NK cells mediate a crucial graft-versus-leukemia effect in haploidentical-HSCT to cure high-risk acute leukemia

by

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Abstract

NK cells are involved in innate defenses against viruses and tumors. Their function is finely tuned by activating and inhibitory receptors. Among the latter, KIRs and CD94/NKG2A recognize HLA-class I molecules, allowing NK cells to discriminate between normal and aberrant cells, as well as, to recognize allogeneic cells, because of their ability to sense HLA polymorphisms. This latter phenomenon plays a key role in HLA-haploidentical hematopoietic stem cell transplantation (haplo-HSCT) for high-risk acute leukemia patients transplanted from an NK-alloreactive donor. Different haplo-HSCT settings have been developed, either T-depleted or T-replete, the latter requiring GvHD prophylaxis. A novel graft manipulation, based on depletion of αβ T cells and B cells, allows infusion of fully mature, including alloreactive, NK cells. The excellent patient clinical outcome underscores the importance of these innate cells in cancer therapy.

Highlights

- T-cell depleted haplo-identical hematopoietic stem cell transplantation (HSCT) represents a novel approach based on grafts depleted of αβT and B cells. This method allows the prompt availability of potent effector cells (i.e. NK and γδT cells), capable of preventing leukemia relapse and controlling infections.

- KIR-mediated recognition of epitopes shared by HLA-Class I alleles is the basis for the NK alloreactivity, which is crucial for GvL effect and prevention of GvHD in T-cell depleted haplo-HSCT.

- CMV infection/reactivation drives the expansion of adaptive NK cell subsets displaying specialized effector function and long-term persistence. These cells may be harnessed as immunotherapeutic tools.

- NK cells may represent a suitable platform for novel therapeutic approaches, such as CAR-engineered NK cells. In addition, the use of monoclonal antibodies against inhibitory checkpoints may unleash anti-tumor NK-cell function.

General physiological aspects of human NK cells

Natural Killer (NK) cells are an important component of innate immunity and are now recognized as part of the broader family of innate lymphoid cells (ILC)[1]. They are involved in early immunity against viruses and in anti-cancer responses, preventing tumor growth and dissemination. While this latter function is primarily related to NK-cell cytolytic activity, the production of chemokines and cytokines promotes early inflammatory responses, complementing the activity of other innate cell types, including dendritic cells (DC), macrophages, stromal cells, eosinophils and neutrophils [2-6]. NK-cell activation and function is regulated by an array of germline-encoded activating and inhibitory receptors. Of great relevance here, NK cells express HLA-Class I-specific inhibitory receptors, particularly CD94/NKG2A and **Killer Immunoglobulin-like receptors** (**KIR**) (see Glossary), the latter sensing HLA allotypic determinants [7].

NK cells originate from hematopoietic stem cells (HSC), primarily in the bone marrow. However, NK-cell precursors at different stages of differentiation can be detected in peripheral blood, thymus, tonsils, lymph nodes, gut-associated lymphoid tissues, liver and decidua (reviewed in Moretta L et al.)[8], suggesting that NK-cell maturation continues in the periphery. NK-cell differentiation from HSC towards mature peripheral NK cells occurs through phenotypically-

identified stages [9]. The progression to $CD56^{bright}$ and, subsequently, to $CD56^{dim}$ and to terminally differentiated NK cells is traced by the gradual downregulation of CD94/NKG2A and the acquisition of CD16, KIRs and CD57 [10] (Figure 1). Remarkably, *in vivo* exposure to **cytomegalovirus (CMV)** accelerates NK-cell maturation, favoring the emergence of highly differentiated NK cells ($KIR^{+}NKG2A^{-}CD57^{+}$)[11]. Studies during the past 15 years revealed that NK cells may play a central role in T-cell depleted **HLA-haploidentical Hematopoietic Stem Cell Transplantation (haplo-HSCT)** setting to cure otherwise fatal leukemia. In particular, HSCT plays a pivotal role to cure leukemia patients either relapsing and/or responding poorly to chemotherapy or carrying high-risk molecular/cytogenetic features. However, the percentages of patients able to locate an HLA-compatible either related or unrelated donor does not exceed 60-70%, thus leaving a remarkable proportion of patients in need of allogeneic HSCT with no effective therapy available. In addition, in the case of HSCT from a matched-unrelated donor (MUD), the time elapsing from the start of the search to transplantation may exceed 2 months. Thus, too high percentages (up to 50%) of patients succumb of their disease or infections before HSCT. HSCT from a haploidentical donor (frequently one parent in pediatric patients) revealed as a promising solution in order to rescue these patients. The continuous improvements in our knowledge on NK receptors, particularly KIRs, as well as in graft manipulation strategies, led in the last few years to truly unthinkable clinical results.

In this review, we outline recent progresses on KIR molecules and their encoding genes, the puzzling role of CMV infection in haplo-HSCT and the new strategies allowing rapid effector cell intervention resulting in a more efficient prevention of both leukemia relapses and life-threating infections.

NK cell receptors and ligands

NK-cell recognition of target cells is tightly tuned by processes involving the integration of signals delivered from multiple inhibitory and activating receptors expressed on NK-cell surface [12]. The principal inhibitory receptors regulating NK-cell function recognize **HLA-Class I** molecules and include inhibitory KIRs (iKIR), CD94/NKG2A, and LILRB1 [7, 13-16]. Thus, since HLA-Class I molecules are ubiquitously expressed on the majority of healthy cells, their interactions with NK inhibitory receptors ensures self-tolerance.

