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Novel insights into NK cell/leukemia molecular interactions and possible tools to potentiate the anti-leukemia NK cell activity

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1. INTRODUCTION

1.1. Natural Killer cells

Natural killer (NK) cells are essential components of innate immunity belonging to the group 1 Innate Lymphoid Cells (ILCs) and are involved in the first line of anti-tumor defense. In healthy individuals, NK cells represent between 5-20% of the lymphocytes in peripheral blood but can be present in lymphoid organs and tissues [1,2]. Based on the cell surface expression of CD56, NK cells are divided into two subpopulations:

- $CD56^{dim}$ NK cells are the major subset in peripheral blood and are well equipped with lytic granules that contain perforin and granzyme B displaying a strong cytotoxic activity. In addition, $CD56^{dim}$ NK cells express high levels of CD16 (FcγRIIIa), which is a potent activating receptor mediating antibody-dependent cellular cytotoxicity (ADCC) [3].

- $CD56^{bright}$ NK cells are more abundant in lymphoid organs, but in peripheral blood only represent 10% of the circulating NK cells. $CD56^{bright}$ NK cells are $CD16^{dim/neg}$, poorly cytotoxic, and are mainly responsible for producing cytokines as IFN-γ and TNF-α, as well as chemokines to guide immune cells to the inflammatory tissues.

NK cells have been found in peripheral blood (PB) and lymphoid and non-lymphoid peripheral tissues as the liver, gut, lung, and placenta. One of the most studied NK cells in tissues are the decidual-resident NK cells (dNK), which play a crucial role in a successful pregnancy, participating in all steps of placentation and vascular remodeling [4].

Human NK cells originate from hematopoietic stem cells (HSC) in the bone marrow. It has been established a classical "linear" model, in which NK cell development occurs through different steps, characterized by the expression of specific surface markers [5]. Stage 1 takes place in the bone marrow, while the other stages are in the secondary lymphoid tissues, the main sites of NK cell development [6]. In the last stage, NK cells acquire the cytotoxic capacity through the formation of lytic granules. The classical "linear" process of NK cell development has been questioned, suggesting that environmental cues can eventually modify the NK cell development [7]. It is now recognized that NK cells not only originate from lymphoid precursors but also from myeloid cells when cultured with NK-supporting cytokines, as well as from other

pathways including the process of "ILC-poiesis" from multipotent ILCPs [8]. Now, it is evident that NK cell development is a "branched" rather than a "linear" process [9].

NK cell effector function is finely regulated by the balance between activating or inhibitory signals, transmitted by an array of receptors upon engagement with specific ligands [1,2,10]. At the immunological synapse, NK cells can release the content of lytic granules, perforin creating pores in the membrane of target cells allowing the entry of granzyme B with caspase activity. Thus, NK cells can lyse the susceptible target cells, as tumor cells, without affecting the surrounding healthy cells. In addition, NK cells can regulate innate and adaptive immune responses interacting with several immune cell types as dendritic cells, macrophages, neutrophils, and T lymphocytes [1].

1.2. NK cell receptors

NK cells keep in check the health of neighboring cells through several germline-encoded receptors, either type I proteins of Ig-like family or type II proteins of C-type lectin-like receptor family, upon engagement with specific ligands. The balance between activating and inhibitory signals transmitted by these receptors finely regulates NK cell function.

1.2.1. Activating receptors

NK cells express an array of activating receptors that promote NK cell-mediated killing of pathological cells over-expressing stress-inducible ligands and induce their physiologic cross-talk with other cell types (Fig. 1). Crucial for relaying the "ON" signal is the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM), more frequently contained within receptor-associated adaptor proteins. The presence of a positively charged aminoacid in the transmembrane domain of the receptor allows this association.

<u>CD16</u>

The low-affinity receptor for the immunoglobulin Fc fragment (FcγRIIIa), also known as CD16, is the potent activating NK cell receptor that mediates ADCC. Upon CD16 engagement, NK cells can eliminate opsonized target cells, as in adoptive immunotherapy using IgG antibodies recognizing tumor-associated antigens [11].

Natural cytotoxicity receptors (NCRs)

NCRs represent major NK cell-activating receptors involved in tumor cell lysis [12]. NCRs include NKp46, NKp30, and NKp44, type I transmembrane molecules that belong to the Ig superfamily [13-15] (Fig. 1). NKp46 and NKp30 are expressed on resting NK cells, whereas NKp44 is acquired after activation [16]. Upon recognition of the ligand, NCRs induce signaling through ITAM-bearing molecules CD3-ζ and/or FcϵRI-γ for NKp46 and NKp30, or DAP12 for NKp44 [12]. NCRs are predominantly expressed on NK cells, but recently the presence of one or another receptor has also been detected on different subsets of ILC. For example, NKp46 is expressed on ILC1 and a subset of ILC3 in mucosa [2]. In addition, $\gamma\delta$ T and CD8$^+$ T cells under interleukin (IL)-15 stimulation can express *de novo* NKp30, acquiring "NK-like" anti-tumor activity [17,18]. Although these receptors play a critical role in natural cytotoxicity, the complete molecular panel of NCR ligands has not yet been discovered [19]. Importantly, surface molecular marker B7-H6 has been identified as NKp30-ligand and it is expressed on tumor cells [20]. A splice variant of mixed-lineage 5 (21spe-MLL5) [21] and HLA-DP401 [22] have been described as membrane-bound NKp44-ligands. Other NCR ligands, such as BAT3/BAG6 [23,24] (NKp30-ligand) and PCNA [25] (NKp44-ligand), are intracellular proteins that may be expressed at the cell surface of tumor or stressed cells. In addition, some molecules of viral origin recognized by NKp46 or NKp44 (e.g., influenza virus-derived hemagglutinin) are capable of triggering NK cell function against infected cells. Finally, NKp46 and NKp44 can also recognize extracellular molecules. In particular, NKp46 can bind to the soluble plasma glycoprotein, the complement factor P/properdin [26], whereas NKp44 can recognize Nidogen-1/Entactin [27] and PDGF-DD [28].

NKG2D

NKG2D is a type II and C-lectin-like molecule that transduces the activating signal through DAP10 adaptor protein (Fig. 1). It is expressed on NK cells and cytotoxic lymphocytes, mainly $\gamma\delta$T cells and CD8$^+$ $\alpha\beta$T cells. Multiple NKG2D-L have been identified and are represented by MHC class I chain-related protein A/B (MICA/B) and UL16 binding proteins (ULBP)1-6. The expression of these ligands is upregulated in stressed, virally infected, and tumor cells [29].

Co-receptors: DNAM-1 and SLAM receptors

NK cells are also equipped with costimulatory receptors, collaborating with other activating receptors to enhance NK cell function. This family include DNAM-1 and SLAM family receptors (e.g. 2B4, NTB-A, etc.) [30-33]. Relevant for anti-tumor activity, DNAM-1 can recognize PVR (CD155) and Nectin-2 (CD112), expressed on various acute leukemias [34]. 2B4 recognizes CD48, ligand exclusively expressed in hematopoietic cells. After ligand recognition, the ITSMs in the 2B4 cytoplasmatic tail become phosphorylated and associate with SLAM-associated protein (SAP). SAP prevents recruitment of SH2 domain-containing protein tyrosine phosphatase-2 (SHP-2) to the cytoplasmic domains of 2B4 receptor leading to NK cell activation. In the absence of SAP, as in X-linked lymphoproliferative (XLP)-1 patients and decidual NK cells, the phosphatases are recruited and, interacting with phosphorylated ITSM, deliver a potent inhibitory signal [35,36]. Patients affected by XLP-1 disease develop a severe immunodeficiency, also impairing NK – T cell development. SAP-deficient NK cells from XLP-1 patients display an impaired cytotoxic activity against CD48^{+} hematopoietic cells (including EBV-transformed B cells) enhancing the patients' immune defect [32,37,38].
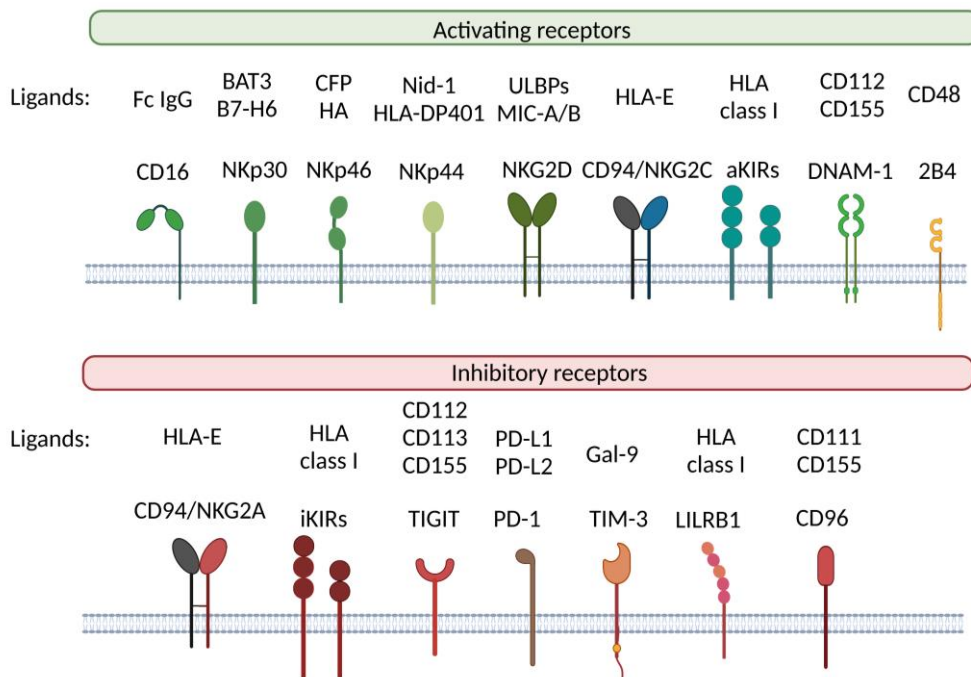
Figure 1. Activating and inhibitory NK cell receptors and their ligands. NK cells express an array of activating receptors that recognize ligands expressed on membrane of stressed target cells (e.g., B7-H6, MIC-A/-B, ULBPs) or soluble ligands such as complement factor P (CFP), and Nidogen-1 (Nid-1). HA; hemagglutinin. Different HLA class I-specific inhibitory and activating receptors recognize epitopes of HLA-A, -B and -C allotypes (i.e., Bw4, C1, and C2) or non-classical HLA class I molecules (HLA-E, HLA-G and HLA-F). Non-HLA specific

inhibitory receptors include TIGIT, PD-1, TIM-3 and CD96. The image was created in BioRender.com and inspired from [39].

1.2.2. HLA-specific inhibitory and activating receptors

Two groups of NK cell receptors can recognize HLA class I molecules, namely Killer Ig-like Receptors (KIR) and CD94/NKG2 heterodimers. They are expressed on NK cells and on a small subset of T cells [40] (Fig. 1).

<u>KIR</u>

KIR are type I molecules, that includes both inhibitory (iKIR) and activating (aKIR) receptors [40]. Their nomenclature indicates the structure and the function of these molecules: KIR2D and KIR3D have two (2D) or three (3D) extracellular domains, followed by L (long) or S (short) intracytoplasmic tail for iKIR and aKIR, respectively [41]. The long cytoplasmatic tail of iKIR contains immunoreceptor tyrosine-based inhibitory motifs (ITIM), that upon phosphorylation, transduce an inhibitory signal by recruiting Src homology region 2-containing protein tyrosine phosphatase (SHP)-1 and SHP-2. Differently, aKIR are characterized by a short cytoplasmic tail without ITIM motifs, transducing activating signals through KARAP/DAP12, an accessory molecule containing ITAM. The polymorphic *KIR* gene family is located on chromosome 19p13.4 and consists of 13 genes and 2 pseudogenes. *KIR* genes are inherited as haplotypes, comprising centromeric (Cen) and telomeric (Tel) regions, delimited by framework genes and separated by a recombination hot spot. Two groups of *KIR* haplotypes have been defined. *KIR* A haplotypes, composed of Cen-A/ Tel-A regions, are characterized by a fixed number of genes mainly encoding iKIR (*KIR2DL1*, *KIR2DL3*, *KIR3DL1*, and *KIR3DL2*) and only one aKIR (*KIR2DS4*), and by a high allelic polymorphism. Differently, *KIR* B haplotypes (including Cen-B-/Tel-A, Cen-A/Tel-B, and Cen-B/Tel-B regions) display a higher gene content diversity, low allelic polymorphism, and a variable number of aKIR (*KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, and *KIR3DS1*) [42].

The main iKIRs recognize specific epitopes by distinct groups of HLA-A, HLA-B and HLA-C allotypes named KIR-ligands (KIR-L). The dimorphism at position 80 of HLA-C defines two groups of ligands: the HLA-C1 group, characterized by asparagine 80 (N80), is recognized by KIR2DL2/L3; the HLA-C2 group, characterized by lysine 80 (K80), is recognized by KIR2DL1 and, to a lesser extent, by KIR2DL2/L3. In addition, KIR3DL1 specifically recognizes HLA-B and HLA-A molecules sharing Bw4 epitope.

Regarding aKIR, KIR2DS1, like the inhibitory counterpart KIR2DL1, recognizes HLA-C2. The ligands of the other aKIR are incompletely characterized [43,44].

CD94/NKG2 receptors

The CD94 glycoprotein may associate with NKG2A or NKG2C C-type lectin molecules, generating receptors with opposite functions. The CD94/NKG2A heterodimer is an inhibitory receptor that recruits SHP-1 and -2 tyrosine phosphatases through the ITIM-bearing NKG2A subunit. The association of CD94 with the NKG2C molecule, highly homologous to NKG2A but lacking ITIMs, forms a receptor triggering NK cell function through the KARAP/DAP12 adaptor and tyrosine kinase pathway [45].

CD94/NKG2A recognizes the nonclassical HLA class I molecule HLA-E, presenting nonameric peptides cleaved from HLA-A, -B, and -C leader sequences (from -22 to -14 residues). Thus, the peptide anchor residue for HLA-E at position 2 corresponds to the residue -21 of the HLA class I leader sequence. All HLA-A and -C allotypes are characterized by methionine at position -21 (-21M). The majority of HLA-B allotypes displays threonine (-21T) and only a minority methionine (-21M) [46,47]. Because -21T HLA-B allotypes do not supply HLA-E binding peptides, the -21 HLA-B dimorphism is relevant for the correct HLA-E folding and expression. According to this dimorphism, the human population can be divided into three groups: homozygotes for -21M HLA-B (M/M), heterozygotes for -21M and -21T HLA-B (M/T), or homozygotes for -21T HLA-B (T/T). It has been reported that individuals carrying at least one copy of -21M HLA-B allotypes (M/x) harbor better educated CD94/NKG2A^{+} NK cells in comparison with T/T individuals [48].

The activating counterpart of CD94/NKG2A, represented by the heterodimer CD94/NKG2C, also binds HLA-E. The infection by cytomegalovirus (HCMV) is associated with a higher expression of CD94/NKG2C. Indeed, expansion of adaptive NK cells, characterized by increased expression of NKG2C, was observed upon HCMV infection. This NKG2C^{+} subset presents a more mature and differentiated phenotype, including the upregulation of CD57, the expression of KIR2DL recognizing self HLA-C, and altered signaling molecules expression that enhances the ADCC potential [49-52].

In addition, NK cells also express leukocyte immunoglobulin-like receptor B1 (LILRB1, also named LIR1 or ILT2) inhibitory receptor that recognizes a broad spectrum of HLA class I molecules [53].

1.2.3. Non-HLA-specific inhibitory receptors

Besides HLA class I specific inhibitory receptors (i.e., KIRs and CD94/NKG2A), NK cells also express other inhibitory checkpoints (IC) responsible for regulating immune cell homeostasis. This group of receptors includes PD-1, TIGIT, CD96, and TIM-3 (Fig.1) [54,55]. Some of these receptors and/or ligands can be up-regulated or *de novo* expressed in the tumor microenvironment, facilitating the tumor escape. For this reason, immune checkpoint inhibitors (ICIs) have been approved for immunotherapy approaches [56,57]. NK cells can express PD-1 that binds ligands, PD-L1 or PD-L2. Different tumor types highly express PD-L1, usually absent on normal healthy cells. PD-1/PD-L1 axis represents an immune escape mechanism in the tumor microenvironment [58]. Other IC that can be present on NK cells are TIGIT and CD96 [59,60], which compete with DNAM-1 for binding their ligands, PVR and Nectin-2 [61], and are usually upregulated in tumor cells. Moreover, TIM-3 is considered a marker of advanced tumors, and its blockade has been reported to enhance NK cell cytotoxicity in preclinical models [62].

1.3. NK cell education and function

As already described, in PB there are two subsets of NK cells: CD56^{bright} and CD56^{dim}. Notably, CD56^{bright} are considered to be the precursors of CD56^{dim} NK cells. The NK cell maturation process occurs through different stages based on phenotypic and functional characteristics. This unidirectional transition is characterized by the gradual acquisition of CD16, KIR, perforin, cytotoxic capacity, decreased surface expression of CD56, and the downregulation of CD94/NKG2A [10,63].

Individual NK cells stochastically express the different KIR, engaging specific KIR-L expressed on the cells. During development, NK cells undergo a process called education, through which the inhibitory interaction between KIRs and self HLA class I ligands will guide the functional capacity of NK cells [64]. NK cells that express at least one inhibitory receptor (KIR or NKG2A) specific for self-HLA molecules become functionally competent. Thus, NK cells are tolerant towards the surrounding healthy cells, expressing normal levels of ligands (i.e., high HLA class I molecules and low activating ligands), and the inhibitory signals through HLA-specific inhibitory receptors

block activation. Conversely, NK cells can kill pathological cells, that have down-regulated HLA class I, reducing the inhibition mediated by HLA-specific inhibitory receptors ("Missing self-recognition") [65], and/or that have over-expressed ligands of activating receptors, increasing the strength of activating interactions ("Induced self-recognition") (Fig. 2).
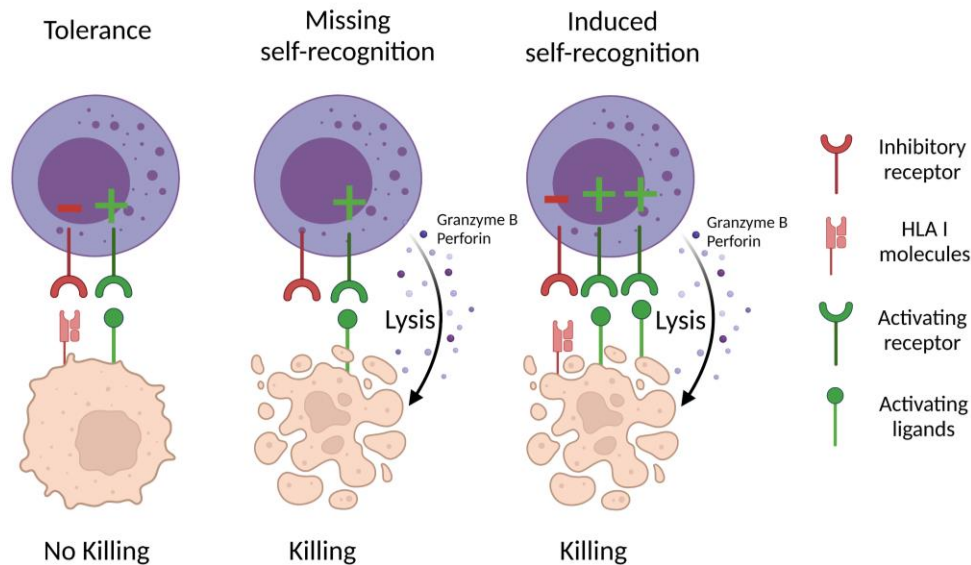
Figure 2. NK cells respond to target cells via balancing signals. NK cells express activating and inhibitory receptors that regulate their function, and the balance of the signaling guides NK cells to tolerance or activation of cytotoxicity against target cells. Figure was done with Biorender.com

In an allogeneic setting, NK alloreactivity can be observed through missing self-recognition. NK cells can be alloreactive when they only express, as inhibitory receptors, a KIR specific for a KIR-L that is present in the donor and missing in allogeneic cells (KIR/KIR-L mismatch). NK alloreactivity is relevant to the clinical context of allogeneic hematopoietic stem cell transplantation (HSCT) when the donor and the recipient are not fully HLA-matched (see 1.4.1. section).

