

At the Bedside: Innate immunity as an immunotherapy tool for hematological malignancies

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ABSTRACT

The identification of an anti-tumor effect displayed by cells of innate immunity has opened new scenarios, not only in the field of allo-HSCT but also for nontransplanted patients with hematological malignancies or solid tumors. Donor-derived NK cells have been shown to contribute to the eradication of malignant cells after allo-HSCT, when recipients lack ligands for their inhibitory receptors. These alloreactive donor NK cells can also kill recipient APCs and CTLs, thus preventing the occurrence of GvHD and graft rejection. The role of activating receptors on the capacity of NK cells to kill leukemia targets has become evident in the last years. The adoptive infusion of ex vivo-activated NK cells has been investigated recently in Phase I/II trials on patients with hematological malignancies and solid tumors, with promising results. $\gamma\delta$ T lymphocytes are also able to display anti-tumor activity—this providing the biological rationale for Phase I/II trials in lymphoproliferative disorders and solid tumors. Aminobisphosphonates are clinically available compounds able to boost $\gamma\delta$ T cell function. As $\gamma\delta$ T cells do not cause GvHD, they could also be transduced with tumor-associated chimeric antigen receptors and safely infused in allo-HSCT recipients. Basic aspects of innate immunity relevant to

the field will be covered by a companion review article. *J. Leukoc. Biol.* **94**: 1141–1157; 2013.

Introduction

allo-HSCT certainly represents the first and most successful example of clinically efficacious, adoptive immunotherapy of cancer [1]. Indeed, several biological and clinical findings support the concept that the therapeutic effect of allo-HSCT is largely dependent on the GvL effect, exerted by donor-derived, immunologically active cells, transferred with the graft or emerging after transplantation [2], which mainly contributes to eradicate tumor cells surviving the preparative regimen to the allograft. In unmanipulated allo-HSCT, the GvL effect is largely dominated by donor-derived T lymphocytes carrying the α and β chains of the TCR that recognize three groups of antigens displayed by malignant cells [2, 3]: (1) the highly polymorphic major histocompatibility antigens, in the case of HLA-mismatched donor/recipient pairs; (2) mHAs, in the case of HLA-matched donor/recipient pairs, represented by polymorphic proteins, expressed by the recipient's cells that give rise to peptides presented in the pocket of HLA molecules to donor T cells [4, 5]; and (3) TSAs or TAAs, which are expressed selectively by tumor cells and not by normal cells or are overexpressed by tumor cells compared with normal cells, respectively [6]. The distribution of these antigens is relevant for the outcome of the donor-derived T cell response, as HLA molecules and mHAs with wide tissue distribution are expressed by malignant cells and normal tissues of the recipients, whereas TSAs/TAAs are restricted to malignant cells [4, 7]. In view of these observations, the GvL effect is often associated with a detrimental donor T cell alloreaction against the host's healthy tissues, known as GvHD [4, 8], which can represent a life-threatening complication of allo-HSCT and/or can markedly impair the patient's quality of life [8, 9].

Abbreviations: 2M3B1-PP=2-methyl-3-butenyl-1-pyrophosphate, ABP=aminobisphosphonate, ALL=acute lymphoblastic leukemia, allo-HSCT=allogeneic hematopoietic stem cell transplantation, ATG=antithymocyte globulin, BM=bone marrow, BrHPP=bromohydrin pyrophosphate, *Cen*=centromeric, CR=complete remission, CY=cyclophosphamide, DLI=donor lymphocyte infusion, DNAM-1=DNAX accessory molecule-1, Flt3=Fms-related tyrosine kinase 3, FLU=fludarabine, GvHD=graft-versus-host disease, GvL=graft-versus-leukemia, HSC=hematopoietic stem cell, IPP=isopentenyl pyrophosphate, KIR=killer Ig-like receptor, LFS=leukemia-free survival, mHA=minor histocompatibility antigen, MM=multiple myeloma, MRD=minimal residual disease, NHL=non-Hodgkin lymphoma, NKAML=pilot study of haploidentical NK cell transplantation for AML, PAg=phosphoantigen, PB=peripheral blood, PP=pyrophosphate, PVR=poliovirus receptor, SCT=stem cell transplantation, TAA=tumor-associated antigen, *Tel*=telomeric, TSA=tumor-specific antigen, UCB=umbilical cord blood, ULBP=UL-16-binding protein

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In allo-HSCT, the recipient and the donor are typed accurately for the most clinically important HLA loci (i.e., A, B, C, DR β 1, and DQB1). Only donors identical or with a single disparity for these loci have been used for years without any form of graft manipulation—the strategies of GvHD prophylaxis—including a calcineurin inhibitor together with short-term methotrexate. HLA haploidentical allo-HSCTs have been performed using T cell depletion of the graft or more recently, adding post-transplantation CY or mAb interfering with T cell functions to the classical scheme of GvHD prophylaxis.