Inhibitory KIRs are characterized by long cytoplasmic tail including **immunoreceptor tyrosine-based inhibitory motifs (ITIM)** that, upon KIR/**KIR-Ligand (KIR-L)** recognition, become

phosphorylated and recruit tyrosine phosphatases able to switch off NK-cell responses [7, 12, 13, 15, 16]. The four main iKIRs recognize polymorphic residues of HLA-Class I α1 domains. They include: KIR2DL1, KIR2DL2/L3 binding to HLA-C allotypes characterized by a Lysine (C2 epitope) or an Asparagine (C1 epitope) at position 80 respectively, KIR3DL1 recognizing HLA-B and HLA-A sharing the Bw4 public epitope, and KIR3DL2 that binds to HLA-A*03, -A*11 allotypes and HLA-F [7, 12-17]. Notably, *KIR* genes are characterized by a high degree of variability at both haplotypic and allelic levels [18-22]. Thus, *KIR* alleles of a given *KIR* gene may code for receptors differing in the extracellular portion or transmembrane/cytoplasmic regions. Polymorphism at each of these regions has been associated with major biological consequences [23-28](Box 1). The non-polymorphic, C-type lectin-like CD94/NKG2A heterodimeric receptor is specific for the non-classical HLA-E molecules that mainly bind peptides derived from the leader sequences of various HLA-A, -B, -C and -G molecules [29]. Therefore, CD94/NKG2A operationally senses the overall HLA-Class I expression on cells. **Activating KIRs (aKIR)**, as well as CD94/NKG2C, represent the activating counterpart of HLA-Class I specific inhibitory receptors [7, 11, 29]. An additional inhibitory receptor is LILRB1 (or ILT-2/LIR-1), that, interacting with conserved α3 domain and β2 microglobulin, recognizes a broad spectrum of classical and non-classical HLA-Class I molecules [30].

There is a high degree of variability of PB NK-cell phenotypes, particularly within the $CD56^{dim}$ subset. NK-cell repertoires are extremely variegated and the diversity is mainly due to the stochastic expression pattern of KIRs and to the *KIR/HLA-Class I* gene variability [31, 32]. Furthermore, an important process referred to as NK-cell "education" or "licensing" has been discovered [33, 34]. NK-cell education, occurring during the developmental process, can ensure that only NK cells expressing surface inhibitory receptor(s) binding self HLA-Class I molecules can be fully functional. Thus, each competent NK cell maintains self-tolerance to autologous healthy cells, while it can recognize and kill abnormal cells that have downregulated HLA-Class I molecules, according to the "missing self" model [35]. By the same mechanism, in a non-autologous setting (such as allogeneic HSCT), NK cells can be alloreactive when they express only "educated" inhibitory KIR(s) that are not engaged by the HLA-Class I molecules present on allogeneic cells. In haplo-HSCT, donor NK alloreactivity may be predicted by investigating both the donor *KIR* gene profile and the donor/recipient KIR-L to identify a possible "KIR/KIR-L mismatch" in graft-versus-host (GvH) direction. Moreover, the donor alloreactive NK subsets can be directly assessed using appropriate anti-KIR and anti-NKG2A mAb combinations (Box 2) [36-38]. Among the aKIRs, the

evaluation of KIR2DS1$^+$ NK cells can be of particular interest. Indeed, in C1$^+$ donor/C2$^+$ recipient pairs, donor KIR2DS1$^+$ NK cells are licensed and capable of recognizing their ligands (i.e. C2 allotypes) on leukemia cells [37, 39] (Box 3).

Another set of NK activating receptors are involved in the process of natural cytotoxicity. Among these receptors, the NCRs play an important role in killing tumor cells of different origin, including leukemia cells [40-42]. Although various NCR ligands have been identified, mainly represented by microbial ligands, our knowledge of NCR ligands on tumor cell membrane is still relatively limited [43]. Thus, while there is functional evidence for a primary role of NKp46 in the NK-cell recognition and killing of leukemia cells, the ligand recognized remains undefined [36]. NKp30, of which different isoforms have been identified [44], recognizes the nucleic factor leukocyte antigen-B-associated transcript 3 (BAT-3) that may be expressed in the cytoplasm of tumor cells, and the cell surface transmembrane protein B7-H6 [45, 46]. NKp30/B7-H6 engagement plays a role in NK-mediated recognition and killing of leukemia cells lines, while B7-H6 expression is infrequent in primary leukemia cells. Since NKp30-mediated killing of different leukemia has been well documented, this implies the existence of other, still undefined, NKp30 ligand(s). NKp44, expressed only upon NK-cell activation, recognizes an isoform of mixed-lineage leukemia-5 (MLL5) molecule. Notably, also soluble NCR ligands have recently been discovered. The complement factor P (CFP) binds to NKp46 while the platelet-derived growth factor P (PDGF)-DD to NKp44 [47, 48]. It is still unknown whether these interactions may occur in leukemia patients, possibly affecting tumor progression or NK-cell activity. This antigen is present on the cell surface of hematopoietic and non-hematopoietic tumor cells, while it is undetectable on normal cells [49]. NKG2D is another important activating receptor involved in tumor cell recognition specific for the stress-inducible molecules MICA/B and ULBPs [50]. In this regard, it is of note that both the surface expression of certain ULBPs on some leukemia blasts and the NKG2D involvement in their killing have been described [36, 51]. Finally, PVR (CD155) and Nectin-2 (CD112) are the ligands of the activating receptor DNAM-1 [52]. These DNAM-1 ligands can be frequently detected on AML and ALL (from both adult and pediatric patients), thus representing relevant functional ligands for a NK-cell activating pathway. However, it should be mentioned that these ligands are also recognized by the inhibitory co-receptors TIGIT and CD96, possibly competing with DNAM-1 [53].

Another relevant activating receptor is CD16 (FcγRIIIa), the low-affinity receptor for the immunoglobulin Fc fragment. Upon CD16 engagement, NK cells, via antibody-dependent cell-mediated cytotoxicity (ADCC), can eliminate opsonized target cells, this observation emphasizing

the concept that NK cells can display a potent anti-tumor effect also in the context of antibody-targeting anticancer therapy [2]. A clear example of the synergistic action of NK cells and mAb is represented by the enhanced NK-mediated lysis of B cells exposed to the anti-CD20 mAb [54]. Although, in the context of haplo-HSCT, a major emphasis has been given to NK-alloreactivity (based on KIR/KIR-L mismatch), it should be underscored that efficient killing of leukemia cells strictly depends on the interaction between several activating receptors and their ligands on target cells.