1.4. NK cell-based therapies in leukemia patients

With the importance of NK cell capacity to kill human tumor cells, several strategies to exploit the potential of NK cells have been used in different clinical contexts, especially to cure hematological malignances.

Allogenic HSCT is a well-established treatment to cure patients with high-risk leukemia, sometimes being the only possible curative therapy [66]. However, it can present clinical complications as graft failure and acute or chronic graft-versus-host disease (GvHD) due to the incompatibility of HLA molecules between donor and recipient. Donor-derived T cells are the principal responsible for GvHD, so different strategies to deplete T cells from the graft were needed. Moreover, the use of fully HLA-matched limits this option for many patients. Finding a fully HLA-matched donor (MUD) is strongly influenced by ethnicity, as 75% probability for Caucasians or less than 20% for African Americans [67]. Alternative to MUD is umbilical cord blood (UCB) or an HLA-haploidentical relative, represented by a sibling or parent sharing one HLA-haplotype with the recipient. The haploidentical hematopoietic stem cell transplantation (haplo-HSCT) offers an important option for most patients because donors can be readily available.

1.4.1. NK cells in haplo-HSCT

The use of haplo-HSCT is being successfully applied in the clinics, following the discovery of suitable strategies to circumvent the problem of the high HLA disparity between donor and recipient [68].

<u>Haplo-HSCT with CD34$^+$ cells</u>

Haplo-HSCT became successful in the '90s combining an intense myeloablative conditioning regimen with the infusion of "mega-doses" of CD34$^+$ cells, thus depleting T cells from the graft to avoid GvHD (Fig. 3). NK cells are the first lymphocyte subset that reconstitutes patients in this transplantation platform. The important observation came with the seminal studies from the Perugia's group in 2002 [69], that demonstrated the importance of NK alloreactivity (according to KIR/KIR-L mismatch in graft-versus-host direction, GvH). Indeed, a better leukemia-free survival (LFS) in high-risk adult acute myeloid leukemia (AML) patients was observed in the presence of donor NK alloreactive cells [70]. Donor-derived alloreactive NK cell subset in the recipient exerts the highest anti-leukemia activity (GvL effect), and it has also been correlated to a

better clinical outcome in pediatric patients with acute lymphoblastic leukemia (ALL) [34]. Donor NK cell alloreactivity can be predicted by analyzing donor *KIR* genotype, and high-resolution HLA class I typing in donor and recipient to search the iKIR(s) specific for KIR-L(s) present in the donor and absent in the patient [71]. Moreover, additional phenotypic and functional analyses are necessary to characterize and quantify the alloreactive NK cell subset.

Even though the results obtained with this platform were positive to avoid GvHD, a further step was needed to control opportunistic infections due to the prolonged lymphopenia and delayed immune system reconstitution.

αβT-cell and B-cell depleted haplo-HSCT

A promising approach with the highest sophistication was found to overcome the limitations of CD34$^+$ haplo-HSCT. The αβT-cell and B-cell depleted haplo-HSCT requires an extensive graft manipulation based on the selective depletion of αβT lymphocytes (to avoid GvHD) and CD19$^+$ B cells (to prevent post-transplant lymphoproliferative disorder). In this transplantation platform, no post-transplant GvHD prophylaxis is necessary. With this approach, in addition to HSCs and myeloid cells, donor-derived NK and γδ T cells are infused in the patient (Fig. 3). Thus, the recipient immediately benefits from high numbers of mature NK and γδT cells, that can exert anti-leukemia activity and control infections, persisting in the recipient [71-73]. The clinical data obtained in a cohort of 80 pediatric patients with ALL or AML were extremely encouraging. The overall survival (OS) of children with ALL was over 70%. The real improvement was observed in AML patients, whose OS was close to 70% as well [74]. Altogether, these data indicate the importance of the infusion within the graft of innate immunity components in addition to HSCs, and underline how this new haplo-HSCT platform significantly improves the clinical outcome in pediatric patients.

Haplo-HSCT with post-transplant cyclophosphamide (PT-Cy)

In the last decades, the use of unmanipulated grafts has been successfully applied, in combination with heavy pharmacological regimens for conditioning and prophylaxis, to overcome the two most common complications: graft rejection and lethal GvHD. In this platform, patients receive a myeloablative conditioning before infusion of the unmanipulated graft, followed by high doses of cyclophosphamide (Cy), at day +3 and day +5, as GvHD prophylaxis (Fig. 3) [75,76]. Cy eliminates highly proliferating cells

with the aim to delete alloreactive T cells. Importantly, HSCs are resistant to Cy because they express high levels of aldehyde dehydrogenase (ALDH), the enzyme that induces Cy detoxification [77,78]. However, recent studies demonstrated that Cy also had a potent cytotoxic effect against NK cells, which probably proliferate early after transplant because of the presence of IL-15 in circulation [79]. The haplo-HSCT with PT-Cy platform is extensively applied because easily manageable by many transplantation centers [80].
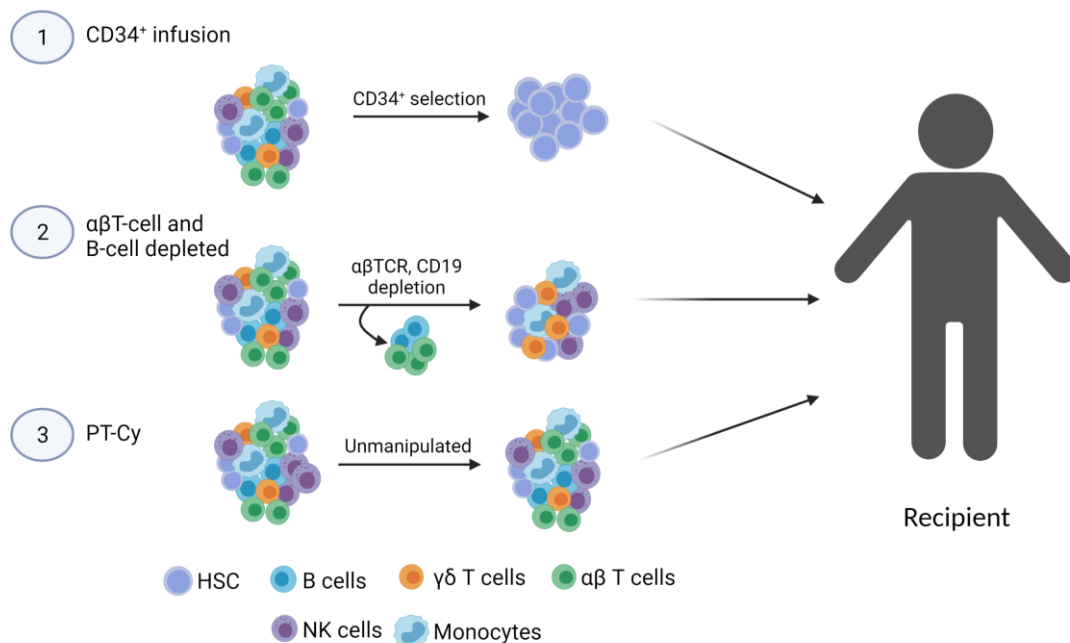

Figure 3. Three different platforms of haplo-HSCT differ on the type of cells infused and the presence or absence of GvHD prophylaxis. 1) Infusion of high doses of CD34$^+$. The first lymphoid cells appearing are CD94/NKG2A$^+$ KIR$^-$ NK cells which are poorly cytolytic. 2) αβT-cell and B-cell depleted haplo-HSCT. This graft manipulation allows the infusion of HSCs, myeloid, fully mature NK cells and γδ T cells. These immune cells can rapidly exert anti-leukemia activity and control infection. 3) Unmanipulated haplo-HSCT and PT-CY. Cells obtained from bone marrow (BM) or peripheral blood stem cells (PBSC) of the donor are directly infused into the recipient without any prior manipulation. Post-transplant high doses of Cy (at day +3 and +5) is a component of GvHD prophylaxis to eliminate alloreactive, highly proliferating αβT cells. The figure created with BioRender.com

1.4.2. Adoptive NK cell therapy

In haplo-HSCT with CD34^{+} and haplo-HSCT and PT-Cy, the appearance of mature NK cells is delayed because they arise from the HSCs. For this reason, the adoptive transfer of NK cells from the donor is an approach currently used in clinics to favor anti-leukemia effect. In a study of AML patients, purified NK cells from the donor were infused after haplo-HSCT without inducing GvHD [81].

Adoptive NK cell therapy can also be applied in a nontransplantation setting. IL-2 activated [82] or resting [83] NK cells can be infused into patients undergoing different immunosuppressive treatments followed by subcutaneous IL-2 injection during a few days. These studies confirmed that haploidentical NK cells persist and expand *in vivo* [82,83].

1.5. Strategies to potentiate the anti-leukemia activity of NK cells

Although adoptive transfer of NK cells shows encouraging results in the clinics to fight cancer, novel strategies are under evaluation to enhance NK cell activity in immunotherapy (Fig. 4) [84,85].
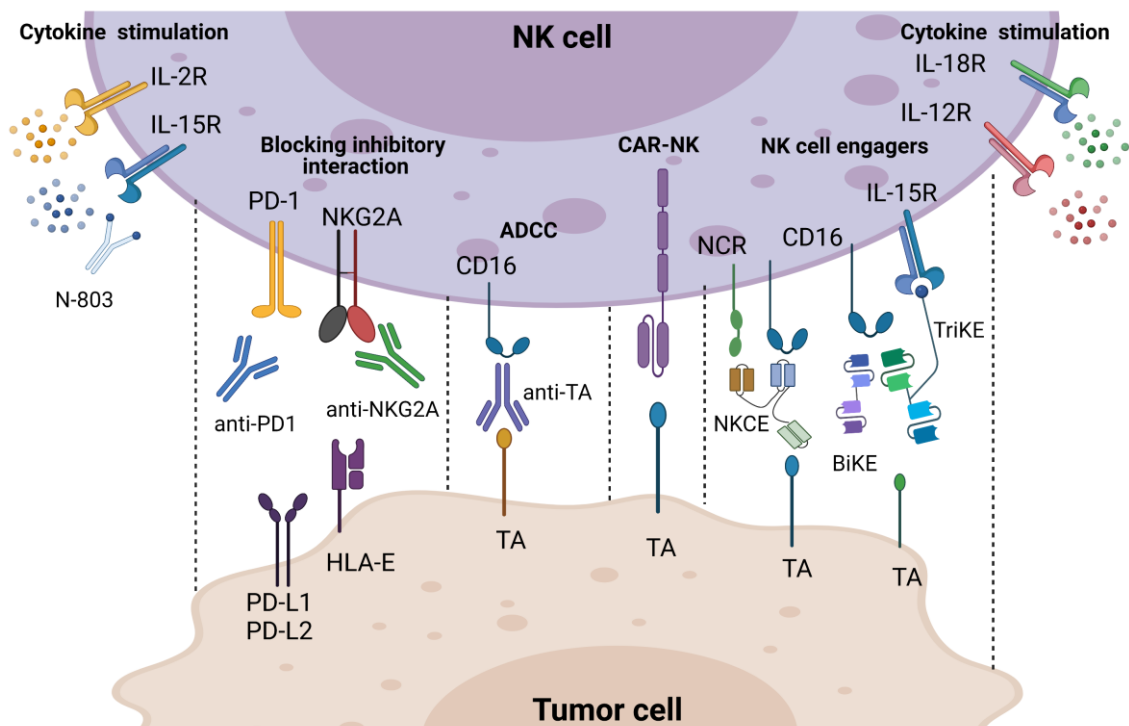
Figure 4. Strategies to potentiate NK cell anti-tumor activity against target cells. Use of cytokines (IL-2, IL-15, IL-12, IL-18), alone or in combination, to enhance NK cell effector function and cytokine secretion. IL-15 is a crucial cytokine for NK cell biology, recombinant IL-15, and the IL-15 superagonist N-803 promote NK cell proliferation and persistence. The use of immune checkpoint blockade (anti-PD1 and anti-NKG2A) potentiates NK cell function. NK

cells can kill tumor cells through ADCC in the presence of monoclonal antibodies directed against a tumor antigen (anti-TA mAb). NK cell engagers can redirect NK cell killing to neoplastic cells triggering CD16 (BiKEs and TriKEs) in addition to NCR (NKCEs) and targeting a specific TA. Genetically modified cell products as chimeric antigen receptor (CAR) NK cells can be used in adoptive NK cell therapies. The figure was done with BioRender.com

1.5.1. Cytokine stimulation

Therapeutic approaches based on cytokine stimulation to enhance NK cell activity and expansion have been used in different preclinical and clinical studies (Fig. 4). Cytokines as IL-2 and IL-15 play an important role in NK cell function; thus, they have been exploited to enhance NK-cell mediated activity against tumors [85].

IL-2 based immunotherapy was the first one applied in the clinics [86]. However, patient survival did not improve, and severe side effects were reported after high-dose IL-2 treatment. Moreover, IL-2 acts on Treg inducing the production of TGF-β that leads to immunosuppression. IL-2 mutants have been developed targeting IL-2Rβγ present on NK cells, and with reduced affinity for IL-2Rα, expressed on Treg. IL-15 appears to be an attractive alternative to IL-2 treatments. Several modifications of IL-15 have been made to enhance its half-life and reduce toxicity. Monomeric recombinant human IL-15 (rhIL-15) treatment showed promising results, either used as a monotherapy or in combination with other therapies [87,88]. N-803 (formerly named ALT-803) is an IL-15 "superagonist", recently developed by binding an IL-15 mutant (with increased affinity for CD122-CD132 receptors) to a soluble dimeric IL-15Rα-Fc fusion protein. N-803 is longer lasting *in vivo* to promote NK cell persistence after transfer into patients. In different mouse models, N-803 showed better survival in comparison with rIL-15 [89,90]. Now, N-803 is used in different clinical trials and in adoptive NK cell therapy [91].

Another promising approach is represented by the "cytokine-induced memory-like NK cells" (CIML-NK). NK cells are pre-activated *ex vivo* with a mixture of cytokines; IL-12, IL-15, and IL-18, before the infusion to the recipient. These cells present high IFN-γ production, high expression of granzyme B and perforin and increased cytotoxicity against tumor cells. Different clinical trials using CIML-NK cells are ongoing [92,93].

1.5.2. Blocking inhibitory checkpoints

NK cells express a variety of inhibitory receptors that lead to NK cell inhibition upon ligand recognition. For this reason, checkpoint blockade approaches have been developed to overcome the inhibition and unleash NK cell activity (Fig. 4).

PD-1 is expressed in NK cells from cancer patients, and it is correlated with a low anti-tumor activity of NK cells upon PD-L1 recognition. In particular, the effect of the IC blockade with nivolumab (anti-PD1) in combination with lirilumab (IPH2101, anti-KIR2D) antibodies in head and neck cancer patients is giving promising results [85].

The KIR family is the most studied group of NK inhibitory receptors. The permanent interaction of KIRs with self HLA-I molecules can result in a reduced anti-tumor activity of NK cells. Anti-KIR antibodies can block this inhibitory interaction. In clinical trials, lirilumab has been also tested against AML, myeloma, and solid tumors with promising results [94,95].

Another inhibitory receptor expressed by NK cells is CD94/NKG2A. Monalizumab (IPH2201), a humanized anti-NKG2A antibody blocking CD94/NKG2A and HLA-E interaction, promotes NK cell anti-tumor activity and it is currently investigated in clinical trials for different types of tumors [96,97] (Fig. 4). In addition, a phase I clinical trial is being performed to evaluate the safety of this antibody after HLA-matched allogenic HSCT (NCT02921685).

1.5.3. CD16-based monoclonal therapies

The use of monoclonal antibodies (mAb) targeting tumor antigens represents a widely used immunotherapeutic approach to cure hematological and solid tumors. These strategies are based on the ability of NK cells to kill tumor cells with antibodies against a specific tumor antigen via ADCC. The first therapeutic molecule used in the clinics was an anti-CD20 mAb, rituximab, developed in 1997 to treat non-Hodgkin's lymphoma patients [98]. This therapy is still a front-line therapy for B cell lymphomas and autoimmune diseases. Indeed, the response of rituximab can be influenced by polymorphisms in the FcγRIIIa receptor. Polymorphisms at the positions 48 and 158 have been correlated with a different binding affinity to rituximab, influencing the ADCC activity. The dimorphism at position 158 results in either a valine or phenylalanine, where the presence of a valine is correlated with a better response to rituximab in non-Hodgkin lymphoma patients [99,100].

1.5.4. NK cell engagers

To specifically redirect NK cell killing to neoplastic cells, molecules termed NK cell engagers (NKCEs) have been produced to improve immunological synapse and cell activation [85,101]. Bispecific killer cell engagers (BiKEs), composed of two single chain variable (scFv) fragments, engage CD16A and target tumor associated antigens (TA) [102]. Trispecific killer engagers (TriKEs), incorporating a modified human interleukin (IL)-15 crosslinker, have been produced and shown to be active in preclinical models [103,104]. The 161533 TriKE is currently being tested in a phase I/II clinical trial (NCT03214666) for the treatment of CD33$^+$ myeloid malignancies. A second-generation CD33-targeting TriKE has been also produced, showing improved functionality *in vitro* and in preclinical mouse models, potentially more efficacious in clinics [105].

Moreover, a tetravalent bispecific antibody that binds CD30 on tumor cells and CD16A on NK cells (AFM13) has been tested in a phase I trial for relapsed/refractory Hodgkin's lymphoma patients [106], either alone or in combination with immune checkpoint inhibitors, in several clinical trials [107]. A recent study described the enhancement of killing in CD30$^+$ malignant cells using *ex vivo* pre-activated NK cells with IL-12, IL-15, and IL-18 and AFM13 [108].

In addition to CD16A, the engagement of other triggering NK cell receptors, such as NKG2D and NCRs, can be extremely valuable [101,109,110]. In line with the notion that full activation of resting NK cells requires the co-engagement of distinct activating receptors [111,112], trifunctional NKCEs referred to as Antibody-based NK cell engager therapeutics (ANKET), that co-engage NKp46 and CD16A on NK cells and bind an antigen on tumor cells (NKp46/CD16A/TA) have been produced. These NKCEs showed more potent activity than therapeutic monoclonal antibodies (e.g., the anti-CD20 rituximab and obinutuzumab) and effectiveness in the control of tumor growth in mouse models [101,113]. At the end of 2021, the first patient was dosed in a phase I/II clinical trial (NCT05086315) with the molecule IPH6101/SAR443579 consisting in NKp46/CD16-NKCE targeting CD123, in patients with different hematological malignancies.

1.5.5. CAR-NK

Genetic engineering therapies are becoming a powerful immunotherapeutic tool used to cure hematological malignancies. Of particular interest is the use of chimeric antigen receptors (CAR) targeting a specific tumor antigen. CAR construct is composed of a genetically modified protein, with an extracellular domain (typically an ScFv) targeting a specific tumor-associated antigen, a transmembrane domain, and an intracellular domain that transduces an activation signal to the effector cell (Fig. 4) [114]. CAR technology was first applied to T cells, but significant complications in the manufacturing process, as high costs and time-consuming cell expansion, have been reported. Moreover, only autologous T cells could be used due to the high risk of GvHD by allogeneic T cells. This problem could be avoided if NK cells were chosen because they do not require HLA matching to be cytotoxic and can be used in allogeneic settings without causing GvHD. Another advantage of NK cells is the expression of activating receptors (e.g., NCR, NKG2D, and DNAM-1), which can be engaged synergistically and independently from CAR, to induce tumor cell killing [115]. Adverse reactions as cytokine release syndrome (CRS) or neurotoxicity have been observed in patients with CAR-T therapies but not found in CAR-NK adoptive therapies in clinical trials.

Thus, CAR-NK therapies could be an alternative to CAR-T. To date, a multitude of CAR-T cell therapies exist, but fewer CAR-NK trials are performed [39]. CAR-NK cells recognized and killed tumor cells and even NCRs were involved in killing the target. A mouse model of human lymphoma showed that the CAR-NK cells can exert *in vivo* strong anti-tumor activity against B-cell malignancies [116].

Different platforms for NK sources are being developed, including some cell lines (NK92 or NKL cell lines) and UCB-derived NK cells. UCB-derived NK cells have been used to generate CAR.CD19 NK cells in the clinics. Recent data demonstrated the efficacy of CAR-NK therapy in CD19-positive cancers, and the administration of CAR-NK was not associated with CRS, neurotoxicity, GvHD, and an increase in the levels of inflammatory cytokines as IL-6. Moreover, CAR-NK cells were expanded and persisted for at least 12 months *in vivo*. Most of the patients had good clinical responses to the CAR-NK treatment (8 from 11 patients) [117,118].

