became fully functional against AML blasts [56]. In fact, we observed that in spite of the persistent overexpression of CD94/NKG2A, the patterns of NK cell receptor expression during the first year after HSCT exhibited great similarity with those of mature NK cells from healthy donors. These NK cells were also functionally competent after UCBT, producing large amounts of IFN- γ , and demonstrated a propensity for degranulation against K562 cells and primary AML blasts comparable to that of normal NK cells. These important clues suggest that the rapid appearance of functional NK cells after UCBT is mainly due to rapid maturation of NK cells. Similarly to CD3/CD19-depleted HSCT, and contrary to CD34⁺-selected HSCT, UCBTs are performed with cells from a particular hematopoietic environment, i.e. especially enriched with stem cells, but also including several other hematopoietic cell subsets at various differentiation stages [56]. Thus, this may suggest that the maturation of NK cells after UCBT occurs under the influence of the microenvironment and the host marrow stromal cells.

Conclusion and Unresolved Questions

The numerous studies investigating the role of alloreactive NK cells in HSCT have revealed major discrepancies regarding the impact of alloreactive NK cells on relapse and survival, as well as on the adverse outcomes related to infection and aGVHD. These discrepancies could be due to differences in donor selection, conditioning regimens, extent of T-cell depletion, hematopoietic stem cell dose, disease state at transplantation, nature of disease, and algorithm of NK cell alloreactivity. For example, Miller et al. [57], in a study with up to 2,000 transplantations, identified that the use of different aGVHD prophylaxis and preparative regimens (e.g., potent T-cell depletion vs. more traditional immunosuppression) may influence NK cell recovery and function and may explain different clinical outcomes. In broad brush strokes, it seems that the most favorable conditions include a myeloablative conditioning regimen, maximal stem cell and minimal T-cell doses, and selection of patients with myeloid disease in remission, as the status of the disease at the time of the transplant appears to be the most important predictive factor on outcome after HSCT. In addition, selecting the most appropriate NK cell alloreactivity model is of major importance. Thus, to assess the presence of NK cell alloreactivity in haploidentical HSCT, it is necessary to analyze donor and recipient HLA class I as well as donor KIR genotypes. In matched HSCT, analysis of the missing ligand in the recipient is necessary in association with the analysis of the donor genotype, in particular the presence and type of activating KIR genes. In addition, the phenotypic analysis of KIR and CD94/NKG2A expressed by donor NK cells can further define the size of the alloreactive NK cell subset. Finally, functional assays testing the ability of NK cells to kill appropriate target cells provide precise information on the degree of alloreactivity of any given NK cell populations. However, NK cell receptor recognition is a complex and incompletely elucidated process, and tests of donor NK cell function against patient blast cells are nearly unresolved. The main developmental challenges for the future are to enhance immune reconstitution and to prevent relapse after HSCT.

Nonetheless, several pressing unanswered fundamental questions will require further biological input.

- What are the ideal conditions for *in vivo* persistence of NK cells after HSCT?
- Should a particular NK cell subset be preferentially expanded after HSCT?

- What are the interactions between NK cells and other hematopoietic cells in the graft that promote the maturation of NK cells after HSCT?
- Which ligands are recognized by activating KIR receptors and what is their relevance in HSCT?
- Is the licensing process fixed and definitive at a particular time, or can NK cells be licensed during their whole lifecycle?

More studies will be needed to answer these questions and expand our understanding of the role of NK cells. This exciting and promising area of research may open many new therapeutic avenues, not only in the field of

hematopoietic disease and transplantation, but also for the treatment of solid tumors, infections and autoimmune diseases.

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