Impact of CMV infection on NK-cell reconstitution in HSCT

NK-cell receptor repertoire is shaped by CMV infection/reactivation, which frequently occurs in patients undergoing allogeneic HSCT [55, 56]. In these immunocompromised individuals, CMV infection has been shown to be a powerful driver of NK-cell maturation [57]. It was also shown that, in umbilical cord blood transplantation (UCBT) patients, CMV infection favours a preferential expansion of highly differentiated NKG2A$^-$ self KIR$^+$ and CD57$^+$ NK cells that express the activating receptor NKG2C [58, 59]. More recently, a remarkable acceleration of NK-cell maturation has been described upon CMV reactivation occurring in pediatric patients receiving $\alpha\beta$T/B-cell depleted haplo-HSCT [60]. These CMV-induced NK cells, that were mainly characterized by a highly differentiated signature (NKG2C$^+$CD57$^+$selfKIR$^+$NKG2A$^-$Siglec-7$^-$NCR^{low}IL18Rα^{low}), persisted over 1 year after transplant and displayed a specialized effector function. Namely, they were capable of killing certain HLA-E$^+$ tumor targets through NKG2C. In addition, they showed an efficient reverse ADCC (i.e. a redirected cytotoxicity assay in which mAbs specific for given NK receptors are bound to FcγR on target cells), but displayed an impaired ability to release IFN-γ upon IL-12+IL-18 exposure. Interestingly, NKG2C$^+$CD57$^+$ NK-cell expansions could be detected also in some recipients who did not reactivate CMV, but had received grafts containing high numbers of mature NK cells derived from a CMV$^+$ donor. A possible explanation is that donor-derived, adoptively transferred NK cells, primed by a previous encounter with CMV in the donor, could have undergone proliferation in the recipient in response to a subclinical virus reactivation [60, 61]. Thus, in response to CMV infection, NK cells show traits typical of adaptive immunity [11]. These include virus-induced, oligoclonal expansion, enhanced effector function, longevity, as well as epigenetic modifications that have recently been described in healthy CMV$^+$ subjects [62, 63]. It has been suggested that these CMV-induced, specialized NK cells, possibly showing memory-like properties, might also exert an anti-leukemic effect preventing leukemia relapse in different HSCT

settings [64, 65]. Previous studies showed that CMV induces a profound downregulation of HLA-Class I, rendering target cells partially resistant to αβT cell-mediated cytotoxicity but susceptible to NK-mediated killing [66]. In line with this concept, it was observed that the CMV-driven expansion of $NKG2C^+CD57^+$ NK cells in HSCT patients correlated with reduced leukemia relapse rates (namely 16% in expanding vs 46% in non-expanding HSCT recipients) [67]. Additionally, high absolute counts of memory-like $NKG2C^+CD57^+KIR^+$ NK cells showed a tendency to correlate with lower relapse incidence also in patients receiving T-replete haplo-HSCT with post-transplant infusion of high-dose cyclophosphamide (Cy)[68]. In view of the features displayed by CMV-induced "adaptive" NK cells, a protocol aimed at expanding and exploiting this specialized subset for cell therapy has been recently developed to treat pediatric ALL since these activated and expanded NK cells were shown to be highly efficient killers of allogeneic pediatric T- and precursor B-cell ALL blasts [69]. Notably, CMV infection is also capable of driving the expansion of NK-cell subsets expressing activating receptors different from NKG2C, such as aKIRs [70, 71]. These receptors could play a relevant role against leukemia, as previously mentioned. Moreover, the presence of aKIRs has been associated with protection against viral infections [72]. In particular, it has been reported that patients receiving haplo-HSCT from donors characterized by the presence of a Tel B/X *KIR* genotype (containing *KIR2DS1* and *KIR3DS1* genes) (Box 1) had reduced infection-related mortality [73].

Although the anti-tumor activity of mature NK cells developing after HSCT has been documented (or suggested) in several reports [74, 75], NK cells with a less mature phenotype have also been reported to be associated with a reduced risk of leukemia relapse. Indeed, NK cells characterized by a $NKG2A^+CD57^-NKG2C^-$ phenotype have been associated with protection from relapses and improved overall survival (OS) in HLA-Class I matched allogeneic HSCT [76]. Notably, however, these findings are compatible with a role played by alloreactive NK cells emerging after CMV-induced differentiation of such $NKG2A^+$ cells towards KIR^+NKG2A^- cells, in the case of a KIR-mismatched haplo-HSCT. Nonetheless, the impact of the repertoire of CMV^+ donor-derived CMV^+ NK cells contained in the grafts on the clinical outcome of patients needs to be specifically investigated in different transplantation settings. This is particularly true in the case of αβ T/B-cell depleted haplo-HSCT, in which it is crucial to optimize donor selection and exploit CMV-induced NK-cell subsets (either adoptively transferred with the graft or *de-novo* generated after HSCT).

NK cells in HLA-haploidentical HSCT

As previously mentioned, the discovery of inhibitory and activating receptors, besides clarifying the molecular mechanism determining NK-cell function and alloreactivity, led to a remarkably rapid exploitation of these findings in the treatment of high-risk acute leukemia. Detailed illustration of haplo-HSCT has been reviewed previously [75, 77]. Thus, here, we will discuss only the highlights and the novelties introduced recently in this transplantation setting that allowed saving thousands of lives in both adult and pediatric patients (Figure 2, Key Figure).

Early studies by Ruggeri et al. reported a 5-year survival probability of approximately 60% in adult AML patients receiving megadoses of highly purified $CD34^+$ cells (T-depleted HSC) from a donor with alloreactive NK cells (according to the KIR/KIR-L mismatch in GvH direction model)[78]. In the absence of NK alloreactivity, the survival rate was markedly lower because of a higher risk of leukemia recurrence. In adults, high-risk ALL were poorly responding also in case of NK alloreactivity [78]. Surprisingly, early studies in pediatric patients showed opposite results. In fact, in case of NK alloreactivity, a good survival rate (approaching 70%) was detected in high-risk ALL but not in AML (where only 40% became long-term survivors). Interestingly, the survival probability in absence of NK alloreactivity was over 35% in ALL. While differences in susceptibility of AML vs ALL in adults or children may be interpreted in view of the different expression of ligands for NK-activating receptors on leukemia cells [51], the better survival rate occurring in children, in the absence of NK alloreactivity, may reflect, at least in part, the additional criteria progressively applied to the selection of the "optimal donor" in each donor/patient pair. Indeed, these criteria were shown to correlate independently with a better prognosis (Box 3). Remarkably, virtually all deaths due to transplant-related mortality or leukemia relapses occurred within few weeks/months after HSCT. As discussed above, NK-cell differentiation from HSC requires 2-3 weeks to reach the maturation stage of $NKG2A^+KIR^-$ cells (i.e. relatively immature cells, poorly cytolytic and with no alloreactivity). Moreover, the first appearance of KIR^+, cytolytic, and potentially alloreactive cells requires 4-6 additional weeks. The post-HSCT immunodeficient status, due both to the absence of adaptive immunity and to the lack of mature NK cells, may be critical by severely impairing control of both leukemia relapses and infections (Figure 2A).