2. AIMS OF THE PROJECT

The objectives of this PhD thesis were to provide novel insights into the molecular interactions that can occur between NK and leukemia cells, and possible tools to potentiate the anti-leukemia NK cell function. Two different clinical contexts were under consideration.

1) **αβT-cell and B-cell depleted haplo-HSCT in pediatric leukemia patients**.

   In this setting, the aims were:

   - Analysis of the NK cell repertoire in donors and patients at different time points after transplantation.

   - Study of the possible role of natural killer cell engagers (NKCEs) to enhance the anti-leukemia activity by donor-derived NK cells. We evaluated the *in vitro* effect of different NK cell engagers (NKCEs), triggering either NKp46 or NKp30 together with CD16, and target either CD19 or CD20 to induce killing of pediatric B cell precursor acute lymphoblastic leukemia (BCP-ALL).

2) **Haplo-HSCT with PT-Cy in adult AML patients**.

   In this setting, the aims were:

   - Phenotypic analysis of NK cells from patients after transplantation compared to the relative donors. Early after haplo-HSCT, patients' NK cells displayed a less mature phenotype, characterized by a high expression of CD94/NKG2A and a low level of KIRs. We investigated if an impaired NK cell function could be restored by blocking CD94/NKG2A and HLA-E inhibitory interaction.

   - Study of the impact of HLA-B -21M/T dimorphism on leukemia HLA-E expression and NKG2A$^+$ NK cell education in our group of patients.

3. αβT-cell and B-cell depleted haplo-HSCT in pediatric leukemia

3.1 Phenotypic and Functional Characterization of NK Cells in αβT-Cell and B-Cell Depleted Haplo-HSCT to Cure Pediatric Patients with Acute Leukemia

The αβT-cell and B-cell depleted haplo-HSCT represents an innovative transplantation strategy to cure high risk leukemia patients. Through this novel graft manipulation method, the recipient immediately benefits from high numbers (20–40 x 10^6/kg) of donor mature NK cells that are not exposed to the effect of post-transplant GvHD prophylaxis by pharmacological immune suppression, which can impair their differentiation and expansion. NK cells can efficiently mediate anti-leukemia activity early after transplantation before the appearance of NK cells differentiating from donor HSC. Patients also benefit from the infusion of γδT cells that can contribute to the anti-infectious activity, in addition to a possible anti-leukemia role [71].

In the paper by Meazza et al. [73] we analyzed a cohort of 80 pediatric leukemia patients receiving αβT-cell and B-cell depleted haplo-HSCT (NCT01810120), whose clinical outcome has been already described [74], providing new insights on NK-cell receptor repertoire of donors and transplanted patients.

In the context of αβT-cell and B-cell depleted haplo-HSCT, choosing the most appropriate donor is crucial to improve the successful outcome of the procedure. In this transplantation setting, NK cells from the donor are infused with the graft. Thus, the donor selection process is highly sophisticated and requires the consideration of multiple factors. In particular, to select the most suitable donor, several NK cell features that have been correlated with a better anti-leukemia potential must be considered. We applied an algorithm based on genetic and phenotypic analyses, taking into account different characteristics of NK cells, prioritizing:

- NK alloreactivity, established according to the KIR/KIR-L mismatch in the GvH direction model. Selection of the donor with a more significant alloreactive NK cell subset [34,69];

- a donor with *KIR* B/X genotype (available in around 75% of cases); aKIR can positively contribute to NK cell-mediated anti-leukemia activity [119];

- the presence of KIR2DS1 (particularly in HLA-C1$^+$ donor and HLA-C2$^+$ patient pairs) can be of interest since NK cells of these donors are "licensed". Indeed, a role of HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1 has been reported [34,120];

- high absolute numbers of NK and γδT lymphocytes can be taken into consideration since these immune effector cells are infused within the graft [121];

- a NKp46^{bright} phenotype can be privileged, considering the relevant role of NKp46 in leukemia recognition [122];

- given its role in the response against CMV, the presence of NKG2C should be checked, to avoid the selection of *NKG2C*$^{-/-}$ individuals [123].

To promote NK-cell mediated anti-leukemia activity, 45% of patients were transplanted with an NK alloreactive donor. *In vitro* functional assays proved the advantage of alloreactivity and the relevance of NCR for NK-mediated anti-leukemia activity.

In the reconstituted NK-cell repertoire in these patients, high percentages of mature and functional KIR$^+$ NKG2A$^-$ CD57$^+$ NK cells, including the alloreactive NK cell subset, were already present one month after HSCT. Thus, the NK cells adoptively infused with the graft persist as mature effectors while new NK cells differentiate from the donor HSCs. Moreover, after the transplant, the alloreactive NK cell subset displayed the highest anti-leukemia activity. Human cytomegalovirus (HCMV) reactivation could influence the NK cell repertoire. The phenotypic pattern of donor NK cells (naïve vs. more differentiated) did not impact post-transplant HCMV reactivation. In the recipients at 6 months after αβT-cell and B-cell depleted haplo-HSCT, HCMV infection/reactivation promoted a more differentiated NK-cell phenotype, variably affecting the size of alloreactive subsets. No significant correlation between naïve or differentiated NK cells and relapse incidence, OS or LFS was observed in this cohort.

Publication:

Meazza, R., M. Falco, F. Loiacono, P. Canevali, M. Della Chiesa, A. Bertaina, D. Pagliara, P. Merli, V. Indio, F. Galaverna, M. Algeri, F. Moretta, N. Colomar-Carando, L. Muccio, S. Sivori, A. Pession, M. C. Mingari, L. Moretta, A. Moretta, F. Locatelli, and D. Pende. **"Phenotypic and Functional Characterization of NK Cells in αβT-Cell and B-Cell Depleted Haplo-HSCT to Cure Pediatric Patients with Acute Leukemia."** Cancers (Basel) 12, no. 8. https://dx.doi.org/10.3390/cancers12082187.

3.2. Exploiting Natural Killer cell engagers to control pediatric B-cell precursor acute lymphoblastic leukemia

3.2.1. Background

ALL is the most common pediatric cancer in developed societies, with a peak of prevalence between 2 and 5 years old. In particular, BCP-ALL is the most represented malignancy among children that arises from an expansion of malignant B cells in the bone marrow [124-126]. Despite the improvement in the treatments, approximately 15% of children with BCP-ALL relapse after frontline chemotherapy [127]. Currently, different strategies to prevent further recurrence in BCP-ALL patients represent an important clinical challenge. NK cells have been shown to exert anti-leukemia activity in the context of haploidentical hematopoietic stem cell transplantation (haplo-HSCT) [115,128]. To enhance NK cell activity against tumor cells, different molecules have been produced to improve NK cell activation. Trifunctional NKCEs that co-engage NKp46 and CD16A on NK cells and bind a tumor antigen (NKp46/CD16A/TA) have been produced. These NKCEs showed more potent activity than the therapeutic monoclonal antibodies (e.g., the anti-CD20 rituximab and obinutuzumab) and effectiveness in the control of tumor growth in mouse models [113].

We report the *in vitro* effect of different NKCEs, which trigger either NKp46 or NKp30 together with CD16A, and target either CD19 or CD20 to induce killing of pediatric BCP-ALL. We tested resting NK cells from healthy donors and pediatric leukemia patients after αβT-cell and B-cell depleted haplo-HSCT.

Publication:

3.2.2. Materials and methods

Healthy donors, leukemia patients, and cell separation

Buffy coats from healthy donors were provided by the blood transfusion center of IRCCS Ospedale Policlinico San Martino (Genoa, Italy), following approved internal operational procedures (IOH78). Peripheral blood (PB) samples were also obtained from donors and pediatric leukemia patients at different time points after αβT-cell and B-cell depleted haplo-HSCT at IRCCS Ospedale Pediatrico Bambino Gesù (OPBG), Rome, Italy. This clinical trial was approved by the Ethical Committee of OPBG (TCR αβ haplo-HSCT-OPBG; Prot. n. 424/2011) and registered at ClinicalTrial.gov website (NCT01810120). All donors for haplo-HSCT are typed for *HLA* class I, and analyzed for KIR-L and *KIR* genotype, as previously described [73].

Primary BCP-ALL blasts were derived from PB or bone marrow (BM) of pediatric patients at diagnosis. PB or BM mononuclear cells (PBMC, BMMC) were isolated by density-gradient centrifugation, and phenotypically characterized by immunofluorescence. NK cell purification was performed using RosetteSep human NK cell enrichment cocktail (StemCell Technologies, Vancouver, Canada) following the manufacturer's instructions. These cells were cryopreserved in FBS containing 10% DMSO. All samples were obtained following written informed consent from donors and patient parents/legal guardians in accordance with the Declaration of Helsinki.

Cell lines

NALM-16 (cat. #ACC-680) and MHH-CALL-4 (cat. #ACC-337), two pediatric BCP-ALL cell lines, were obtained from DSMZ (Braunschweig, Germany). DNA of MHH-CALL-4 cell line was extracted using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), and analyzed for KIR-L expression by SSP-PCR using Olerup KIR HLA ligand kit (GenoVision, Saltsjoebaden, Sweden). In addition, the erythroleukemia K562 and the lung carcinoma A549 cell lines were certified by STR analysis performed by ICLC Italian cell line collection (ICLC, www.iclc.it) in accordance to published profiles. The cell lines were cultured in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% FBS (with the exception of 20% FBS for MHH-CALL-4) (Euroclone, Milan, Italy), 2mM L-glutamine (Lonza), and 100 U/mL penicillin-streptomycin (Lonza) in 5% CO_2 incubator at 37ºC.

Production and purification of NKCEs targeting CD19 or CD20

NKp46- and NKp30-NKCEs were generated under the previously described multifunctional format NKCE-2 [113]. In brief, the sequences encoding the three different fragments of each multispecific molecule were inserted into the pTT-5 vector between the HindIII and BamHI restriction sites. Expression vectors were used to cotransfect EXPI-293F cells (Life Technologies) in the presence of PEI (37°C, 5% CO_2, 150 rpm). The cells were used to seed culture flasks at a density of 1 x 10^6 cells per mL and were cultured in EXPI293 medium (Gibco) supplemented with valproic acid (final concentration 0.5 mM), glucose (4 g/L), and tryptone N1 (0.5%). The supernatants were harvested after six days and passed through a Stericup filter with 0.22 μm pores. Multispecific molecules were purified with Protein A beads (250 μL/50 mL SN), eluted with 0.1 M sodium citrate buffer at pH 3 and immediately neutralized with 1 M Tris pH 8. The proteins were then dialyzed overnight against 1 x PBS at 4°C and concentrated to 10 mg/mL before loading on an S200 Increase 10/300 column, and the proteins yielding a peak at the expected size were harvested. Alternatively, NKCE molecules were purified by ion-exchange chromatography on a MonoS 4.6/100PE column (GE Healthcare). All the purified molecules were stored in 1X PBS and analyzed to check for the absence of aggregates and endotoxins.

Antibodies, immunofluorescence, and cytofluorimetric analysis

All antibodies used in this study are detailed in Table 1. Surface phenotype of NK cells derived from healthy donors and patients after αβT-cell and B-cell depleted haplo-HSCT was analyzed on freshly isolated PBMC by multi-parametric flow cytometry. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD, Biosciences, San Jose, CA, USA), washed twice with the Perm/Wash Buffer (BD Biosciences) followed by staining with anti-Perforin and anti-Granzyme B or the corresponding isotype control mAbs. Leukemia cell lines were analyzed by indirect immunofluorescence using specific mAb and appropriate secondary reagents (Southern Biotech, Birmingham, AL). Primary BCP-ALL samples were analyzed by multi-parametric flow cytometry using CD19-PE-Cy7, CD20-V450, CD3-BV510, CD45-APC-Vio770 mAb. HLA class I expression was evaluated by indirect immunofluorescence using W6/32 mAb and FITC-conjugated anti-IgG2a secondary reagent (Southern Biotech).

Samples were acquired using Gallios (Beckman Coulter, Brea, CA, USA) or MACSQuant-analyzer (Miltenyi-Biotech, Bergisch Gladbach, Germany) and analyzed with FlowJo, Version 10.7 (BD Biosciences).

Cytotoxicity assays

Cryopreserved or freshly isolated NK cells were incubated overnight with complete medium (10% FBS) and then tested in functional assays against different target cells. Effector and target cells were co-cultured for 4 hours at 37ºC using an E:T ratio of 10:1, unless differently specified. NKCEs at different concentrations (from 10^0 to 10^{-4} µg/mL) have been added in the co-culture. Standard ^{51}Chromium (^{51}Cr)-release assay was performed using target cell lines [34]. Due to new limitations in the use of radiolabeled material and difficulties on ^{51}Cr-labeling of primary leukemia, we setup cytotoxicity assays using 7AAD/AnnexinV (7AAD/AnnV) staining. Briefly, 0,4-1x10^6 resting NK cells were labeled with Cell Trace Violet (CTV, Thermo Fisher, Waltham MA, USA) at 37°C for 15 min, according to manufacturer's instruction. After washing in PBS, CTV-labeled NK cells were resuspended at 1x10^6/ml in complete medium; 100.000 CTV-labeled NK cells were co-cultured for 4 hours with 10.000 target cells in U bottom 96-well plate, in the presence of NKCEs at different concentrations, as indicated. Wells with target cells alone were added. Apoptosis of target cells (gated as CTV-negative cells) was analyzed by assessing 7AAD (BD Biosciences) and AnnexinV FITC (Thermo Fisher) expression by flow cytometry. Percentage of specific lysis was calculated following this formula:

$$\% \; specific \; lysis = \frac{\% \, 7AAD^+AnnV^+ target \, cells \, in \, coculture - \% \, 7AAD^+AnnV^+ \, target \, cells \, alone}{100 - \% \, 7AAD^+AnnV^+ \, target \, cells \, alone} \; x \; 100$$

Degranulation assay and IFN-γ production

NK cell activation by NKCEs against BCP-ALL was evaluated as CD107a expression and, in some experiments, IFN-γ production by flow cytometry. Resting human NK cells from healthy donors or PBMC from transplanted patients (primarily in the first trimester post-transplant) were cultured for 4 hours in the presence or absence of BCP-ALL cells at E:T 1:1 at 37ºC, using the indicated concentration of NKCEs. Golgi Stop (BD Biosciences) was added after the first hour of incubation. Thereafter, cells were washed and stained with Live/Dead Fixable Aqua stain (Thermo Fisher), anti-CD3, -

CD56 and -CD107a mAbs. Appropriate antibody combinations allowing the identification of NK cell subsets (sKIR2DL1$^+$, KIR2DL3/3DL1$^+$, and KIR$^-$NKG2A$^+$) were used, and the gating strategy is shown in Fig. S1. For the simultaneous analysis of CD107a and IFN-γ expression, 6-hour co-culture was performed, and Golgi Plug (BD Bioscience) was also added after the first hour of incubation. After the surface staining, as above, cells were fixed and permeabilized with Cytofix/Cytoperm, washed twice with the Perm/Wash Buffer (both from BD Biosciences) and stained with anti-IFN-γ mAb. MAbs used in these assays are described in Table 1. Samples were analyzed using Gallios or MACSQuant-analyzer. CD107a and IFN-γ data referred to as % positive cells represent the difference between the % of CD107a$^+$ (or IFN-γ$^+$) NK cells co-cultured with target cells and the % of CD107a$^+$ (or IFN-γ$^+$) NK cells cultured with medium alone.

Statistical analysis

Graphical representation and statistical analysis were performed with Prism software, Version 9.0.2 (GraphPad Software, San Diego CA, USA). We used Mann-Whitney tests to compare two groups with non-normally distributed variables. Two-way ANOVA followed by Tukey's comparison test was used to analyze experiments with more than two groups. Significance is indicated as: *p ≤ 0,05; ** p ≤ 0,01; *** p ≤ 0.001. N is the number of samples used in the experiments. The means are shown, and bars indicate SEM.

Table 1: Antibodies used in immunofluorescence and flow cytometry

Clone	Specificity	Fluorochrome	Supplier
UCHT1	CD3	PE-CF594, BV510	BD Bioscience, San Jose, CA USA
HIB19	CD19	PE-CF594	BD Bioscience, San Jose, CA USA
3G8	CD16	FITC	BD Bioscience, San Jose, CA USA
NCAM 16.2	CD56	BV421	BD Bioscience, San Jose, CA USA
CHL	KIR2DL2/S2/L3	FITC	BD Bioscience, San Jose, CA USA
H4A3	CD107a	FITC, PE	BD Bioscience, San Jose, CA USA
B27	IFN-γ	PE	BD Bioscience, San Jose, CA USA
L27	CD20	V450	BD Bioscience, San Jose, CA USA
L27	CD20	Unconjugated (IgG1)	BD Bioscience, San Jose, CA USA
N901	CD56	PE-Cy7	Beckman Coulter, Brea, CA USA
EB6B	KIR2DL1/S1 and KIR2DL3*005	PE, PE-Cy7	Beckman Coulter, Brea, CA USA
GL183	KIR2DL2/S2/L3	PE-Cy7	Beckman Coulter, Brea, CA USA
Z199	NKG2A	APC	Beckman Coulter, Brea, CA USA
IMMU510	TCR PAN γδ	FITC	Beckman Coulter, Brea, CA USA
BW264/56	CD3	VioBlue	Miltenyi Biotech, Bergisch Gladbach Germany
5B1	CD45	APC-Vio 770	Miltenyi Biotech, Bergisch Gladbach Germany
9E2	NKp46 (CD335)	PE	Miltenyi Biotech, Bergisch Gladbach Germany
DX9	KIR3DL1	FITC, PE-Vio770	Miltenyi Biotech, Bergisch Gladbach Germany
143211	KIR2DL1, 2DS5	PE	R&D systems, Minneapolis, MN USA
GB11	Granzyme B	Alexa Fluor 647	Biolegend, San Diego, CA USA
MOPC-21	IgG1 isotype control	Alexa Fluor 647	Biolegend, San Diego, CA USA
P30-15	NKp30	Alexa Fluor 647	Biolegend, San Diego, CA USA
δG9	Perforin	PE	Ancell, Stillwater, MN USA
Isotype Control	IgG2b	PE	Ancell, Stillwater, MN USA
SJ25C1	CD19	PE-Cy7	Thermo Fisher, Waltham MA, USA
W6/32	HLA class I	Unconjugated (IgG2a)	Our Laboratory
BU19	CD19	Unconjugated (IgG1)	Our Laboratory

3.2.3. Results

NKp46- and NKp30-NKCEs potentiate NK cell activation against BCP-ALL cell lines

Trifunctional NKp46/Fc/CD19 NKCE2 (hereafter referred to as CD19-NKp46-NKCE) and NKp46/Fc/CD20 NKCE2 (hereafter referred to as CD20-NKp46-NKCE), co-engaging NKp46 and CD16A activating receptors and targeting either CD19 or CD20, have been proved extremely efficient in enhancing NK cell killing of lymphoma cell lines [113]. New NKCEs containing the Fc portion and triggering NKp30 have been also produced targeting CD19 (i.e., CD19-NKp30-NKCE) or CD20 (i.e., CD20-NKp30-NKCE) with the same trifunctional antibody format. Control molecules were also generated by the replacement of the anti-TA (CD19 or CD20) with isotype control (IC-NKp46-NKCE, IC-NKp30-NKCE) and used in all functional assays.