Although the overall survival rate of $CD34^+$ haplo-HSCT patients was good, considering the extremely poor prognosis of patients not receiving HSCT, a further improvement was clearly needed, particularly for pediatric AML patients (in whom an OS rate of ~30% was recorded). Thus, to improve the clinical outcome, a promising approach was offered by a newly developed method of graft manipulation based on the negative selection of αβT lymphocytes (responsible of **graft-**

versus-host disease, **GvHD**) and $CD19^+$ B cells (from which EBV-related post-transplant lymphoproliferative disorders, PTLD, may develop when efficient T-cell immune-surveillance is lacking) [79]. Accordingly, after this more selective and refined manipulation, the graft infused, in addition to HSC, contains different cell types, including mature NK cells and γδT lymphocytes, which can exert a strong anti-leukemia activity. In particular, through the approach of selective αβT- and B-cell depletion, the recipient immediately benefits from high numbers (on average 20-40 millions per Kg of recipient body weight) of donor mature NK cells [80] that can fully display their activity, because not exposed to the effect of pharmacological prophylaxis of GvHD, which can impair differentiation/expansion of this lymphocyte subset (Figure 2B). Patients also benefit from the infusion of several millions of γδT cells, which have no alloreactive capacity, but contribute an important anti-infectious activity, in addition to a possible anti-leukemia role [81-84]. Importantly, the clinical outcome of children with acute leukemia given an αβT- and B-cell depleted haplo-HSCT was particularly good. Indeed, the OS probability in patients with high-risk ALL was over 70%. Even more relevant was the improvement in AML patients, their OS being close to 70% [85]. Altogether, these data indicate that the infusion of cells belonging to innate immunity, together with high numbers of committed hematopoietic progenitors (and monocyte/DC), may contribute to greatly improve the clinical outcome of pediatric patients with high-risk ALL and AML.

Another approach of haplo-HSCT has been developed in the last decade based on the finding that post-transplant administration of high doses of Cy leads to selective elimination of alloreactive T cells, undergoing rapid proliferation in this highly incompatible T-replete transplantation setting.

Thus, while in the haplo-HSCT approaches described above, GvHD prophylaxis is based on the extensive removal of αβ T cells, the haplo-HSCT based on post-transplant Cy administration allows the selective removal of T cells that rapidly proliferate in response to patient's alloantigens. Notably, the infusion of unmanipulated transplants requires limited graft processing and maintains T cells potentially active against post-transplant infections. However, some relevant open questions and concerns remain unanswered. It is well established that alloreactive T cells display a major anti-leukemia activity in those transplantation settings containing "programmed" numbers of T cells allowing a relatively limited risk of GvHD. Indeed, non-alloreactive, leukemia-specific T cells are infrequent and a net distinction between T cell-mediated GvL (graft-versus-leukemia) and GvH effect is always questionable. In contrast, in the classical haplo-HSCT described above, NK cells exert GvL, without causing GvHD. Thus, an important question is whether NK activity, and

particularly alloreactivity, is maintained in patients receiving post-transplantation Cy in a haplo-HSCT setting. This question has been recently addressed by Russo et al. [68]. They showed that an active donor NK-cell proliferation occurs immediately after HSCT, possibly favored by high levels of IL-15 serum concentration, and that post-transplant (on days +3 and +4) Cy administration causes a profound NK-cell depletion, including the alloreactive population. The Cy-induced removal of mature, cytolytic (possibly alloreactive) NK cells would result in a compromised NK-mediated anti-leukemic effect (Figure 2C). Indeed, in the above study, no significant difference was reported in progression-free survival between patients with or without putative NK alloreactivity. Thus, the question remains of which cells are actually responsible of the anti-leukemia effect in the early time window following HSCT. At later time intervals, when the effect of Cy is vanished, one may speculate that a selective growth of the rare leukemia-specific T cell clones may occur. In addition, it is conceivable that NK cells undergoing differentiation from HSC or common lymphoid precursors, originally present in the graft, may undergo maturation and exert anti-leukemia activity. Additional studies are clearly necessary to provide further knowledge on the NK cell (and γδT lymphocyte) dynamics in post-transplantation Cy haplo-HSCT setting.

Concluding remarks

In recent years, Immunology has provided particularly powerful tools to successfully fight cancer. Major achievements have been obtained by haplo-HSCT to cure high-risk hematologic malignancies, contributing to save thousands of lives. Different strategies, described in this review, have been successfully applied to prevent GvHD without impairing GvL effect. Many clinical studies aimed at exploring the role of adoptive immunotherapy with NK cells in patients with hematological and non-hematological malignancies are also ongoing. While some studies reported encouraging responses in patients with hematological malignancies [86, 87], the real clinical benefit deriving from this approach in patients with solid tumors remains to be proven. In this context, these difficulties could reflect: i) differences in tumor burden residual after chemo/radiotherapy, ii) difficulty in NK-cell trafficking to sites of solid tumors, and iii) the highly immunosuppressive microenvironment of solid tumors.

The development of novel combination therapies (e.g. the adoptive transfer of T cells engineered with a suicide gene)[88], the use of T or NK cells equipped with chimeric antigen receptors (CAR) targeting tumor antigens, as well as the blockade of checkpoint inhibitors (e.g. anti-PD-1, anti-PD-

L1, anti-CTLA4, anti-NKG2A, and/or anti-KIR mAbs) will provide a therapeutic armamentarium, that was indeed unthinkable until recently (see Outstanding Questions Box).