We tested the *in vitro* effect of these NKCEs to potentiate NK cell activity against leukemia cells. We selected two pediatric BCP-ALL cell lines, MHH-CALL-4 and NALM-16, that were analyzed for the expression of the relevant target antigens. While high levels of CD19 expression were detected on the cell surface of both MHH-CALL-4 and NALM-16, only weak CD20 staining was observed (Fig. 1A).
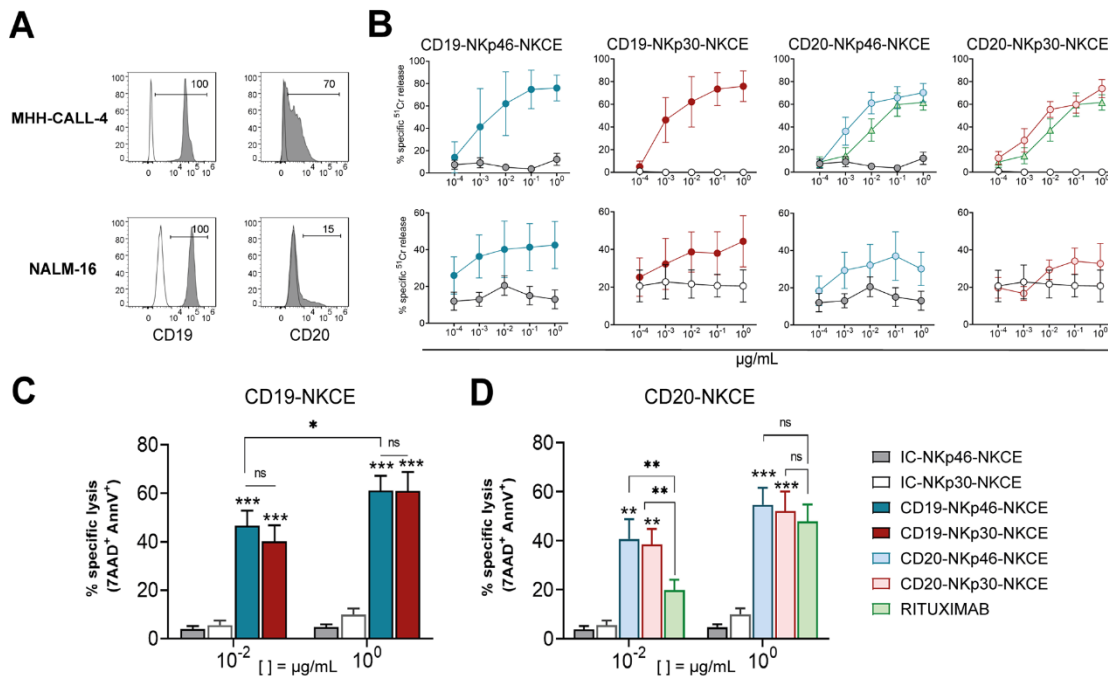

Figure 1. Effect of NKCEs targeting CD19 or CD20 on BCP-ALL cell lines. A) Two pediatric BCP-ALL cell lines were characterized for the surface expression of CD19 and CD20. Numbers represent the percentage of positive cells. **B)** Comparison of cytotoxicity (^{51}Cr-release

assay) of resting NK cells from healthy donors against MHH-CALL-4 (n= 2-6), NALM-16 (n= 3) in the presence of NKCEs at different concentrations, as indicated (see legend for color symbols in panel D). E:T ratio were 10:1 or 5:1 for MHH-CALL-4 and NALM-16, respectively. **C, D)** % of specific lysis (7AAD/AnnV staining) of MHH-CALL-4 co-cultured with resting NK cells from healthy donors (n= 4-9) in the presence of CD19-NKCEs or IC-NKCEs **(C)** or CD20-NKCEs or IC-NKCEs or rituximab **(D)** at 10^{-2} µg/mL and 10^{0} µg/mL. Results from 6-10 independent experiments are reported.

Then, we assessed the anti-leukemia efficacy of resting NK cells from healthy donors against the two BCP-ALL cell lines in the presence of different concentration of NKp46-NKCE or NKp30-NKCE, targeting either CD19 or CD20 (Fig. 1B). NK cell-mediated killing of both cell lines was induced using all NKCEs, starting from a concentration of 10^{-3} µg/ml and reaching the peak of activity at 10^{-1} or 10^{0} µg/ml. Moreover, the effect induced by NKCEs towards MHH-CALL-4 cells was particularly evident, given the resistance of this cell line to NK cell lysis. Conversely, NALM-16 cells display more susceptibility to NK-mediated killing. Consistent with the different expression levels of the target molecules, CD19-NKCEs appears to be better than CD20. In parallel to CD20-NKCEs, the effect of the anti-CD20 rituximab was also tested. In further experiments, we focused on MHH-CALL-4 target cells and we used a cytotoxicity assay based on 7AAD/AnnexinV staining and cytofluorimetric analysis that led to similar results of ^{51}Cr-release assay (Fig. S2). Thereafter, we selected two NKCE concentrations, namely 10^{0} µg/mL (i.e., optimal) or 10^{-2} µg/mL (i.e., just sub-optimal). Also, in this cytofluorimetric/cytotoxicity assay, NKCEs based on the engagement of either NKp46 or NKp30 appeared equally efficient (Fig. 1C, D). Targeting CD20, we found that the CD20-NKCEs at 10^{-2} µg/mL were significantly more efficient than rituximab to induce MHH-CALL-4 killing (41 ± 6,99 with CD20-NKp46-NKCE and 39 ± 6,77 with CD20-NKp30-NKCE vs. 20 ± 13,97 with rituximab, as mean % lysis ± SEM) (Fig. 1D). Moreover, to prove the specific effect of these NKCEs, we documented that they did not enhance the killing of cell lines lacking CD19 or CD20, namely K562 and A549 (Fig. S3). Finally, we investigated the effect of NKCEs on NK cell activation against MHH-CALL-4, analyzing CD107a degranulation and cytokine production by resting NK cell in the presence of NKCEs. Both NKp30- or NKp46-NKCEs equally displayed a strong capacity to induce CD107a degranulation (Fig. 2A, B) and IFN-γ expression (Fig. 2A, C). Moreover, targeting CD19 appeared more efficient than CD20 because the CD20-NKCEs showed a significantly reduced activity

at the lower concentration. Altogether, these data show that both CD19-NKp46-NKCE and CD19-NKp30-NKCE potentiate NK cell activity against BCP-ALL cell lines.
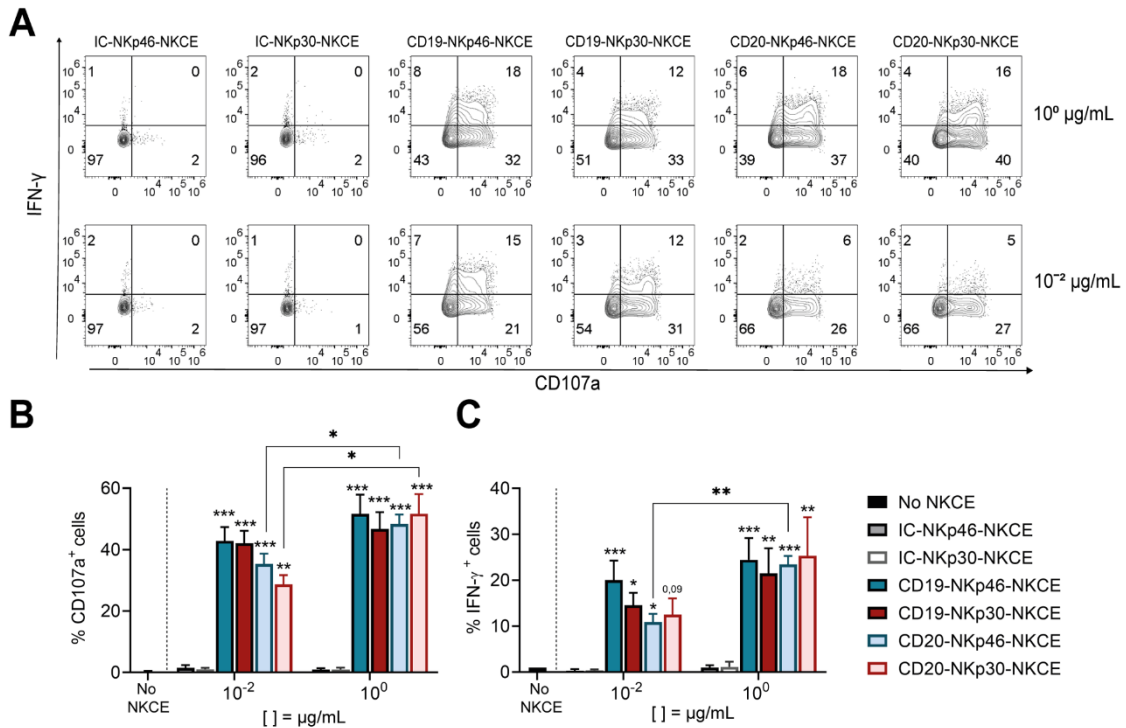

Figure 2. NKCEs enhance NK cell activity against MHH-CALL-4 cell line. CD107a expression and IFN-γ production by resting NK cells from healthy donors (*n*= 3-6) co-cultured with MHH-CALL-4 cell line in the absence or presence of CD19-NKCEs, CD20-NKCEs, or the control molecules (IC-NKp46-NKCE, IC-NKp30-NKCE) at 10^{-2} µg/mL and 10^0 µg/mL. **A)** Flow cytometry analysis of a representative experiment is shown. **B)** CD107a expression and (**C**) IFN-γ production were performed in 3-6 independent experiments.

NKCEs promote NK cell mediated lytic effect against primary BCP-ALL

Primary ALL blasts are known to be resistant to lysis by NK cells [34], particularly when NK cells are employed at resting state without any previous activation by cytokines (e.g. IL-2 or IL-15). Therefore, we investigated the effect of NKCEs against a panel of primary BCP-ALL obtained from pediatric patients at diagnosis. PBMC or BMMC were collected and samples containing ≥50% leukemia blasts were selected. By flow cytometry, we analyzed the expression of CD45, CD19, CD20 (Fig. 3A), CD3 (Fig S4) and HLA class I molecules (Fig. S5). BCP-ALL blasts could be identified as CD45^{dim} cells, which brightly expressed CD19, while CD20 was very low or almost negative (Fig. 3A). Conversely, healthy B lymphocytes in these samples showed the following phenotype CD45^{bright} CD19$^+$ CD20$^+$ cells. CD3$^+$ cells were all CD45^{bright} (Fig S4). In ALL#06, 96% BCP-ALL blasts displaying CD45^{dim} CD19$^+$ CD20$^-$ phenotype,

and only 1% healthy B cells were present. We tested in parallel the efficacy of CD19-NKp46-NKCE and CD20-NKp46-NKCE at the optimal concentration (10^0 µg/ml) to promote NK cell activity against this target cell. Remarkably, CD19-NKp46-NKCE was able to potentiate both NK cell cytotoxicity (Fig. 3B) and CD107a degranulation capacity (Fig. 3C) against ALL#06, while, according to its lack of CD20 expression, CD20-NKp46-NKCE did not exert any effect. Therefore, in further experiments with primary BCP-ALL, the use of NKCEs targeting CD19 at the optimal concentration was privileged. Resting NK cells from healthy donors were challenged against different BCP-ALL primary blasts, using CD19-NKCEs in cytotoxicity and degranulation assays (Fig. 3D).
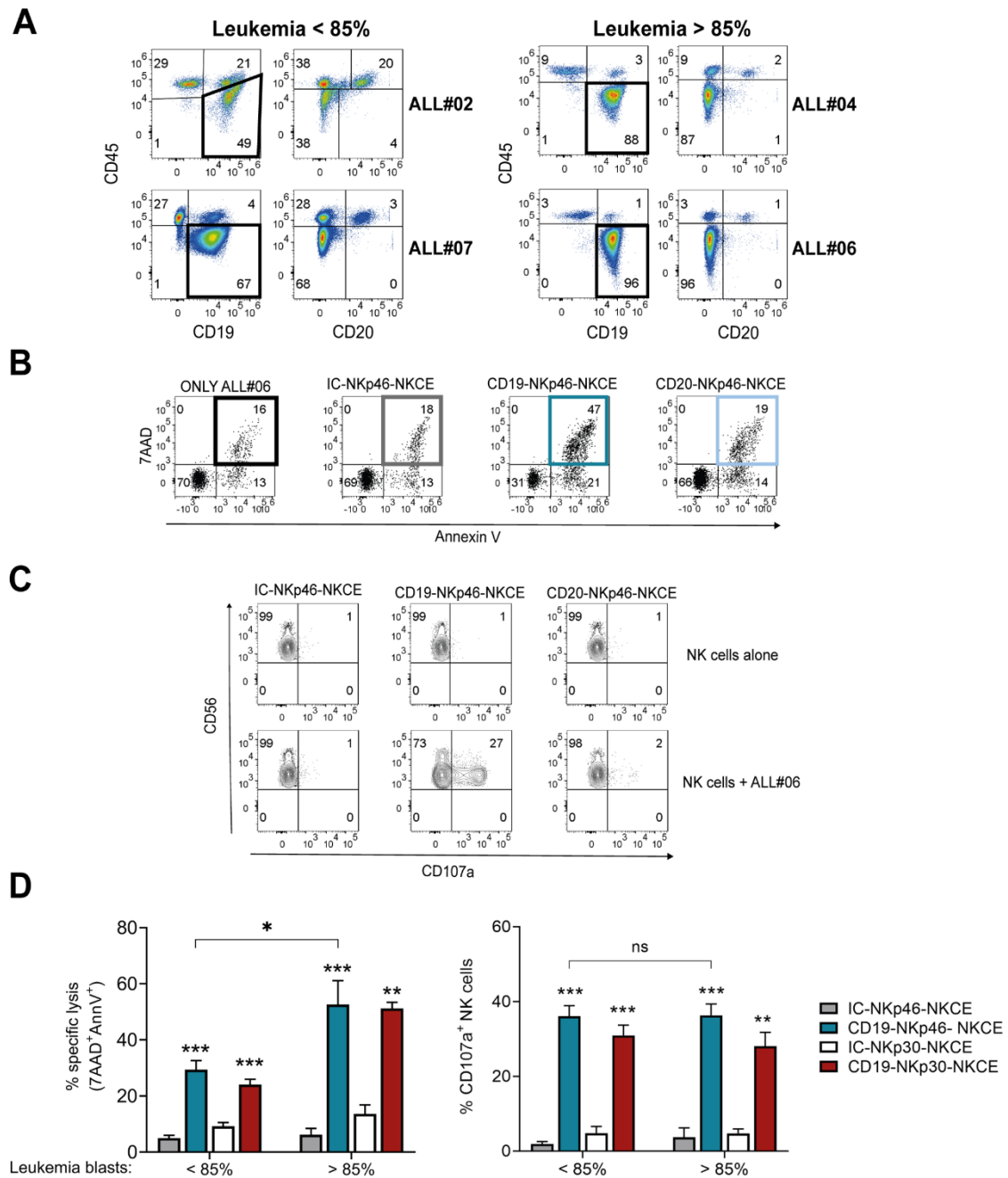
Figure 3. Effect of NKCEs targeting CD19 or CD20 on primary BCP-ALL. A) Phenotypic characterization of four BCP-ALL primary leukemia, evaluating CD45, CD19, and CD20 expression. **B)** 7AAD/AnnV staining of ALL#06 cells cultured either alone (only ALL#06) or with resting NK cells from a representative healthy donor and the indicated NKCEs (10^0µg/ml). **C)** CD107a expression of NK cells cultured either alone or with ALL#06 in the presence of IC-NKp46, CD19-NKp46 or CD20-NKp46-NKCE (10^0µg/ml). A representative experiment is shown. **D)** % of specific lysis of CD19^{+} leukemia blasts (left panel) and CD107a degranulation activity (right panel) of resting NK cells from healthy donors (*n*= 3-6) upon co-culture with primary leukemia blasts, induced by NKCEs at 10^0 µg/mL, as indicated. Data obtained with target cells containing leukemia blasts <85% (ALL#02 and ALL#07) or >85% (ALL#04 and ALL#06) are pooled.

Our data indicate that both CD19-NKp46-NKCE and CD19-NKp30-NKCE potentiate the NK cell-mediated anti-leukemia effect inducing leukemia cell killing. Moreover, a higher percentage of target cell lysis was obtained in samples with higher content of leukemia blasts. This difference was not observed in degranulation assays; the NKCEs induced efficient NK cell activation against target cells, containing either less or more than 85% primary leukemia.

These results provide evidence that CD19-NKCEs can engage resting NK cells, inducing the killing of primary BCP-ALL blasts.

CD19-NKp46-NKCE enhances anti-leukemia activity of NK cells after αβT-cell and B-cell depleted haplo-HSCT

We evaluated the possible effect of NKCEs to potentiate the anti-leukemia activity of NK cells derived from leukemia pediatric patients after αβT-cell and B-cell depleted haplo-HSCT. This transplantation setting is based on a graft manipulation strategy that allows the infusion of mature immune cells, mainly NK and γδT cells in addition to hematopoietic stem cells. Indeed, engrafted mature and functional NK cells persist in the peripheral blood of the recipient for at least one month, particularly when high numbers of NK cells have been infused within the graft [34,73]. At 1-3 months after transplantation, NK cells represent the most abundant lymphocyte subset, T (mainly γδT cells) cells are present, while B cells are almost absent (Fig. S6). By flow cytometry, we characterized NK cell phenotype in the patient reconstituted repertoire (a representative case is shown in Fig. S5B) and in healthy donors, evaluating surface staining of NKp46, NKp30, CD16 as well as the intracellular staining of perforin and granzyme B (Fig. 4A). Higher expression of NKp46 and NKp30 while lower expression of CD16 was observed in NK cells from transplanted patients in comparison with the donors. No significant differences were detected in the levels of perforin and granzyme B. Therefore, we challenged NK cells from these transplanted patients against MHH-CALL-4 leukemia cell line and primary BCP-ALL containing more than 85% of blasts, with the addition of NKCEs. When MHH-CALL-4 were used as target cells, CD19-NKp46-NKCE and CD19-NKp30-NKCE were equally able to enhance NK cell killing (Fig. 4B,C) and degranulation (Fig. 4D), consistent with the data obtained with healthy donor-derived NK cells (Fig. 1C and Fig. 2B).
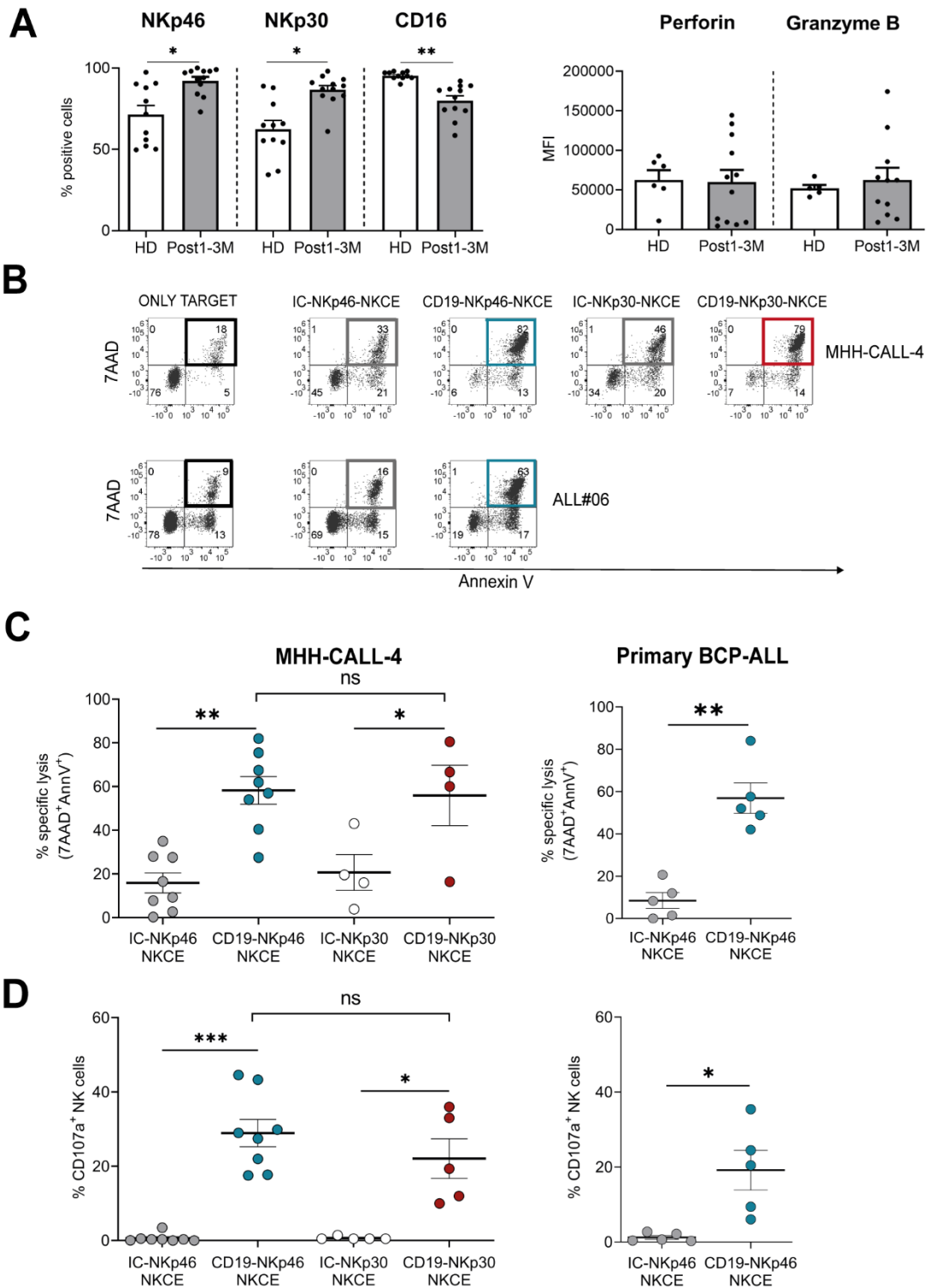
Figure 4. CD19-NKCEs efficiently potentiate NK cell anti-leukemia activity in a transplantation setting. A) Evaluation of NKp46, NKp30, CD16, Perforin, and Granzyme B expression of NK cells from transplanted patients and healthy donors (HD). **B)** Representative experiment of cytotoxicity (7AAD/AnnV staining). MHH-CALL-4 cell line or ALL#06 primary leukemia target cells were cultured either alone (only target) or with resting NK cells from a transplanted patient (3 months after haplo-HSCT) and the indicated NKCEs (10^0μg/ml). **C)** % of specific lysis (7AAD/AnnV staining) and **(D)** CD107a degranulation assay of resting NK cells from transplanted patients against MHH-CALL-4 (left panels) or primary leukemia blasts

(ALL#04 and ALL#06) (right panels) in the presence of the indicated NKCE at 10^0 µg/mL. Pooled data obtained with primary leukemia blasts are shown.

We focused on CD19-NKp46-NKCE to be tested in assays using primary BCP-ALL as target cells, due to limited cell availability from patient samples. Remarkably, CD19-NKp46-NKCE induced a potent enhancement of NK cell killing of BCP-ALL blasts (Fig. 4B-D), at levels comparable to those obtained with healthy NK cells (Fig. 3D). These data support that NK cells of αβT-cell and B-cell depleted haplo-HSCT recipients are equipped with an adequate pattern of triggering receptors and lytic machinery. Through NKCE engagement, these patient NK cells can efficiently kill BCP-ALL, both cell lines and primary blasts.

The effect of NKCE can override the HLA-specific inhibitory interactions

We investigated if the activating effect of NKCEs might be affected by HLA-specific inhibitory interactions between KIR or NKG2A on effector NK cells and their cognate HLA class I ligands on BCP-ALL target cells. As shown in Fig. S4, MHH-CALL-4 cell line and primary leukemia blasts (gated as CD45^{dim} cells) express high HLA class I levels, which are, in most cases, similar to the healthy counterpart (gated as CD45^{bright} cells). The analysis of KIR-L in MHH-CALL-4 cell line indicated the presence of HLA-C alleles with only C1 epitope, and Bw4 epitope carried by HLA-B (T^{80}) and HLA-A alleles. Then, to obtain NK cells, we selected donors characterized by an *HLA* class I typing coding for all KIR-L (i.e., C1, C2, and Bw4) and a *KIR* gene repertoire indicating the presence of an A/A genotype. In these individuals, KIR2DL1, KIR2DL3, and KIR3DL1 were educated, and functional tests were not interfered with the presence of the activating counterparts (namely KIR2DS1, KIR2DS2, and KIR3DS1), potentially contributing to triggering pathways. In addition, NK cells from these donors, having a C2 HLA mismatch versus MHH-CALL-4, could contain the alloreactive subset (i.e., Allo C2) [73]. NK cells from three healthy donors (Fig. 5A,B) and one patient who received αβT-cell and B-cell depleted haplo-HSCT from an Allo C2 donor (Fig. 5C,D) were selected. We could evaluate the NKCE-induced degranulation capacity of different NK cell subsets, either expressing only KIR2DL3 and/or KIR3DL1 (i.e., KIR2DL3/KIR3DL1$^+$) recognizing C1 and Bw4, expressing only CD94/NKG2A (i.e., KIR$^-$NKG2A$^+$) recognizing HLA-E, or expressing only KIR2DL1 (i.e., sKIR2DL1$^+$, Allo C2 subset) recognizing no HLA molecules on MHH-CALL-4. These three NK cell

subsets were well represented in all individuals evaluated, including the donor-derived alloreactive subset in the reconstituted repertoire of the transplanted patient. We demonstrated that CD19-NKp46-NKCE could promote the degranulation of all NK cell subsets that would be otherwise unable to exert any activity against MHH-CALL-4 (Fig. 5).
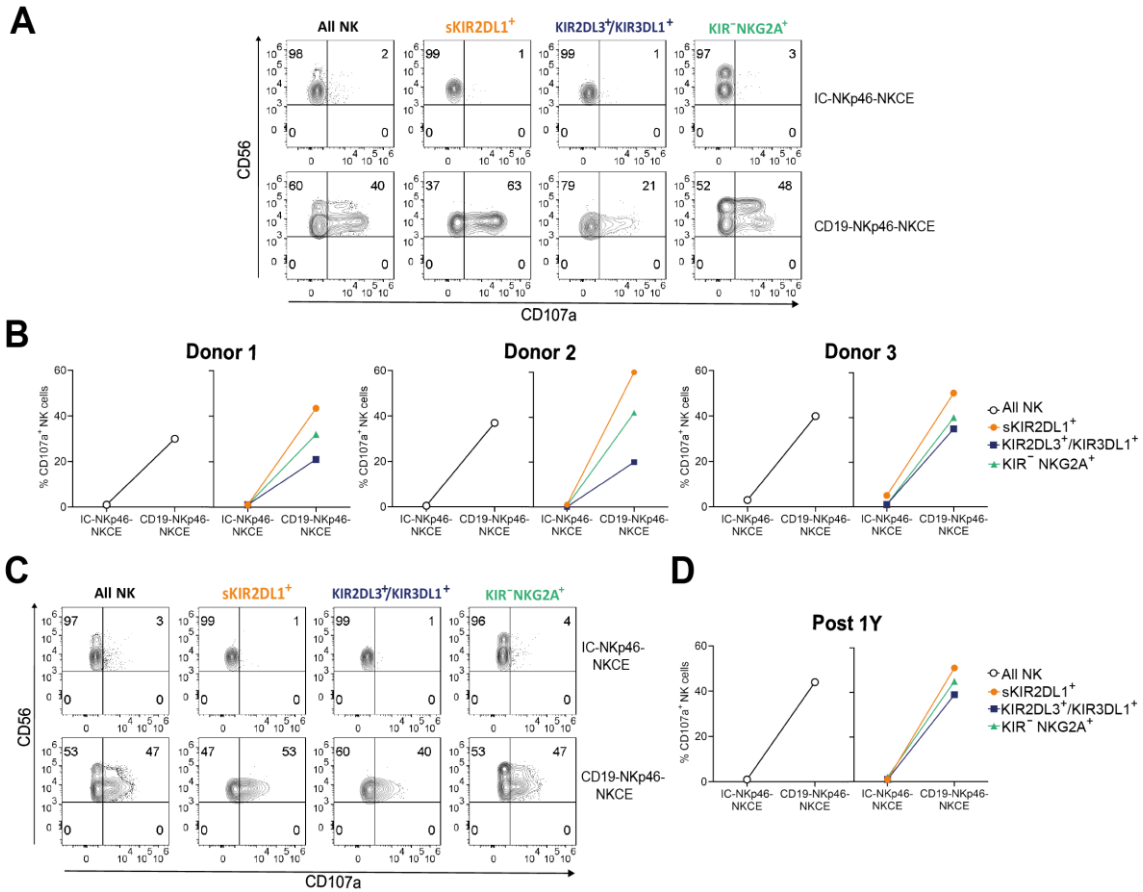
Figure 5. NKCEs override HLA-I inhibitory interactions. Degranulation activity of NK cells (All NK) and different NK cell subsets (sKIR2DL1$^+$, KIR2DL3$^+$/KIR3DL1$^+$, and KIR$^-$NKG2A$^+$) from healthy donors or transplanted patient induced by MHH-CALL-4 and IC-NKp46- or CD19-NKp46-NKCE at 10^0 µg/mL. **A)** Contour plot of a representative donor showing the degranulation activity of the different NK cell subsets in the presence of the target cell and NKCEs, as indicated. **B)** Data from three healthy donors are reported. **C-D)** Degranulation capacity of NK cell subsets from one patient at 1-year after haplo-HSCT. **C)** Contour plot of flow cytometry (raw data). **D)** Graphical representation.

These data indicate that the activation induced by the NKCE against BCP-ALL can override the inhibitory signal(s) derived by the interaction(s) between iKIR/KIR-L and/or NKG2A/HLA-E. In all cases, there was an efficiency ranking among the subsets. The sKIR2DL1$^+$ cells (with no inhibitory HLA specific interactions) showed the highest activity, followed by KIR$^-$NKG2A$^+$, and by KIR2DL3/KIR3DL1$^+$ cells, suggesting residual inhibition, lower by NKG2A than by the two KIRs recognizing HLA molecules on target cells.

3.2.4. Discussion

NK cells are becoming more and more attractive in immunotherapy approaches, particularly in the context of hematological malignancies [115]. Several strategies have been exploited to enhance their efficacy, often translating to NK cells the experience obtained on T cells. In addition to cell engineering through CAR constructs producing CAR-NK cells [118,129], different engagers have been developed to specifically "arm" NK cells and to selectively redirect them towards tumor cells. Here, we provide evidence that NKCEs, especially CD19-NKp46-NKCE, can engage resting NK cells derived from healthy controls and transplanted patients. These molecules promote the lysis of BCP-ALL, which otherwise would be resistant. Indeed, primary pediatric BCP-ALL blasts are characterized by the expression of CD19, while CD20 is virtually negative, leading to lower interest on CD20-NKCEs as well as possible therapies with anti-CD20 mAb like rituximab. Considering the known surface NK cell receptor ligands, BCP-ALL display in general high levels of HLA class I molecules (as also shown in Fig. S4), presence of the DNAM-1 ligand Nectin-2, and absence of all NKG2D ligands by phenotypic analysis. In addition, functional data suggest that BCP-ALL also express NKp46 ligands, although their molecular identification has still not been achieved [73,122]. Although freshly isolated NK cells are equipped with lytic granules and can lyse tumor cells without any prior tumor-specific sensitization, priming with pro-inflammatory cytokines (e.g., IL-2 or IL-15) upregulates NK cell activity. Besides the prototypic NK cell susceptible leukemia cell line K562 (HLA class I negative), here we describe that resting NK cells can display some killing of the NALM-16 BCP-ALL cell line, carrying a hemizygous HLA haplotype and expressing many surface activating receptor ligands (i.e., PVR, Nectin-2, MICA and ULBP1-3) [73] . In contrast, MHH-CALL-4 cell line resembled more the primary BCP-ALL blasts, both in terms of phenotypic features and resistance to lysis by resting NK cells. Indeed, we previously showed that primary leukemia blasts could be killed only by cytokine-activated NK cells, particularly in case of alloreactive NK cells that are not inhibited by HLA class I molecules on target cells [34,73]. In T-cell depleted haplo-HSCT, the KIR/KIR-L mismatch in GvH direction has been associated to a reduced risk of disease recurrence in patients with acute leukemia thanks to an alloreactive NK cell-mediated GvL effect, both in adult and pediatric patients [69,130]. Thus, we consider NK alloreactivity of first priority in donor selection criteria in the context of αβT-cell

and B-cell depleted haplo-HSCT to cure pediatric patients with high-risk leukemia, including BCP-ALL. This transplantation platform allows the engraftment, together with HSC, of mature NK and γδT cells that persist for some time in the patient circulation as immunocompetent cells [73,74]. The αβT-cell and B-cell depleted haplo-HSCT represents an important therapeutic option for patients with BCP-ALL, that is the most common pediatric leukemia. We tested the *in vitro* effect of NKCEs on NK cells derived from this cohort of donors and post-transplant patients. The ability of the NKCEs to ligate both CD16A and NKp46 is relevant in this clinical context, because NK cells in the reconstituted repertoire, containing NK cells at different stages of maturation, can present higher proportions of CD56^{bright} CD16[dim/neg] cells compared to healthy PB NK cells. However, both CD56^{bright} and CD56^{dim} NK cells usually express high levels of NKp46 (Fig. 4A). It is also worth mentioning that an additional donor selection criterion accounts on NCR[bright] phenotype of NK cells, possibly related to high anti-leukemia activity [73]. Notably, we documented that CD19-NKp46-NKCE was efficient in triggering the lysis of primary BCP-ALL blasts even by resting NK cells derived from transplanted patients. These experiments are challenging because few PBMCs are obtained from small blood samples in children. In degranulation assays, we could circumvent the problem of NK cell purification using total PBMC from patients at early time points after transplant, characterized by a predominance of NK cells and absence of B cells. We also provided evidence that the effect of NKCEs can override the inhibition delivered by iKIR and/or NKG2A upon interaction with their ligands, even though the alloreactive subset always exerted the highest activity (Fig. 5).

NKp30-NKCEs appeared to be as efficient as NKp46-NKCEs, consistent with their equal expression and function on NK cells [15]. Recently, bispecific immunoligands via NKp30 engagement were more efficient against EGFR-overexpressing tumor cells than the clinically approved cetuximab [110]. NKp30 expression has been also described on γδ T and CD8^{+} T cells, upon culture in IL-15 [17,18]. Considering that γδ T cells are well represented in αβT-cell and B-cell depleted haplo-HSCT, also these lymphocytes might benefit from NKp30-NKCEs. We performed preliminary experiments using samples from transplanted patients, and sometimes we could detect low NKp30 expression and little effect of CD19-NKp30-NKCE in killing MHH-CALL-4 cell line (Fig S7). This finding deserves further investigation.

The 161519 TriKE was shown to induce NK cell proliferation, to be more effective than rituximab in inducing the *in vitro* killing of Raji lymphoma cell line and primary CLL targets by healthy NK cells, and to restore functionality of NK cells from CLL patients [104]. Moreover, in xenograft model involving human PBMC and Namalwa lymphoma cells, treatment with 161519 TriKE induced more sustained lasting antitumor activity compared to 1619 BiKE, whose activity could be enhanced by combined use of IL-2 [131].

NKCEs targeting CD19 or CD20 were demonstrated to be more potent *in vitro* than rituximab against Daudi lymphoma cell line, as well as we found in our present study using BCP-ALL cell lines. In mouse models, NKCE targeting CD20 controlled the growth of Raji lymphoma cell line, showing *in vivo* pharmacokinetics similar to obinutuzumab, and no off-target effects [132]. Our *in vitro* data are encouraging to envisage the use of CD19-NKp46-NKCE in clinical practice to fight BCP-ALL in children with relapsed/refractory BCP-ALL or with disease relapsing after allogeneic HSCT. In case of relapse after haplo-HSCT, to further increment the activity of NK cells already present in the patient reconstituted repertoire, new NK cells can be infused, derived from the same haploidentical donor of the graft, possibly also benefiting from NK alloreactivity. NKCE can be used together with adoptive NK cell transfer, only requiring purification procedures without any cell manipulation. A more effective approach might require the infusion of cytokine activated NK cells *ex vivo* precomplexed with the engager, as recently proposed for AFM13 and IL12/15/18 pre-activated NK cells to treat CD30^{+} malignancies [108]. The approach based on CD19-NKp46-NKCE and NK cells would be characterized by safety and feasibility, and might complement other existing treatments for BCP-ALL as blinatumomab that act on T cells [133-137].