BOX 1- KIR polymorphism

The *KIR* gene family consists in 13 polymorphic genes and 2 pseudogenes. It is characterized by an extremely high degree of diversity occurring from both *KIR* gene content variability and *KIR* allelic polymorphism [18-22].

KIR genes are organized in haplotypes and, although more than 80 different *KIR* haplotypes have been reported, two distinct groups (termed A and B) have been identified. The A haplotypes are characterized by a fixed number of *KIR* genes including several iKIR (*KIR3DL3*, *KIR2DL3*, *KIR2DL1*, *KIR2DL4*, *KIR3DL1*, and *KIR3DL2*), only one aKIR (*KIR2DS4*), and the two pseudogenes (*KIR2DP1* and *KIR3DP1*). In contrast, B haplotypes have variable and greater gene content, and are characterized by the presence of at least one of the following genes: *KIR2DS2*, *KIR2DL2, KIR2DL5B, KIR3DS1*, *KIR2DL5A, KIR2DS3*, *KIR2DS5*, and *KIR2DS1.* Structurally a high recombination hot spot divides almost all *KIR* haplotypes into two regions (referred to as centromeric and telomeric) each bordered by two framework genes (*KIR3DL3* - *KIR3DP1* and *KIR2DL4* - *KIR3DL2* for the centromeric and the telomeric regions respectively). Notably, in Caucasian, the most common haplotypes (present in ~95%) combine one of the most frequent centromeric regions with one of the most frequent telomeric regions depicted in Figure I [22].

While all A haplotypes combine a Cen-A motif with and a Tel-A motif, B haplotypes include haplotypes comprising at least a Cen-B or a Tel-B region. Two *KIR* haplotypes give rise to A/A and B/X (including both A/B and B/B) genotypes. Due to the level of heterogeneity characterizing B haplotypes, Cooley et al. proposed an elegant method for further stratifying the B/X genotypes through the B content value calculation. This score is assessed determining the number of centromeric and telomeric B regions included in the genotype [89].

More than 900 alleles have been detected (https://www.ebi.ac.uk/ipd/kir-release July 2017) (Figure II) and the allelic variability within iKIRs is higher than that reported among aKIRs.

Several allelic polymorphisms have been associated with KIR molecules characterized by relevant biological differences. Indeed, polymorphisms have been reported causing: i) amino acidic substitution determining the intracellular retention of KIR receptors or their low expression [25, 26, 90, 91], ii) a premature termination codon (i.e. *KIR* Null alleles), iii) deletion producing

frameshift and consequently production of “soluble receptors” [92], iv) variability in ligand affinity [23, 24, 27, 28, 93], v) diversity in signal transduction capability [94]. Notably, recent clinical studies underline the relevance of *KIR2DL1* and *KIR3DL1* polymorphisms [95, 96], suggesting novel criteria to improve HSCT donor selection.

BOX 2-Alloreactive NK-cell subsets

Haplo-HSCT retrospective studies revealed that the presence of donor NK alloreactivity correlated with a better clinical outcome in both adult AML patients and pediatric ALL patients [37, 75, 78, 97]. Thus, in prospective studies, when alternative donors are present and therefore donor selection can be performed, the presence of donor NK alloreactivity can be included in positive selection criteria (Box 3).

Presence of donor NK alloreactivity can be predicted analyzing: i) donor *KIR* genotype, ii) *HLA-Class I* typing in both donor and recipient, and searching donor iKIR(s) specific for KIR-L(s) present in the donor and absent in the patient. In particular, three different NK alloreactive subsets can be identified according to the missing KIR-L in the recipient. The three iKIRs relevant for the NK alloreactivity are: KIR2DL1, KIR2DL2/L3, and KIR3DL1 (Figure III). Notably, while almost all the individuals have haplotypes carrying *KIR2DL2/L3* gene, ~5% of the population lacks *KIR2DL1* gene having two *KIR* haplotypes characterized by Cen-B2 regions (Box 1). *KIR* genotype analysis of potential Bw4 alloreactive donors requires an even more accurate analysis. Indeed *KIR3DL1* and *KIR3DS1* are alleles of the same locus and, therefore, individuals characterized by two Tel-B regions are *KIR3DL1* negative. Moreover, some *KIR3DL1* alleles code for a misfolded receptors that are retained into the cytoplasm [90]. In this regard, a recent study indicated that ~12.2% of the *KIR3DL1*^{pos} donors lack the KIR3DL1 surface receptor [98] .

A precise characterization of potential donors requires combining genotypic and phenotypic analyses. Thus, the effective presence, as well as the size, of the alloreactive NK subset should be evaluated by flow-cytometry analysis. In particular, alloreactive NK cells can be identified as the subset expressing only iKIR(s) specific for the mismatched KIR-L(s) and lacking the CD94/NKG2A heterodimer. Notably, clinical studies demonstrated that the size of the alloreactive NK subset, correlating with the ability to kill mismatched leukemia cells *in vitro* [37], influenced clinical outcome [99].

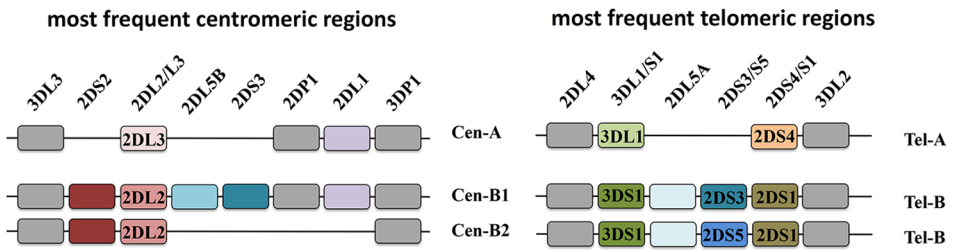
Although it is well established that NK cells expressing iKIR for self-cognate ligand (i.e. licensed) are fully competent and therefore more responsive than their unlicensed counterpart [33, 34], the

cytokine storm, induced upon HSCT, may activate the unlicensed NK cell subset that in turn "become" alloreactive [96, 100]. Thus, a different model, based only on the lack of one or more KIR-L in the recipient and the presence in the donor of the iKIR(s) recognizing the KIR-L(s) missing in the patient, has been also proposed.

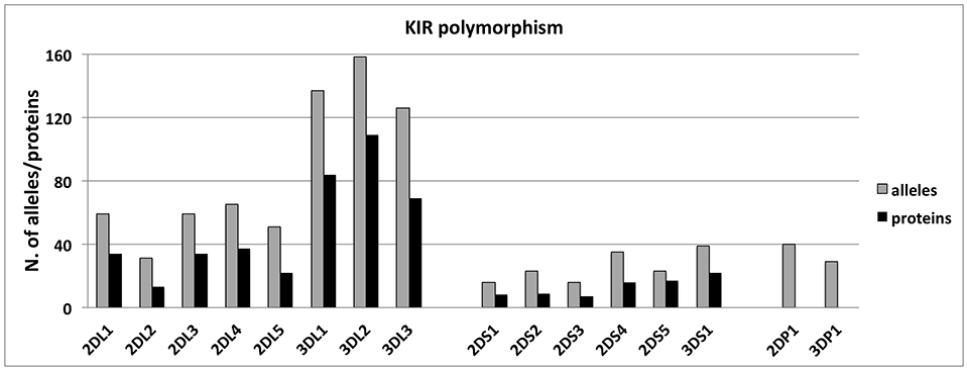
BOX 3-Donor selection criteria

In T-cell depleted haplo-HSCT, the choice of the best available donor is crucial for optimizing the success rate of the procedure. This selection process is highly complex and sophisticated, taking into account several variables. In particular, donor NK alloreactivity established according to the KIR/KIR-L mismatch in GvH direction model is prioritized and it occurs in approximately 50% of the donor/recipient pairs. In view of the advantage offered in terms of reduction of both infection-related mortality [73] and leukemia recurrence [101], *KIR* B/X genotype donor (available in around 75% of cases) should be privileged, as well. For HLA-$C2^+$ recipients, the choice of an HLA-$C1^+$/$KIR2DS1^+$ donor can be of particular interest, since NK cells of these donors are licensed and capable of recognizing their ligands on leukemia cells. The donor/recipient CMV serology has also a great relevance. Patients seropositive for the virus should not be transplanted, whenever possible, from a seronegative donor, because of the intrinsic difficulties in mounting an efficient anti-CMV primary T-cell response in the context of a state of profound immune-deficiency, like that characterizing the immediate post-transplant period of T-cell depleted haplo-HSCT. There is also a considerable body of evidence suggesting that, at least in children and young adults, the mother of the patient should be preferred as donor for haplo-HSCT, since maternal grafts may exert a more potent alloreactive effect preferentially active against leukemia cells in comparison with the father [102]. In αβT cell and B cell-depleted haplo-HSCT, since mature NK and γδT lymphocytes are infused, a high absolute cell number of these efficient immune effector cells can be taken into consideration. In addition, it is worth evaluating the NK cell expression of NKp46 and NKG2C triggering receptors. An $NKp46^{bright}$ phenotype can be privileged, considering the relevant role of NKp46 in leukemia recognition [36]. Due to its role in the response against CMV, the presence of NKG2C should be checked, trying to avoid the selection of $NKG2C^{-/-}$ individuals [60]. These donor selection criteria have been recently reported by Locatelli et al. [85].

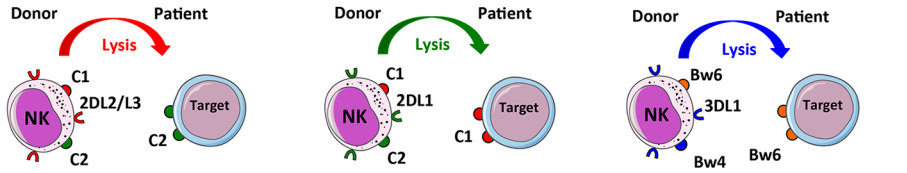
Box 1



Box 2



Box 3



C1 alloreactivity

KIR receptor	ligand
2DL2/L3	HLA-C ^{Asn80} (C1) HLA-B*46:01,*73:01 (C1)

C2 alloreactivity

KIR receptor	ligand
2DL1	HLA-C ^{Lys80} (C2)

Bw4 alloreactivity

KIR receptor	ligand
3DL1	HLA-B and –A (Bw4)

Acknowledgments

Dedicated to Alessandro Moretta

After the submission of this review, sadly Alessandro passed away on February 17 2018 at age of 64. His discoveries of KIR and of the main activating NK receptors involved in tumor cell killing (the NCR) represent most important milestones in Immunology. The major achievements in the cure of high-risk leukemias in the haplo-HSCT setting, described in this review, stem from Alessandro's seminal discoveries. Alessandro was not only a great scientist but also an exceptional person. We sorely miss his scientific insight as well as his uncommon humanity, irony and smile.

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The authors apologize for the inability to reference all relevant publications because of text limitations.

Conflict-of-interest disclosure

A.M. is founder and shareholder of Innate-Pharma (Marseille, France). The remaining authors have no conflicting financial interests.

Figure legends

Figure 1

Stages of human peripheral NK-cell differentiation

Unidirectional progression through NK-cell maturation stages can be identified based on phenotypic and functional criteria. Note that the progression of $CD56^{dim}$ towards terminally differentiated NK cells is accompanied by the progressive acquisition of more efficient cytolytic activity, paralleled by loss of proliferative capability. Different maturation stages are primarily identified according to the progressive downregulation of CD94/NKG2A and the expression of KIRs and CD16. Note that CD57 marks only late stages of NK-cell differentiation. The expression of CD94/NKG2C can be observed in small proportions at each stage, but it is particularly favored by CMV infection on mature NK cells.

Figure 2 /Key figure

Strategies for HSCT from a HLA-haploidentical donor: possible role of NK cells.

Three different haplo-HSCT settings that differ for the type of infused cells and presence/absence of GvHD prophylaxis are depicted.

A) Infusion of "pure" $CD34^+$ cells in high numbers ("megadoses"). The first lymphoid cells appearing early after transplantation in the recipient are immature CD94/$NKG2A^+KIR^-$ NK cells. These cells are poorly cytolytic and lack alloreactivity. The generation of mature KIR^+ (potentially alloreactive) cells requires additional weeks. These cells may efficiently eliminate patient residual leukemia blasts, DC and T lymphocytes.