3.2.5. Supplementary information
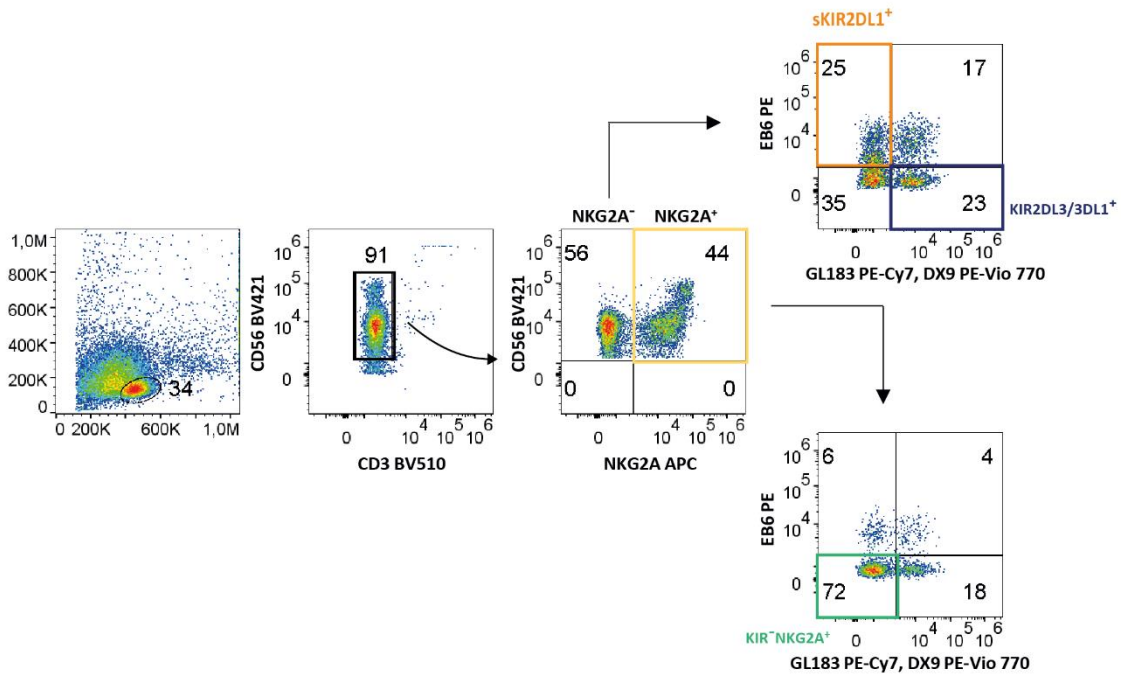
Figure S1. Gating strategy to define NK cell subsets in degranulation assay. Purified NK cells from donors characterized by an A/A *KIR* genotype were stained with the following mAb combination to identify sKIR2DL1[+], KIR2DL3[+]/KIR3DL1[+], and KIR[-]NKG2A^{+} NK cell subsets: CD3-BV510, CD56-BV421, NKG2A-APC, EB6- or 143211-PE, GL183-Pc7, DX9-PE-Vio-770 and CD107a-FITC. In some experiments, the mAb combination was modified using CD107a-PE, and, consequently, EB6-Pc7, CHL- and DX9-FITC were used.
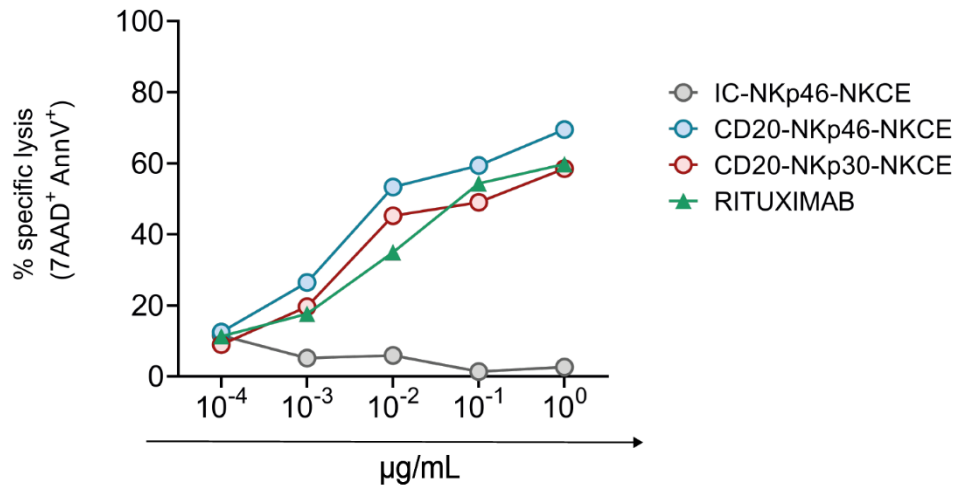
Figure S2. Cytotoxicity assay based on **7**AAD/Annexin V staining presents similar results to ^{51}Cr-release assay (reported in Fig. 1B). Resting NK cells from a representative healthy donor were tested against MHH-CALL-4 using different concentrations of CD20-NKCEs or rituximab. E:T ratio 10:1.
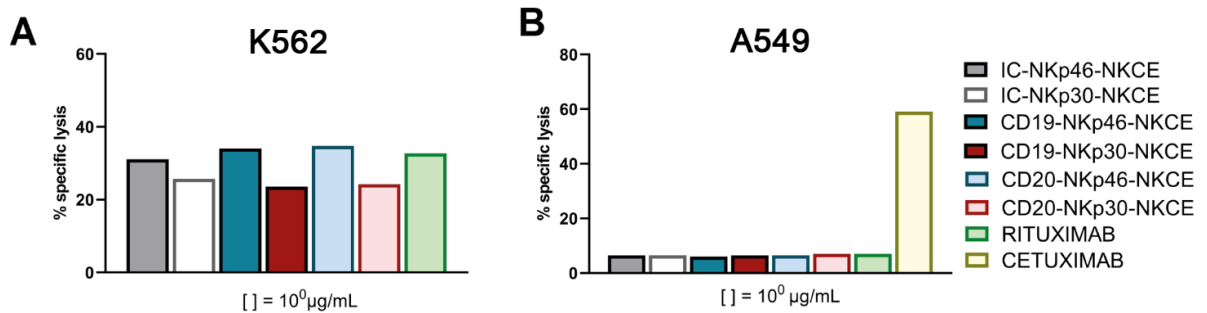
Figure S3. CD19- and CD20-NKCEs do not enhance the killing of CD19$^-$ and CD20$^-$ cell lines, K562 **(A)** and A549 **(B)**. E:T ratio 10:1 (K562) and 5:1 (A549). Results from a representative donor are shown.
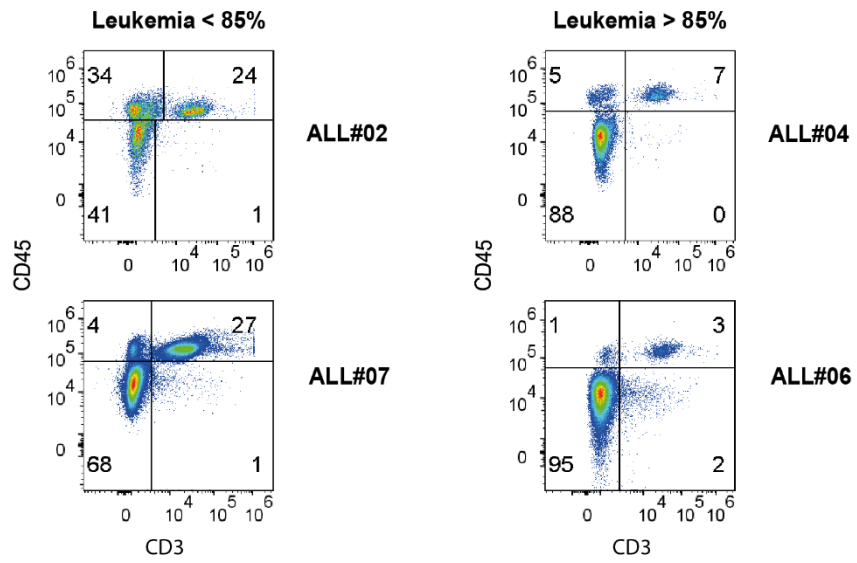
Figure S4. Analysis of CD45 and CD3 expression on samples obtained from four leukemia patients at disease onset. CD3^{+} cells, which are all CD45^{bright}, represent the healthy counterpart. Numbers indicate the percentage of cells in each quadrant.
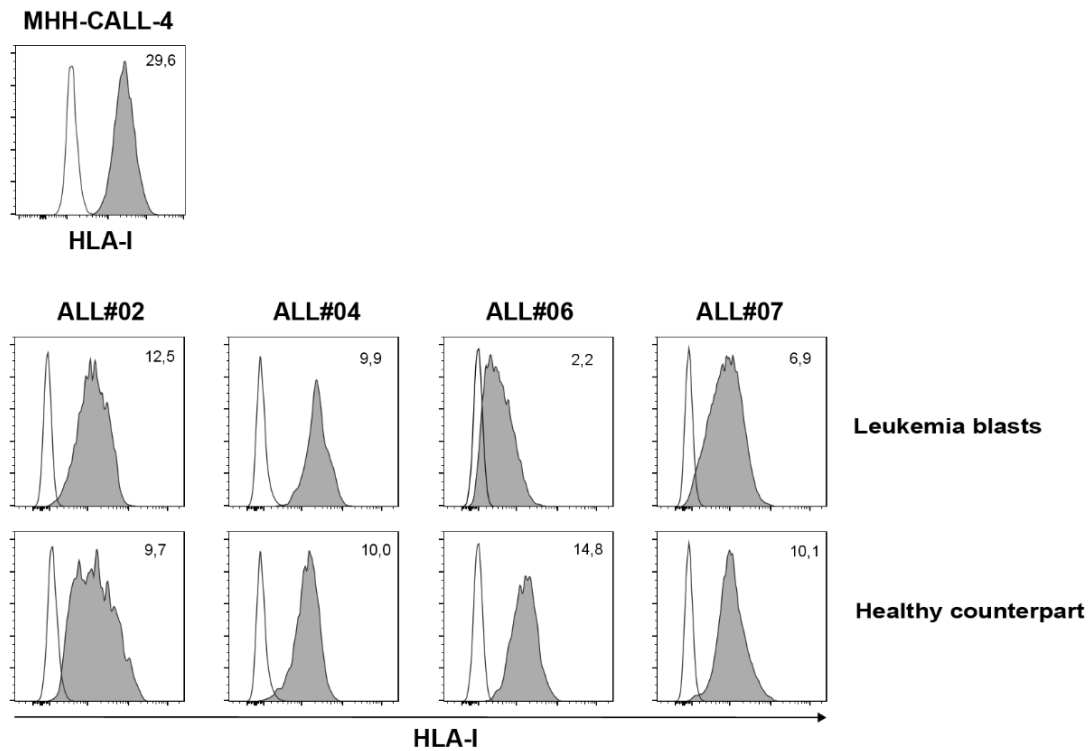
Figure S5. Analysis of HLA class I (HLA-I) expression on MHH-CALL-4 cell line and samples obtained from four leukemia patients at disease onset. Immunofluorescence was performed using W6/32 mAb in combination with anti-IgG2a FITC secondary reagent (gray histograms). Negative controls with the secondary reagent were also performed (white histograms). The expression of HLA-I on leukemia blasts (CD45^{dim}) is compared to the healthy counterpart (CD45^{bright}) by an appropriate gating strategy. Staining index (SI) is indicated.
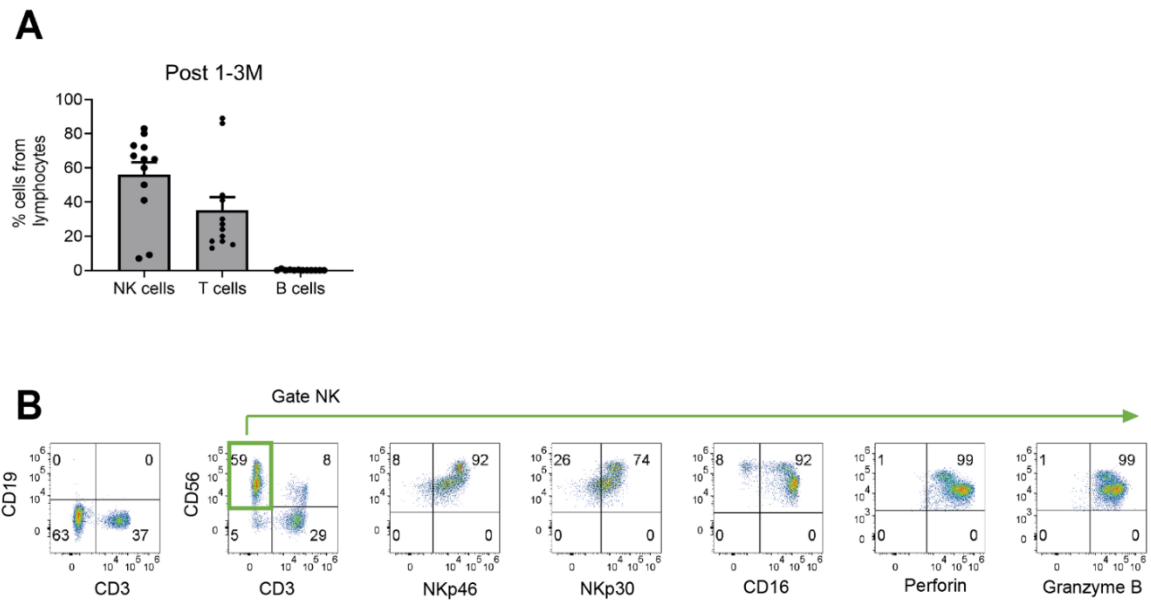
Figure S6. Immunological reconstitution in patients at early time points after αβT-cell and B-cell depleted haplo-HSCT. A) % of NK cells (CD56$^+$CD3$^-$), T cells (CD3$^+$ CD56$^-$), and B cells (CD19$^+$CD3$^-$) in patients at 1 or 3 months after haplo-HSCT (Post 1-3M). **B)** Representative flow cytometry analysis of PBMC of a patient (post-3M): identification of different lymphocyte subsets, and characterization of the expression of NKp46, NKp30, CD16, Perforin and Granzyme B on NK cells (gating on CD56$^+$CD3$^-$).
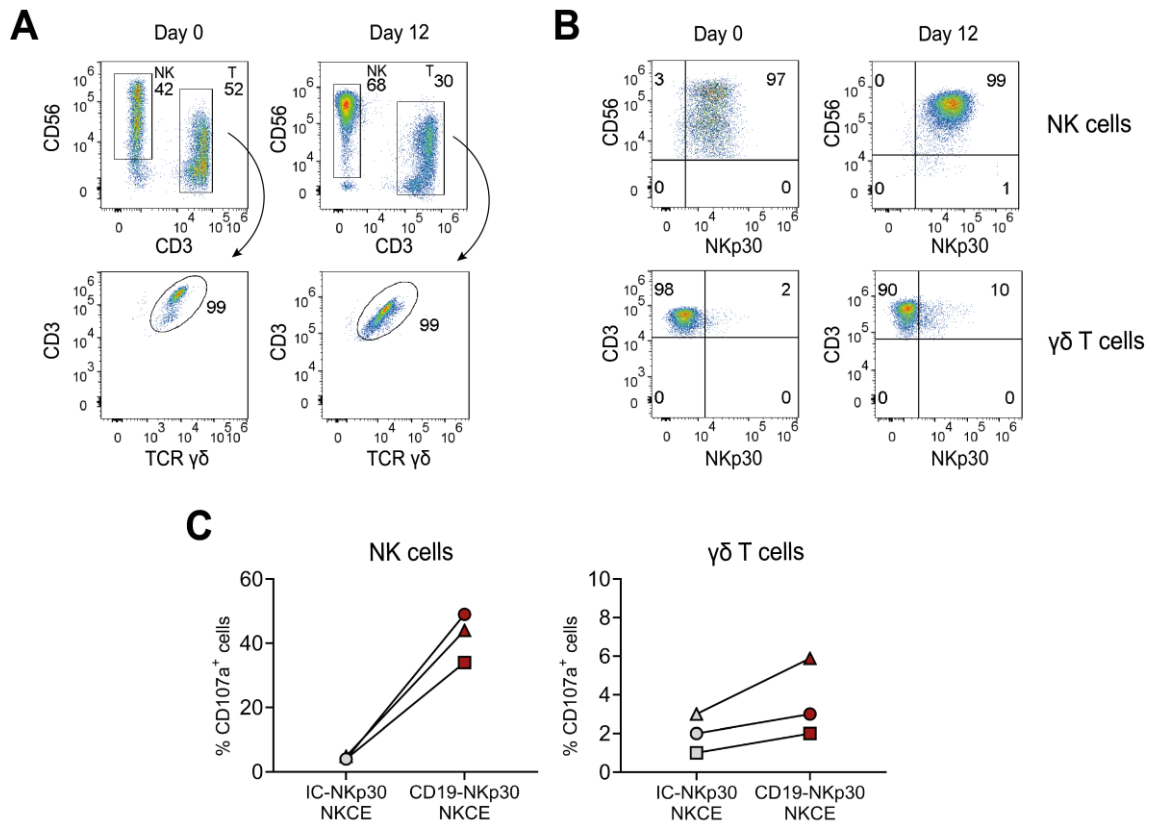
Figure S7. Effect of CD19-NKp30-NKCE on γδ T cells. A-B) Flow cytometry analyses performed on PBMC either freshly isolated (day 0) or cultured 12 days with IL-15 at 10 ng/mL (day 12) in a representative patient at 1 month after haplo-HSCT. **A)** % of NK cells (CD56$^+$ CD3$^-$) and T cells (CD3$^+$) of lymphocytes at day 0 and day 12. γδ T cells were identified gating on CD3$^+$ cells and represented the totality of the CD3$^+$ population (lower panels). **B)** Analysis of NKp30 expression on NK cells (CD56$^+$ CD3$^-$) and γδ T cells (CD3$^+$) at day 0 and day 12. **C)** CD107a degranulation assay of IL-15 cultured (day 12) NK cells or γδ T cells from three transplanted patients against MHH-CALL-4 in the presence of the indicated NKCEs at 10^0 µg/mL. NK cells were identified gating on CD56$^+$CD3$^-$; while γδ T cells gating on CD3$^+$ TCR γδ$^+$ cells. The triangle corresponds to the representative patient shown in panels A and B.

4. Haplo-HSCT with PT-Cy in adult AML patients

4.1. Background

AML is characterized by a rapid expansion of myeloid cells in the bone marrow and peripheral blood and represents about 25% of childhood leukemias. However, its incidence increases with age, and it is the most common acute leukemia in adults with a median age of onset at around 68 years old [138]. Although new pharmacological treatments are applied, the outcome is still poor, and most of the patients relapse within the first three years [139-141].

Allogeneic HSCT represents a valid therapeutic option for patients with high-risk or chemorefractory AML [142]. In the past, the applicability of allo-HSCT has been limited, as only 30%-35% of patients have HLA-matched siblings and outcomes using other donor types have been influenced by high toxicity, graft failure, graft-versus-host disease (GvHD), and consequently non-relapse mortality. Improvements in HLA typing, GvHD prophylaxis, and the use of other transplantation settings, also with haploidentical donors, have addressed these historical challenges [143].

In the last years, it has been developed an haplo-HSCT approach with minimal requirements in terms of graft processing and specific expertise from the transplant team. This transplantation setting is based on the infusion of unmanipulated grafts and the post-transplant administration of high doses of cyclophosphamide (PT-Cy), as part of GvHD prophylaxis [75]. The PT-Cy strategy leads to the elimination of alloreactive T cells undergoing rapid proliferation in response to patient's alloantigens in this transplantation setting [75,144]. ALDH is a known enzyme that confers resistance to Cy and CD34$^+$ stem cells present high levels of this enzyme, meaning that are resistant to Cy lysis [77]. The effect of PT-Cy on NK cells and NK cell alloreactivity has been partially investigated. Russo et al. performed the characterization of NK cell phenotype after PT-Cy haplo-HSCT [79]. They observed that immediately after HSCT, active proliferation of donor-derived NK cells that do not express ALDH and can be killed by Cy. Moreover, Cy-induced elimination of mature, cytolytic NK cells (including the alloreactive NK cell population) would decrease the NK cell-mediated anti-leukemic effect. Indeed, no significant impact of putative NK alloreactivity on progression-free survival was observed in this cohort of patients [79].

We analyzed the NK cell repertoire in AML patients who received haplo-HSCT and PT-Cy in our hospital. The first characteristic to highlight was the high expression of CD94/NKG2A, virtually present on all NK cells after transplantation. Thus, we

performed functional assays to evaluate whether blocking the inhibitory interaction between CD94/NKG2A and HLA-E could enhance transplanted NK cell activity against target cells. Our data suggested that inhibitory checkpoint blockade could increase the anti-leukemia activity of NK cells at early time points after transplantation. CD94/NKG2A is involved in NK cell education and regulates the NK cell function [64]. It has been reported that NKG2A education is influenced by the M/T dimorphism in -21 residue of HLA-B leader sequences. Indeed, the presence of -21M correlated with a higher expression of HLA-E and with functionally more efficient CD94/NKG2A^{+} NK cells [48]. Hallner and colleagues studied the impact of this dimorphism on NK cell education and NK cell-mediated anti-leukemia activity in AML patients. They reported that -21 M/x individuals harbor a better-educated NKG2A^{+} NK cell subset with superior degranulation capacity against AML. In the same study, they also correlated -21 M/x AML patients receiving histamine dihydrochloride and low-dose of IL-2 with a better clinical outcome (in terms of leukemia-free survival and overall survival) in comparison with T/T patients [145].

Taking advantage of these findings, we found it worth investigating the impact of -21 HLA-B dimorphism on leukemia HLA-E expression and NKG2A^{+} NK cell education in our group of AML patients receiving haplo-HSCT with PT-Cy.

4.2. Materials and methods

Donors, leukemia patients, cell separation and culture

Peripheral blood (PB) samples from donors and adult patients after haplo-HSCT and PT-Cy were obtained at IRCCS Ospedale Policlinico San Martino (Genoa, Italy), and this study was approved by CER Liguria Ethical Committee (415REG2015). All donors for haplo-HSCT were typed for HLA class I and analyzed for KIR-L expression and KIR genotype, as previously described [73]. Cells were cryopreserved in FBS containing 10% DMSO.

Primary AML blasts were derived from PB or bone marrow (BM) of adult patients at diagnosis. Written informed consents were provided according to the Declaration of Helsinki. PB or BM mononuclear cells (PBMC, BMMC) were isolated by density-gradient centrifugation, phenotypically characterized by immunofluorescence and cryopreserved.

Buffy coats from healthy donors were provided by the blood transfusion center of IRCCS Ospedale Policlinico San Martino (Genoa, Italy), following approved internal operational procedures (IOH78). NK cells were isolated using the RosetteSep method (StemCell Technologies, Vancouver, BC, Canada). Purified NK cells were incubated with saturating amounts of a mixture of mAb, including 11PB6 (anti-KIR2DL1/S1, IgG1), GL-183 (anti-KIR2DL2/L3/S2, IgG1), AZ158 (anti-KIR3DL1/S1/L2, IgG2a), F278 (anti-LIR-1, IgG1), followed by incubation with anti-IgG-PE second reagent. Then, cells were sorted by negative selection using FACSAria II (BD Biosciences), to deplete NK cells expressing KIR and/or LIR-1. The selected cells were highly enriched in NK cells expressing only CD94/NKG2A as HLA-specific receptor and cultured on irradiated feeder cells in the presence of 1.5 ng/mL phytohemagglutinin (Life Technologies, Paisley, Scotland) and IL-2 to obtain proliferation of KIR^- $NKG2A^+$ NK cells.

Cell lines

K562, K562-HLA-E^G (kindly provided by Dr. E.H. Weiss, Ludwig-Maximilians-Universität, Munich, Germany), P815 and MOLM-14 cell lines were certified by STR analysis performed by ICLC Italian cell line collection (ICLC, www.iclc.it) in accordance with published profiles. The cell lines were cultured in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% FBS (Euroclone, Milan, Italy), 2mM L-

glutamine (Lonza) and 100 U/mL penicillin-streptomycin (Lonza) in a 5% CO_2 incubator at 37ºC.

K562-HLA-E^G cells were obtained upon transfection with HLA-E*01033 as previously reported [146]. Cells were maintained in RPMI-1640 complete medium and G-418 selection (Roche, Basel, Switzerland). Synthetic leader sequence peptides from HLA-B (VMAPRTVLL and VTAPRTVLL) were purchased from Biosyntan (Berlin, Germany). As described, to stabilize HLA-E expression, 10^6/mL cells were incubated with 100 µM of peptide overnight at 37ºC, 5% CO_2 with serum-free RPMI-1640. After the incubation, HLA-E expression was detected by indirect immunofluorescence using 3D12 mAb and PE-conjugated anti-IgM.

Antibodies, immunofluorescence and cytofluorimetric analysis

All antibodies used in this study are detailed in Table 1. The surface phenotype of NK cells from healthy donors and patients after HSCT was analyzed on freshly isolated PBMC by multi-parametric flow cytometry. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm kit, washed twice with the Perm/Wash Buffer (both from BD Biosciences, San José, CA) followed by staining with anti-perforin, anti-granzyme B and anti-SAP or the corresponding isotype control mAbs. Primary AML samples were analyzed by multi-parametric flow cytometry using fluorochrome-conjugated mAb. CD48 expression was evaluated by indirect immunofluorescence using CD48 mAb and FITC-conjugated anti-IgM second reagent (Southern Biotech). MFI values were calculated by subtracting the value of the isotype or unstained control.

Samples were acquired using Gallios (Beckman Coulter, Brea, CA, USA) or MACSQuant-analyzer (Miltenyi-Biotech, Bergisch Gladbach, Germany) and analyzed with FlowJo Version 10.7 (BD Biosciences).

Degranulation assay and IFN-γ production

PBMC from donors or transplanted patients were incubated overnight with IL-2 (600U/mL). Then, PBMC were cultured with the target cells at effector: target (E: T) 2:1 for 2 hours either in the presence or absence of Y9 mAb (anti-CD94, IgM). In the absence of Y9 an IgM isotype control (anti-CD1a) was used. Golgi Stop (BD Biosciences) was added after the first hour of incubation. Thereafter, cells were washed and stained with anti-CD3, -CD56 and -CD107a mAbs. Data referred to % CD107a$^+$

cells represent the difference between the % of CD107a$^+$ NK cells co-cultured with target cells and the % of CD107a$^+$ NK cells cultured with medium alone. To detect intracellular production of IFN-γ, PBMC were overnight cultured either with medium alone or with IL-12 (2 ng/mL) and IL-18 (20 ng/mL). Then, Golgi Plug (BD Bioscience) was added for 5 hours. After the surface staining with anti-CD3 and anti-CD56, cells were fixed and permeabilized with Cytofix/Cytoperm, washed twice with the Perm/Wash Buffer (both from BD Biosciences) and stained with anti-IFN-γ mAb.

In Reverse Antibody-dependent cellular cytotoxicity (R-ADCC) assays, thawed PBMC were cultured overnight in the presence of IL-2 and then incubated with the FcγR$^+$ p815 cell line either with the presence of PP35 (anti-2B4, IgG1) and/or BAB281 mAb (anti-NKp46, IgG1) as previously described [37]. Samples were analyzed using Gallios (Beckman Coulter, Brea, CA, USA) or MACSQuant-analyzer (Miltenyi-Biotech, Bergisch Gladbach, Germany).

Cytotoxicity assay

Polyclonal activated KIR$^-$ NKG2A$^+$ NK cells were tested for cytolytic activity in a 4-hour ^{51}Cr-release assay to analyze the susceptibility to lysis of primary AML at E:T 10:1, as previously described [122]. For masking experiments, Y9 mAb (anti-CD94, IgM) at a concentration of 10 μg/mL was added in the assay.

Statistical analysis

Unpaired t-test was used to compare two groups, either for normally distributed variables or Mann-Whitney tests with non-normally distributed variables. Significance is indicated as: *p ≤ 0,05; ** p ≤ 0,01; *** p ≤ 0.001, **** p ≤ 0,0001. N is the number of samples used in the experiments. The means are shown, and bars indicate SEM. Graphic representation and statistical analysis were performed with GraphPad Prism software version 9 (GraphPad Software, La Jolla, CA).

Table 1. Antibodies used in immunofluorescence and flow cytometry

Clone	Specificity	Fluorochrome	Supplier
UCHT1	CD3	PE-CF594, BV510	BD Bioscience, San José, CA USA
3G8	CD16	FITC	BD Bioscience, San José, CA USA
NCAM 16.2	CD56	BV421	BD Bioscience, San José, CA USA
H4A3	CD107a	PE	BD Bioscience, San José, CA USA
B27	IFN-γ	PE	BD Bioscience, San José, CA USA
TU145	CD48	IgM	BD Bioscience, San José, CA USA
N901	CD56	PE-Cy7	Beckman Coulter, Brea, CA USA
EB6B	KIR2DL1/S1 and KIR2DL3*005	APC	Beckman Coulter, Brea, CA USA
GL-183	KIR2DL2/S2/L3	APC	Beckman Coulter, Brea, CA USA
Z27	KIR3DL1/S1	APC	Beckman Coulter, Brea, CA USA
Z199	NKG2A	APC	Beckman Coulter, Brea, CA USA
BW264/56	CD3	VioBlue	Miltenyi Biotech, Bergisch Gladbach Germany
5B1	CD45	APC-Vio770	Miltenyi Biotech, Bergisch Gladbach Germany
9E2	NKp46 (CD335)	PE	Miltenyi Biotech, Bergisch Gladbach Germany
134591	NKG2C	AlexaFluo488	R&D systems, Minneapolis, MN USA
GB11	Granzyme B	Alexa Fluor 647	Biolegend, San Diego, CA USA
MOPC-21	IgG1 isotype control	Alexa Fluor 647	Biolegend, San Diego, CA USA
3D12	HLA-E	Unconjugated (IgM)	Biolegend, San Diego, CA USA
δG9	Perforin	PE	Ancell, Stillwater, MN USA
Isotype Control	IgG2b	PE	Ancell, Stillwater, MN USA
SJ25C1	CD19	PE-Cy7	Thermo Fisher, Waltham MA, USA
1C9	SH2D1A	Unconjugated (IgG2a)	Abnova, Taipei, TWN
W6/32	HLA class I	Unconjugated (IgG2a)	Our Laboratory
BU19	CD19	Unconjugated (IgG1)	Our Laboratory
BAB281	NKp46	Unconjugated (IgG1)	Our Laboratory
PP35	2B4	Unconjugated (IgG1)	Our Laboratory
Y9	CD94	Unconjugated (IgM)	Our Laboratory
FM184/703	CD1a	Unconjugated (IgM)	Our Laboratory

4.3. Results

NK cells after transplantation display a less mature phenotype.

In haplo-HSCT with PT-Cy, we investigated the NK cell reconstitution and the NK cell phenotype in patients after 1-month of transplantation (hereafter referred to post-1M) in comparison with the relative donors by multi-parametric flow cytometry (Fig. 1).
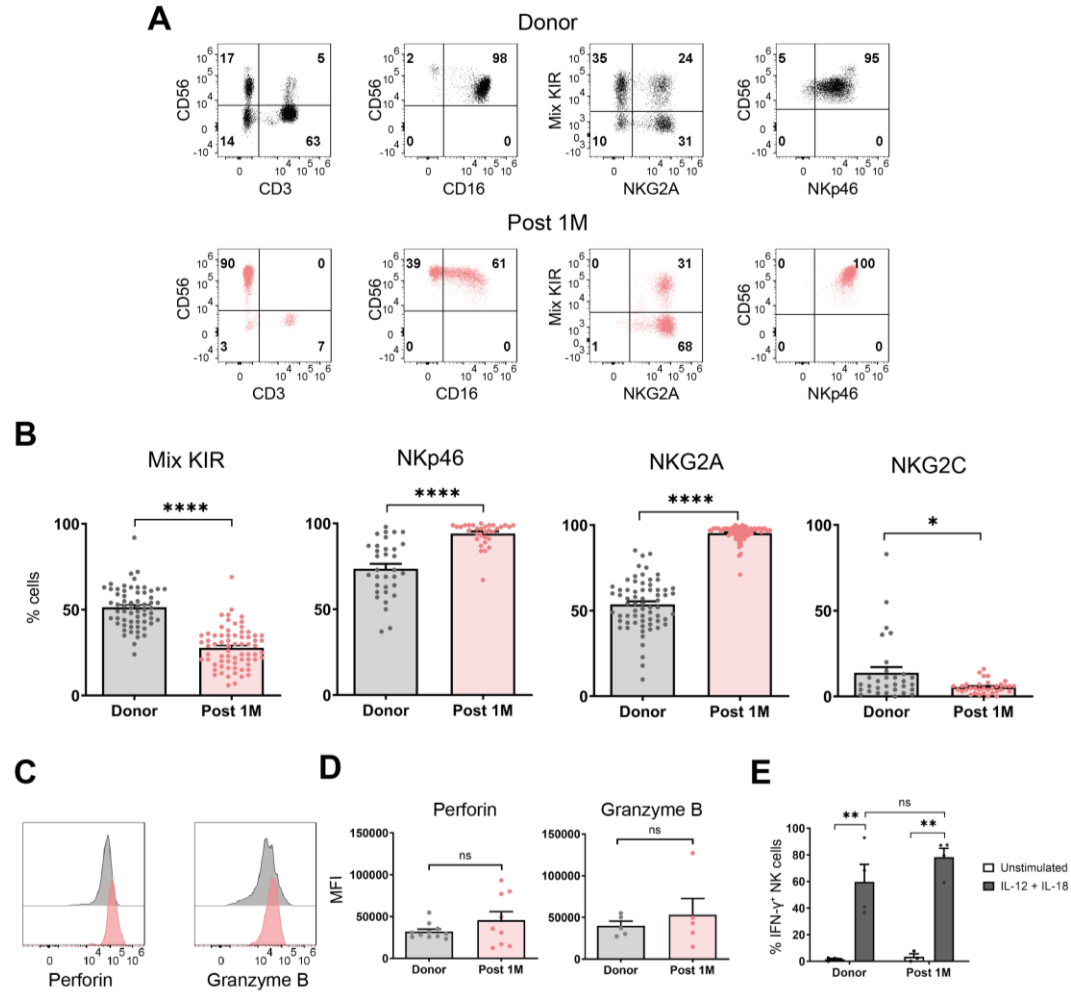
Figure 1. NK cells at early time points after transplantation display a less mature phenotype. A. Representative dot plots showing the cell surface expression of CD3, CD56 on PBMC, and, after gating CD3⁻CD56⁺, the CD16, KIR, NKG2A and NKp46 expression on NK cells from a relative donor and a post-1 month (post-1M) patient. **B.** Cumulative data analyzing fresh NK cells from relative donors (n=63) and post-1M patients (n=70) for KIR, NKG2A, NKG2C and NKp46 cell surface expression. **C.** Representative histograms of intra-cytoplasmic perforin and granzyme B content in NK cells from a representative relative donor (grey) and post-1M patient (pink). **D.** Perforin (donors n=11; post-1M n=9) and granzyme B (n=5, both donors and post-1M patients) intra-cytoplasmic expression in fresh NK cells (gating CD3⁻ CD56⁺ cells). **E.** IFN-γ production from donor (n=4) or post-1M (n=4) NK cells, either incubated overnight with medium (Unstimulated) or with IL-12 and IL-18 (2 ng/mL and 20 ng/mL, respectively).

First, we observed that NK cells were the most representative subset in peripheral blood of post-1M patients. The phenotypic features of these NK cells differed from the relative donors. Post-1M NK cells were mostly CD56^{bright}, CD16^{neg} or CD16^{dim} in contrast to the main CD56^{dim} CD16^{pos} phenotype of healthy NK cells. Regarding the HLA-specific inhibitory receptors, in the reconstituted repertoire the percentage of KIRs was very low while the expression of NKG2A was expressed on virtually all NK cells. Focusing on the activating receptors, high expression of NKp46 and low presence of NKG2C was found on post-1M NK cells (Fig. 1A-B).

Then, to evaluate the cytolytic potential of NK cells, we measured the content of perforin and granzyme B (Fig. 1C-D). By intracellular staining, no differences in the expression of perforin and granzyme B were found in comparison with donors. We evaluated the IFN-γ production of donor and post-transplant NK cells upon cytokine stimulation, and both were equally efficient (Fig. 1E). Taking all these data together, we can point that post-1M NK cells might be functionally competent.

SAP deficiency can be an indicator of immature stage of NK cells

In the literature, the absence of SAP has been documented in immature stages of NK cell development [31]. As post-1M NK cells present a less mature phenotype, we investigated the expression of SAP by intracellular staining. We observed a substantial SAP$^-$ population in post-1M NK cells, mainly confined in CD56^{bright} cells (Fig. 2A-B). In healthy donors NK cells represent a minority (around 10%) and very few SAP$^-$ could be detected. Thus, we suggest SAP deficiency as an indicator of immature stage of NK cells at early time points after transplantation.
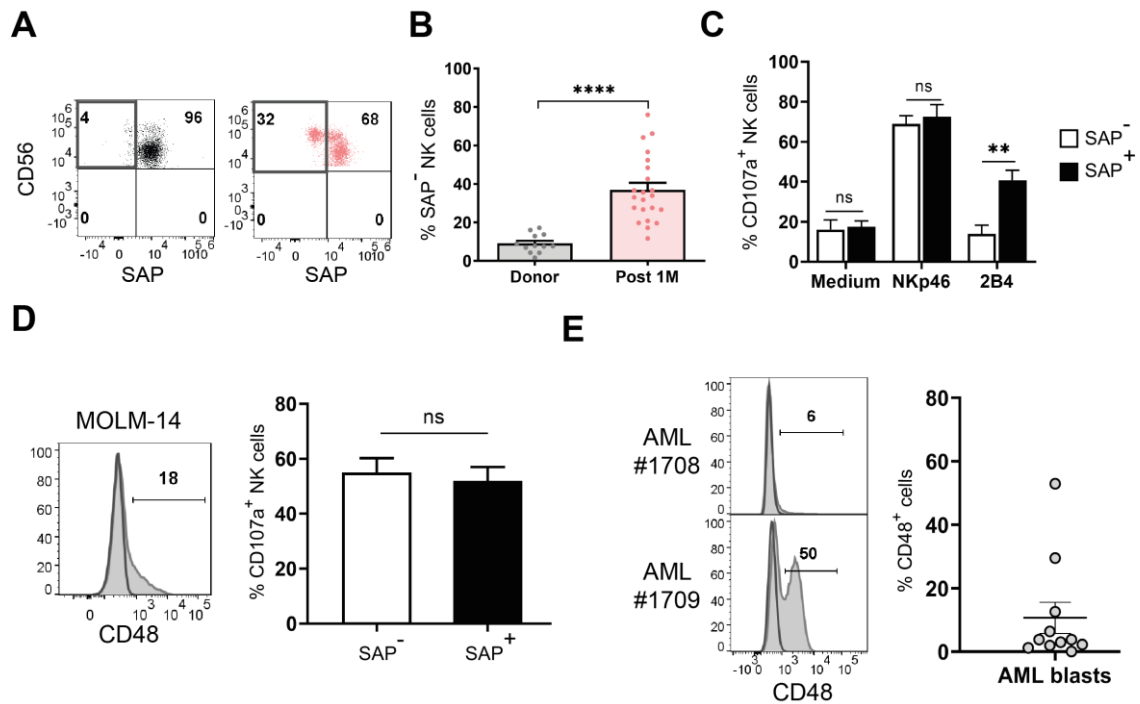
Figure 2. SAP as an indicator of an immature stage of NK cells at early time points after transplantation. A. Representative dot plots of SAP expression from donor and post-1M NK cells. **B.** Percentage of SAP⁻ cells in different donor (n=12) and patient (n=22) pairs gating on NK cells. **C.** IL-2 stimulated NK cells from post-1M patients were analyzed for degranulation activity in R-ADCC assay against the FcγR⁺ p815 target cell line in the presence or absence of anti-NKp46 or anti-2B4 specific mAb (n=4). **D.** CD48 expression on MOLM-14 (left panel) and degranulation response of NK cells stimulated overnight with IL-2 from post-1M patients (n=3) against MOLM-14 cell line. **E.** Expression of CD48 surface marker in adult primary AML blasts (n=11). Two representative AML samples are shown in the left histograms.

2B4 recognizes CD48, a ligand exclusively expressed in hematopoietic cells. After ligand recognition, the ITSMs present in the 2B4 cytoplasmatic tail become phosphorylated and associate with SAP, activating downstream signalling pathways that result in NK cell activation. In the absence of SAP, the phosphatases are recruited, can interact with phosphorylated ITSM, delivering the inhibitory signal [36]. Differentially gating on SAP⁺ or SAP⁻ subsets of post-1M NK cells, we evaluated the activity of NKp46 and 2B4 by R-ADCC assay, engaging post-1M NK cells with specific antibodies (Fig. 2C). While NKp46-mediated activation was equally efficient in both subsets, 2B4 engagement induced activation of SAP⁺ but not of SAP⁻ population. These data suggested that the cytolytic activity of SAP⁻ NK cells might be impaired against CD48⁺ hematopoietic target cells. However, it was already described that CD48 could be downregulated in AML primary blasts compared to healthy PBMC counterpart [122]. We analysed the representative AML cell line MOLM-14, and we found that

CD48 expression was very low. We performed degranulation assays to evaluate the impact of the SAP⁻ subset in the activity against MOLM-14 (Fig. 2D). We observed that both SAP⁻ and SAP⁺ subsets were equally effective in inducing NK cell degranulation. Then, consistent with previous data, a new cohort of primary AML blasts mostly showed a low/negative expression of CD48 (Fig. 2E). From these data we can infer that SAP⁻ NK cells shouldn't have an impaired anti-leukemia activity.

NK cells from transplanted patients enhance the anti-leukemia activity once NKG2A/HLA-E interaction is blocked

The -21 HLA-B dimorphism influences the HLA-E expression and -21 M/x individuals have cells with a higher HLA-E expression than -21 T/T [48]. We used K562-E^G cell line loaded with either VMAPRTVLL or VTAPRTVLL peptide corresponding to the two different HLA-B leader sequences (position 2 of the peptide corresponds to position -21 of the HLA-B leader sequence). After overnight incubation at 37°C with the peptide, HLA-E expression was evaluated by immunofluorescence. As already described, K562-E^G cells loaded with the VMAPRTVLL peptide presented a higher HLA-E expression as compared to VTAPRTVLL [146] (Fig. 3A).
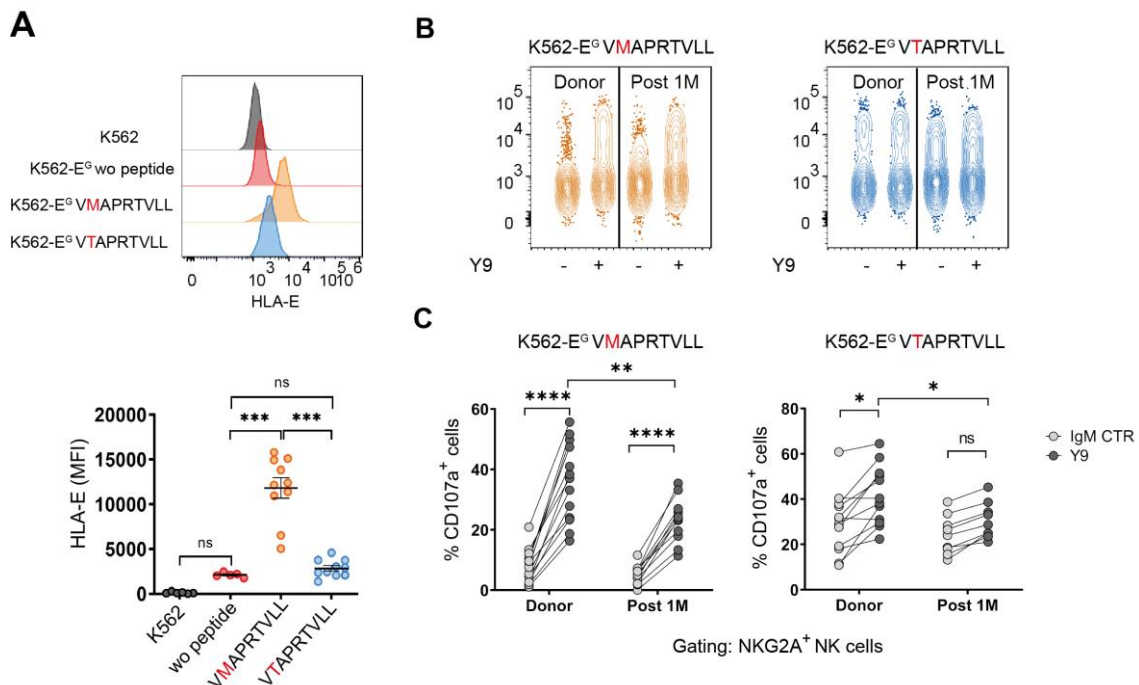
Figure 3. NK cell activity is enhanced once NKG2A/HLA-E interaction is blocked. A. HLA-E expression on K562 and K562-E^G cell line, incubated alone (wo peptide) or pulsed with VMAPRTVLL or VTAPRTVLL peptide. A representative experiment (upper panel), and data from cumulative experiments (n=5-10) (lower panel) are shown. **B-C.** Degranulation capability of IL-2 stimulated NK cells from relative donors and post-1M patients against K562-E^G pulsed

with VMAPRTVLL or VTAPRTVLL peptide, in the absence (IgM CTR) or presence of anti-CD94 specific mAb of IgM isotype (Y9). Representative experiment **(B)** and cumulative experiments (donor n=13; post-1M n=11) **(C)** are shown. CD107a expression was evaluated on CD3$^-$CD56$^+$ NKG2A$^+$ NK cells.