B) αβ T/$CD19^+$ B cells depleted haplo-HSCT. This graft manipulation allows infusion not only of HSC, but also of fully mature donor NK cells and γδT cells. Both these effector cells may rapidly mediate killing of residual leukemia blasts, DC and T cells, drastically reducing both leukemia relapse and transplant-related mortality, with greatly improved survival. This graft also includes myeloid cells (M).

C) Un-manipulated haplo-HSCT and PT-Cy. This graft contains αβ T cells possibly responsible of severe GvHD. Alloreactive, highly proliferating, αβ T cells are eliminated by Cy administrated early after transplantation, thus avoiding severe GvHD. Infused NK cells (including mature KIR^+, alloreactive subsets) undergo post-transplant proliferation and are profoundly depleted by Cy administration. Low-frequency, leukemia-specific T cells and/or low-affinity alloreactive T cells (collectively indicated as leukemia-reactive T cells) could exert an anti-leukemia activity. In

addition, it is conceivable that NK cells developing from HSC, when the Cy effect is vanished, may also play a relevant role. Remarkably, in this transplantation setting, control of infections is exerted primarily by non-alloreactive T cells.

Figure 1

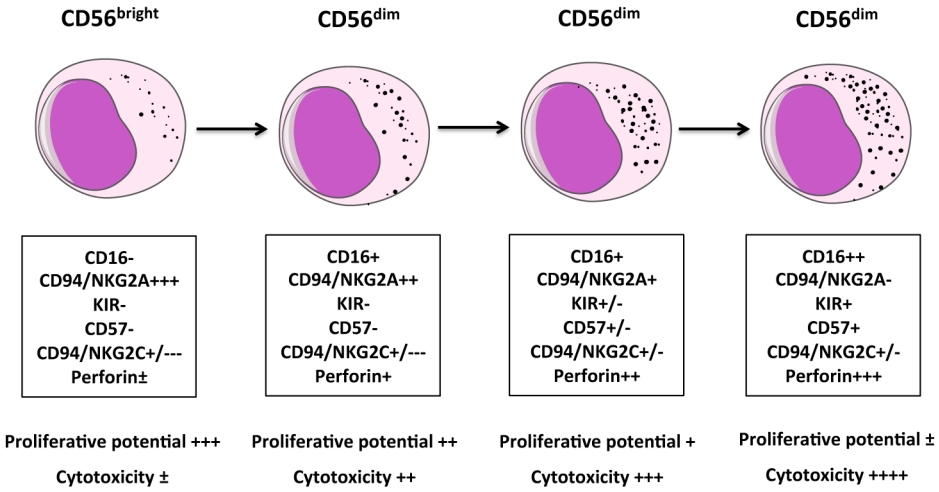
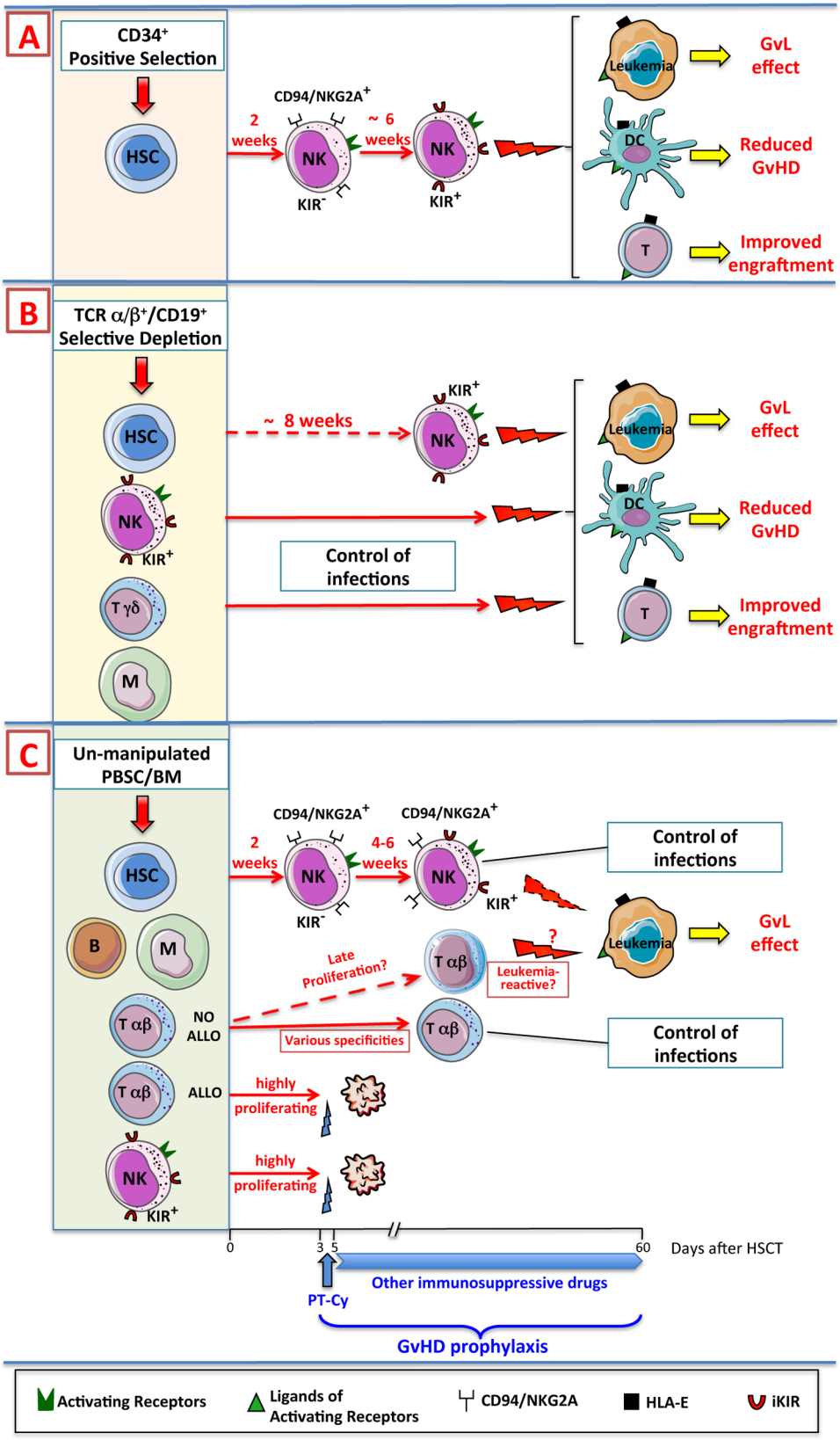