To evaluate the effect of NKG2A/HLA-E inhibitory interaction, we performed blocking assays challenging IL-2 pre-stimulated NK cells against K562-E^G pulsed either with VMAPRTVLL or VTAPRTVLL in the presence or absence of anti-CD94 (Y9, IgM). For a better comparison, only NKG2A$^+$ NK cells from healthy donors (i.e., a variable fraction) and post-1M patients (i.e., almost the totality) were considered. When K562-E^G was loaded with the VMAPRTVLL peptide, NK cells displayed a very low degranulation activity due to the high interaction between NKG2A and HLA-E. Indeed, by blocking this inhibitory interaction, we could restore the degranulation capability in post-1M and donor NK cells. When VTAPRTVLL was loaded, only donor NK cells could significantly enhance their activity upon blocking the NKG2A/HLA-E interaction. In general, NK cells from post-1M showed lower activity in comparison with the donors (Fig. 3B, C). Data using sorted KIR$^-$ NKG2A$^+$ polyclonal activated NK cells from healthy donors against primary AML blasts by ^{51}Cr-release-assay showed, in some cases, an increase of killing of the blasts using anti-CD94 mAb. These data can reflect the heterogeneity of HLA-E expression on AML blasts (Fig. 4).
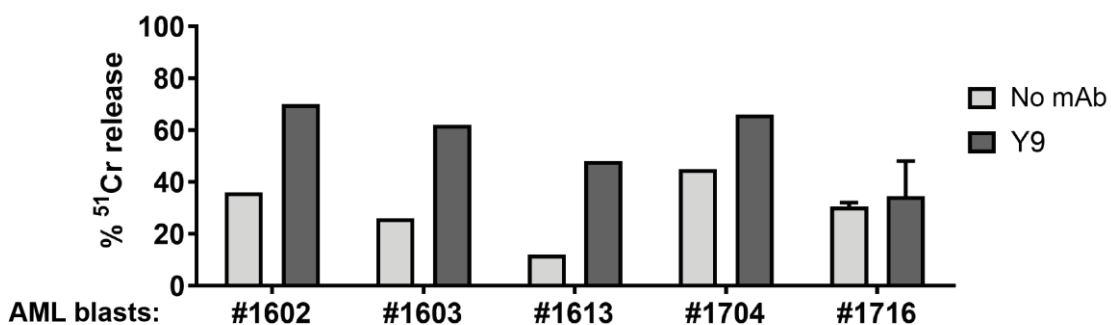

Figure 4. NK cell activity against primary AML blasts. Cytotoxicity was assessed using 4h ^{51}Cr-release-assay using primary AML blasts as target cells. Polyclonal activated KIR$^-$ NKG2A$^+$ NK cells from healthy donors were used as effector cells at E:T 10:1 in the presence or absence of Y9 mAb. In the case of AML#1716, two independent experiments were performed.

In the post-transplant reconstituted repertoire: are -21M/x NK cells more efficient than T/T?

Taking advantage on the functional studies described on NK cells from healthy donors [48] and from AML patients [145], we wondered if we could correlate -21M/x HLA-B genotype with a superior degranulation capacity of NKG2A$^+$ NK cells in our group of transplanted patients. Considering the -21 HLA-B M/x or T/T status of the relative donors, we stratified our patients into two groups. First, we compared the granzyme B content by intracellular staining in both groups, and no significant differences were found, although a tendency of M/x higher than T/T could be observed (Fig. 5A).
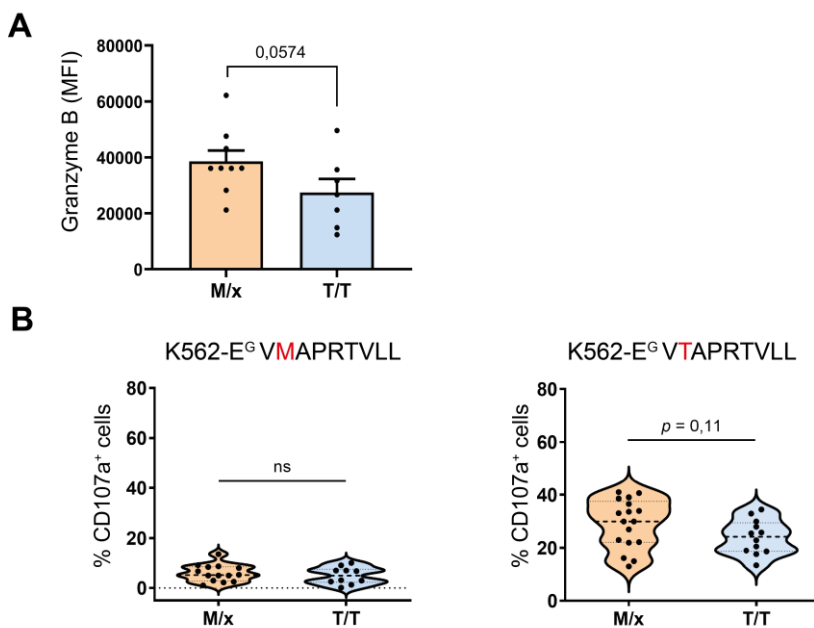
Figure 5. Functional potential of NK cells in patient reconstituted repertoire in relation to M/x or T/T status of related donors. A. Granzyme B intracellular expression in post-1M NK cells transplanted from either M/x (n=9) or T/T (n=7) donors. **B.** Frequency of CD107$^+$ post-1M NK cells from either M/x (n=17) or T/T (n=12) donors towards K562-E^G pulsed with either VMAPRTVLL or VTAPRTVLL peptide.

Then, we challenged NK cells overnight incubated with IL-2 against K562-E^G pulsed with either VMAPRTVLL or VTAPRTVLL to evaluate their degranulation capacity (Fig. 5B). Although it is not statistically significant, we observed that NK cells in the reconstituted repertoire of patients transplanted from M/x donors, as compared to T/T donors, displayed a higher degranulation capacity against VTAPRTVLL-pulsed K562-E^G. This tendency was not observed when NK cells were cultured against VMAPRTVLL-pulsed K562- E^G, in the presence of a strong inhibitory NKG2A/HLA-E interaction.

4.4. Discussion

In this study, we integrated phenotypic and functional analyses to investigate the reconstituted NK cell repertoire of AML patients at early time points after haplo-HSCT with PT-Cy. These NK cells displayed less mature features, characterized by a predominant CD56^{bright} phenotype, high expression of CD94/NKG2A and a low level of KIRs, as already reported. Our original data regarded the observation of high expression of the activating receptor NKp46, high levels of perforin and granzyme B as well as good IFN-γ production upon cytokine stimulation, suggesting that NK cells can be functionally competent. In the NK cell characterization, we also included the intracellular staining of SAP, proposing SAP$^-$ NK cells as particularly immature cells but potentially effective against AML blasts, often showing a CD48$^{low/neg}$ phenotype. We investigated if blocking the inhibitory checkpoint NKG2A could unleash the anti-leukemia activity of NK cells after haplo-HSCT and PT-Cy, supporting the usefulness of the therapy with monalizumab in these patients. Finally, we wondered whether the dimorphism at -21 HLA-B could affect the functionality of NKG2A$^+$ NK cells in this transplantation setting.

The immature features of NK cells in patients transplanted with haplo-HSCT with PT-Cy have been already described by Russo et al. [79], showing that high dose of Cy given as GvHD prophylaxis at days +3 and +5 eliminates not only the infused donor-derived alloreactive T cells but also all mature NK cells. High levels of IL-15 serum concentrations immediately after HSCT can support the proliferation of NK cells, which, because of their lack of ALDH, become susceptible to Cy effect and quickly eliminated. After day 15, circulating NK cells are newly originated from the HSCs, and thus display the immature phenotype. The persistence for 2-3 months, in the reconstituted repertoire, of NK cells characterized by CD56^{bright} and NKG2A$^+$ phenotype can also be due to the effects of other immunosuppressive drugs included in the GvHD prophylaxis. *In vitro* assays showed that Cyclosporine A (CsA) reduced proliferation of CD56^{dim} CD16$^+$ KIR$^+$ NK cells, while CD56^{bright} NK cells were relatively resistant to CsA [147]. Thus, the CD56^{bright} KIR$^-$ NKG2A$^+$ phenotype might reflect a status of immature NK cells and/or be consequent to the effect of a drug as CsA.

To improve the characterization of NK cells after haplo-HSCT with PT-Cy, we analyzed the intracellular SAP expression, according to previous studies correlating the lack of SAP in NK cells to immature stages of their development [31]. In the absence of SAP, SLAM receptors deliver inhibitory instead of activating signals upon engagement with their ligands. In pathological conditions, like XLP-1 patients ($SH2D1A^-$), SAP$^-$ NK cells have an impaired cytolytic response against B-EBV cells, mainly due to 2B4/CD48 inhibitory interaction [38]. We observed a SAP$^-$ phenotype in a variable but consistent fraction of post-1M NK cells, always confined in CD56^{bright} cells. In healthy donors, SAP$^-$ CD56^{bright} NK cells could be only minimally detected. We believe that SAP$^-$ marker can be helpful to identify immature NK cells in the reconstituted repertoire of transplanted patients. We wondered whether this feature, with the consequent lack of 2B4 activating pathway, could impair the anti-leukemia NK cell activity. In this context, it is essential to highlight that the expression of CD48 on AML was almost negative, differently from the CD48$^+$ healthy hematopoietic counterpart. These data suggest that even the SAP$^-$ NK cells might preserve the anti-leukemia activity, as we observed using MOLM-14 cell line, as target.

Since NK cells early after transplantation showed a high expression of CD94/NKG2A, we thought that blocking this inhibitory immune checkpoint could enhance their anti-leukemia activity. We studied the function of NKG2A$^+$ NK cells using as target cells K562-E^G, in which the HLA-E expression can be variable depending on the peptide used for pulsing. Although less efficient than NK cells from healthy donors, a significant increase of post-1M NK cell degranulation was observed upon blocking the interaction between NKG2A and the highly expressed HLA-E, when VMAPRTVLL-pulsed K562-E^G was used as target cell. This is a positive result supporting that blocking of this immune checkpoint in this transplantation setting could be beneficial. Indeed, different clinical trials based on the use of the anti-NKG2A (monalizumab) are ongoing. A phase I clinical trial in an allogeneic HSCT setting showed to be safe in patients with hematological malignancies as AML, ALL and CLL (NCT02921685). Moreover, a phase II clinical trial will start in haplo-HSCT with PT-Cy (EudraCT Number: 2020-005902-24) to treat AML and myelodysplastic syndrome.

We are aware that the levels of HLA-E expression on K562-E^G pulsed with peptides are not physiological, and we would extend our analysis to primary adult AML blasts. In our experience, however, the HLA-E staining on PBMC is quite low, and it's not easy to compare with the leukemia cells included in the same sample. Nevertheless, in some

cases AML blasts expressed even lower levels of HLA-E than the healthy counterpart. We tested some primary AML samples as target cells in cytotoxicity assays, using polyclonal activated KIR^- $NKG2A^+$ NK cells from healthy donors as effectors, in the presence or absence of Y9 mAb. We had the functional evidence of HLA-E heterogeneity on different AML blasts, protecting or not their killing from NK cells expressing exclusively NKG2A as inhibitory receptor.

In this study, we also investigated the possible impact of -21 HLA-B dimorphism in our group of patients. Data from Hallner et al. showed a better clinical outcome (leukemia-free survival and overall survival) in -21M/x AML patients receiving histamine dihydrochloride and low-dose IL-2 compared to T/T patients [145]. Our *in vitro* data indicated a tendency that patients transplanted from -21M/x donors, as compared with T/T donors, harbored more efficient NK cells against VTAPRTVLL-pulsed K562-E^G, but without reaching a significant difference (Fig. 5). Our study on this issue is rather preliminary, because a greater number of cases should be analyzed. It's known that -21M HLA-B allele is less frequent (37,7%) than the -21T HLA-B, which is the most common one with a frequency of 93,3%. In fact, only 6,1% are -21M/M, 31,6% are M/T, and 62,3% are T/T [48]. With these frequencies, the probability to obtain a sample from an M/x individual is reduced in comparison to T/T, representing a challenge to get the same number of cases. We also aim to perform a retrospective study, analyzing a large cohort of AML patients who received haplo-HSCT with PT-Cy, to understand if there is any correlation between the -21 HLA-B dimorphism and leukemia-free survival. No difference in outcome in terms of LFS or OS between the M/x and T/T genotypes has been reported in patients receiving fully matched allo-HSCTs where donor and recipient have the same -21 HLA-B genotype [145]. In our cohort of patients receiving haplo-HSCT with PT-Cy, this study requires the analysis of HLA-B alleles from both donors and patients, stratifying into -21M/x or T/T. We hypothesize that favorable donor/recipient pairs would consist of -21M/x donors, harboring better educated $NKG2A^+$ NK cells, and -21T/T recipients, whose eventually relapsing AML blasts might display low levels of HLA-E.

5. CONCLUDING REMARKS

In this thesis, I analyzed the reconstituted NK cells in two contexts of haplo-HSCT, and I reported *in vitro* evidence of different strategies to potentiate anti-leukemia NK cell functions.

1. In $\alpha\beta$T-cell and B-cell depleted haplo-HSCT to cure pediatric patients:
   - Mature NK cells infused within the graft persist in transplanted patients
   - CD19- and CD20-NKCEs enhance NK cell activity against BCP-ALL cell lines, CD19-NKCEs against primary BCP-ALL blasts.
   - NK cells from transplanted patients are potentiated in killing BCP-ALL through CD19-NKCEs.
   - NKCE-induced activation overrides HLA-I inhibitory interactions.

2. In haplo-HSCT with PT-Cy in adult AML patients:
   - Post-1M NK cells present some immaturity features but can be functional competent.
   - Post-1M NK cells are NKG2A$^+$, and their activity against HLA-E$^+$ leukemia target cells is enhanced once NKG2A is blocked.
   - More experiments are ongoing to evaluate the impact of -21 HLA-B dimorphism in NK cell activity from patients at early time points after transplantation.

Proposed tools to potentiate the anti-leukemia NK cell activity
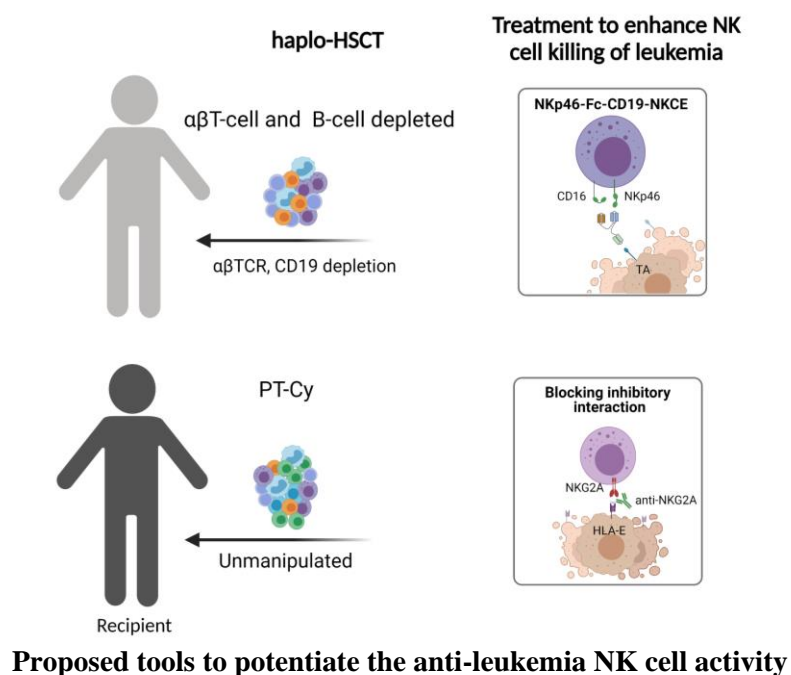
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7. PhD PORTFOLIO

<div style="text-align:center">Publications</div>

- Raffaella Meazza, Michela Falco, Fabrizio Loiacono, Paolo Canevali, Mariella Della Chiesa, Alice Bertaina, Daria Pagliara, Pietro Merli, Valentina Indio, Federica Galaverna, Mattia Algeri, Francesca Moretta, <u>Natalia Colomar-Carando</u>, Letizia Muccio, Simona Sivori, Andrea Pession, Maria Cristina Mingari, Lorenzo Moretta, Alessandro Moretta, Franco Locatelli[§], and Daniela Pende[§]. ([§]F.L. and D.P. equally contributed to this study.) **Phenotypic and functional characterization of NK cells in αβT-cell and B-cell depleted haplo-HSCT to cure pediatric patients with acute leukemia.** *Cancers* 2020;12(8):E2187.

- <u>Natalia Colomar-Carando</u>, Laurent Gauthier, Pietro Merli, Fabrizio Loiacono, Paolo Canevali, Michela Falco, Federica Galaverna, Benjamin Rossi, Frédéric Bosco, Mélody Caratini, Maria Cristina Mingari, Franco Locatelli, Eric Vivier, Raffaella Meazza[§], Daniela Pende[§]. ([§] R.M. and D.P. share last authorship). **Exploiting Natural Killer cell engagers to control pediatric B-cell precursor acute lymphoblastic leukemia.** *Cancer Immunol Res* 2022, *10*, 291-302, doi:10.1158/2326-6066.CIR-21-0843.

- Raffaella Meazza[§], Michela Falco[§], Paolo Canevali, Fabrizio Loiacono, <u>Natalia Colomar-Carando</u>, Aura Muntasell, Anna Rea, Maria Cristina Mingari, Franco Locatelli, Lorenzo Moretta, Miguel Lopez-Botet*, Daniela Pende*. ([§] R.M. and M.F. share first authorship and * M.L.-B. and D.P. share last authorship). **Characterization of KIR[+] NK cell subsets with a monoclonal antibody selectively recognizing KIR2DL1 and blocking the specific interaction with HLA-C.** *(HLA, Manuscript under revision).*

Training activities and courses

- Course in Clinical Genetics (Jan. 2019, Giannina Gaslini Institute, Genoa)
- European advanced course bridging Basic & Clinical Immunology (May 2019, Florence, Italy).
- 9th Workshop on 3D advanced in-vitro Models (Jul. 2019 - IVTech and University of Genoa, Genoa, Italy)
- The SIICA School of Immunology 2020 Viral Immunology & Vaccinology (Apr. 2020, Online).
- Coursera Online Courses:
    - Innovation in Healthcare and Global healthcare (Aug. 2020)
    - Fundamentals of Immunology: Innate Immunity and B-Cell Function (Jul. 2020).
    - Writing in Science (May 2021)
- Special Application Training Flow Cytometry - Miltenyi Biotech (Jun. 2020, Virtual).
- Project/task managing attitude (Dr Roberta Tasso) – University of Genoa (Jun. 2020, Virtual).

National and International conferences

- KIR Workshop 2018 (Oct. 2018, Camogli, Italy).
- NK meeting 2019 (Sept. 2019, Luxembourg). Poster
- The International Retreat of PhD Students in Immunology – SIICA (Dec. 2019, Camogli, Italy). Oral presentation
- SIICA XII NATIONAL CONGRESS (May 2021, Virtual)

MATURE-NK project meetings and training

- MATURE-NK Kick-off meeting and training on "Teamwork, Leadership and Entrepreneurship" (Apr. 2019, Bergisch Gladbach, Germany).
- MATURE-NK 2nd Consortium meeting and training on "Patent and Intellectual property, part I" (Sept. 2019, Barcelona, Spain) and "Bioinformatics" (Sept. 2019, Vienna, Austria)

- MATURE-NK 3rd Consortium meeting and training on "Business course, part I" (Sept. 2020, Virtual).

- MATURE-NK 4th Consortium meeting and training on "Patent and Intellectual property, part II" (Apr. 2021, Virtual)

- MATURE-NK 5th Consortium meeting, and training on "Process and Development" organized by Glycostem, Fraunhofer Institute and Miltenyi Biotech (Oct. 2021, Virtual)

Secondments

- Secondment "HP-DM1 mAb characterization" Immunology Lab, Universitat Pompeu Fabra, Barcelona, Spain. Supervisors: Prof. Miguel-López Botet and Dr. Aura Muntasell (Sept. 2020).

- Secondment "NKCEs to control BCP-ALL" Innate Pharma, Marseille, France. Supervisors: Prof. Eric Vivier and Dr. Laurent Gauthier (Virtual).