Figure 2


A
CD34+ Positive Selection
HSC
2 weeks
CD94/NKG2A+
NK
KIR-
~ 6 weeks
NK
KIR+
Leukemia
GvL effect
DC
Reduced GvHD
T
Improved engraftment
B
TCR α/β+/CD19+ Selective Depletion
HSC
NK
KIR+
T γδ
M
~ 8 weeks
KIR+
NK
Control of infections
Leukemia
GvL effect
DC
Reduced GvHD
T
Improved engraftment
C
Un-manipulated PBSC/BM
HSC
B
M
T αβ
NO ALLO
T αβ
ALLO
NK
KIR+
2 weeks
CD94/NKG2A+
NK
KIR-
4-6 weeks
CD94/NKG2A+
NK
KIR+
Control of infections
Late Proliferation?
T αβ
Leukemia-reactive?
?
Leukemia
GvL effect
Various specificities
T αβ
Control of infections
highly proliferating
highly proliferating
0
3
5
60
Days after HSCT
PT-Cy
Other immunosuppressive drugs
GvHD prophylaxis
Activating Receptors
Ligands of Activating Receptors
CD94/NKG2A
HLA-E
iKIR

GLOSSARY

Cytomegalovirus (CMV): β-herpesvirus generally acquired early in life. In healthy individuals, infection is asymptomatic or mild, but CMV is never completely eliminated. In immunocompromised subjects, such as HSCT recipients, CMV infection/reactivation becomes an important cause of morbidity. Pre-emptive (i.e. pre-symptomatic) anti-viral therapy in HSCT recipients developing CMV infection/reactivation can efficiently prevent the most severe complications.

Graft-versus-host disease (GvHD): major complication of hematopoietic transplantation in which donor T cells present in the graft attack host tissues due to recognition of non-shared histocompatibility antigens. GvHD occurs most often when donor and patient did not display a perfect HLA matched typing. There are two forms of GvHD: an early form, namely acute GvHD, that occurs soon after transplantation, and a late form called chronic GvHD. T-cell depletion or immunosuppressive drugs administration can avoid/reduce the occurrence of this immune-mediated complication of allogeneic HSCT.

Hematopoietic Stem Cell Transplantation (HSCT): utilized in the therapy of non-malignant (e.g. primary immunodeficiencies) or malignant diseases (e.g. high-risk acute leukemias) to reconstitute lympho/hemopoiesis compromised either by diseases or by myeloablative therapy.

HLA-haploidentical HSCT: when the patient shares only an HLA-haplotype with the donor (e.g. a parent for pediatric patients). Strictly necessary in patients needing HSCT in the absence of an HLA-compatible donor. This transplantation setting strictly requires a deep depletion of αβT cells (*ex vivo* by graft manipulation or *in vivo* by the use of PT-Cy and immunosuppressive treatment) to avoid severe GvHD.

Human Leukocyte Antigens (HLA): refers to major histocompatibilty complex (MHC) in human. HLA molecules play a relevant role allowing the immune system to discriminate between “self” and “non-self”. The classical HLA-Class I molecules: i) include HLA-A, -B, and –C, ii) present antigens to $CD8^+$ T cells, ii) are recognized by iKIRs.

Immunoreceptor tyrosine-based inhibition motif (ITIM): conserved sequence of amino acids (S/I/V/LxYxxI/V/L) present in the cytoplasmic tails of many immune inhibitory receptors. Upon receptor/ligand recognition, Tyrosine, included in ITIMs, becomes phosphorylated, allowing the recruitment of tyrosine phosphatase (i.e. SHP-1 and SHP-2).

Killer Immunoglobulin-like receptors (KIR): type I surface molecules, encoded by a polymorphic gene family located on chromosome 19p13.4. They are expressed by NK cells and a subset of T lymphocytes. KIRs nomenclature is based on the number of the extracellular domains (two or three, KIR2D or KIR3D respectively) and on the length of the cytoplasmic tail (long for inhibitory KIR2DL, KIR3DL receptors or short for activating KIR2DS, KIR3DS receptors), thus providing information on both structure and function.

KIR activating receptors (aKIR): carry a positively charge aminoacidic residue in the transmembrane region that allows their interaction with an adaptor molecule (DAP-12) relevant for activating signal transduction.

KIR-ligand (KIR-L): allotypic determinants shared by groups of classical HLA-Class I molecules and recognized by KIRs.

Outstanding questions box

- Will novel genetic engineering strategies be capable to improve the anti-tumor activity displayed by NK cells?

- Will haplo-HSCT in combination with CAR-T cells or CAR-NK cells and/or checkpoint inhibitors further improve the survival rate in patients with high-risk acute leukemia?

- Could similar transplantation strategies and/or NK or (CAR-NK) cell infusion be successfully applied to the therapy of other hematologic malignancies or solid tumors?

- Will third-party CAR-NK cells be able to improve the logistics of delivering this therapy to large numbers of patients, a major limitation to current CAR-T cell therapies?

- Since NK-cell triggering requires recognition of ligands for activating receptors, would the identification of novel or still unknown ligands by high-throughput approaches, such as RNA-seq analysis and proteomic studies, be useful to improve NK-mediated leukemia blasts killing?

- Would drug-induced up-regulation of relevant ligands on tumor cells enhance their susceptibility to the NK cell-mediated lysis?

- Could CMV-driven NK-cell maturation in HSCT patients be exploited *in vivo* to favor the generation of long-living, anti-viral and/or anti-leukemic effector NK cells?

- Given the positive correlation between the presence of aKIRs (in particular with a high B-content value) and the favorable clinical outcome, would a better knowledge of aKIR ligands permit to improve donor selection criteria?

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