Over the last 45 years, a large proportion of the thousands of patients affected by hematological malignant disorders transplanted from a HLA-matched—related or unrelated—donor has been cured of their original disease [1]. Despite this remarkable success, only 25% of patients in need of an allograft have a HLA identical sibling available, and for <70% of the remaining patients, a suitable, HLA-compatible, unrelated volunteer can be found [10]. This proportion can be even lower for patients belonging to ethnic groups poorly represented in the registries. In the absence of a HLA-matched donor, alternative donors/sources of HSCs, such as unrelated UCB and HLA haploidentical relatives, are being used increasingly to offer the chance of an allograft to any patient in need to be transplanted [10]. In particular, the majority of patients has a family member, identical for one HLA haplotype and fully mismatched for the other (i.e., haploidentical), who can serve immediately as donor [3, 11, 12].

For many years, relevant obstacles to a wide use of HLA haploidentical family donors were represented by GvHD and graft rejection, mediated by donor and host alloreactive T cell response, respectively [11, 12]. A major breakthrough in the history of successful haploidentical allo-HSCT was the demonstration that an efficient T cell depletion of the graft prevents GvHD [11, 12]. However, the extensive T cell depletion of the graft shifts the balance between competing host and donor T cells in favor of the unopposed host-versus-graft rejection. To overcome this hurdle, the use of “megadoses” of G-CSF-mobilized PB-derived HSCs was shown in animal models to be able to elude the residual, antidonor T lymphocyte reactivity of the recipient [13]. An effective clinical translation of this approach was then obtained by the Perugia group in adult patients with high-risk, acute leukemia [14]. The infusion of megadoses of T cell-depleted HSCs from G-CSF-mobilized PB without any subsequent pharmacological GvHD prophylaxis resulted into an engraftment rate >90% and a cumulative incidence of Grade II–IV acute and chronic GvHD <10% [14]. Subsequent studies performed using purified CD34⁺ HSCs confirmed that sustained engraftment of donor hematopoiesis, without the occurrence of GvHD, can be obtained in the majority of patients with acute leukemia, given HLA haploidentical allo-HSCT, and that a substantial proportion of them became long-term survivors [14–19].

In view of the role played by donor T cells in mediating the GvL effect, it was anticipated that a relevant proportion of patients with acute leukemia, given this type of allograft, would have experienced disease relapse. This expectation was only partly confirmed by the clinical results, as it became evident initially in adult patients affected by AML [15, 20] and more

recently, in children with ALL [16, 17, 21] that a subgroup of patients was characterized by a particularly low risk of relapse [11, 12]. These patients belonged to the group transplanted from a donor having NK cells that were “alloreactive” toward recipient targets.

In this review, we will discuss the most relevant studies supporting the role played by NK cells in eradication of hematological malignancies. We will also analyze the peculiarities of the $\gamma\delta$ T lymphocyte subset in an anticancer perspective, paying particular attention to the aspects that are crucial for the optimal clinical exploitation of these cells of innate immunity.

IMPACT OF NK CELL ALLOREACTIVITY AND OF NK-ACTIVATING RECEPTORS ON THE OUTCOME OF PATIENTS GIVEN ALLO-HSCT

NK cell function is finely regulated by an array of receptors transducing inhibitory or activating signals [22]. The inhibitory signals represent a peculiarity of NK cells (see also the companion review by Norell and coworkers [23]) compared with the function of T and B lymphocytes, which are regulated only by activating signals [22, 24]. Seminal studies have shown that the signals delivered by inhibitory receptors, present on the surface of NK lymphocytes, are even more important than the activating signals [22, 24]. Among receptors that negatively regulate NK cell function, a crucial role is played by those interacting with MHC class I molecules. These receptors include KIRs, specific in humans for determinants shared by groups of HLA-A, -B, and -C allotypes (referred to as KIR ligands), and CD94/NKG2A heterodimer, specific for the nonclassical, class I molecule HLA-E. They avoid that NK cells attack autologous normal cells and allow that cells, in which MHC class I expression is down-regulated (e.g., by tumor transformation or viral infection), be killed [22, 25–28]. In an allo-HSCT setting, NK cells can kill nonself cells through the mechanism of “missing-self recognition”, provided that the donor: (1) expresses a KIR ligand missing in the recipient HLA genotype and (2) expresses the specific KIR, leading to a KIR/KIR ligand mismatch in the graft-versus-host direction (**Fig. 1**) [15, 29–31]. In humans, according to the concept of missing-self recognition, donor NK cell alloreactivity can be predicted to occur in ~50% of patients given a HLA disparate family donor allo-HSCT [29]. A recent, further evolution in the possibility of exploiting the NK cell-mediated lytic effect against leukemia blasts derives from the creation of what can be called a sort of “artificial alloreactivity” [32]. Indeed, a fully human mAb, 1-7F9, which cross-reacts with KIR2DL1, -2, and -3 receptors and prevents their inhibitory signaling, has been engineered [32, 33]. This mAb has been shown to augment NK cell-mediated lysis of HLA-C-expressing tumor cells, including autologous AML blasts, in vitro and in animal models, without inducing killing of normal PBMCs, suggesting a therapeutic window for harnessing NK cell cytotoxicity against malignant targets [32, 33].

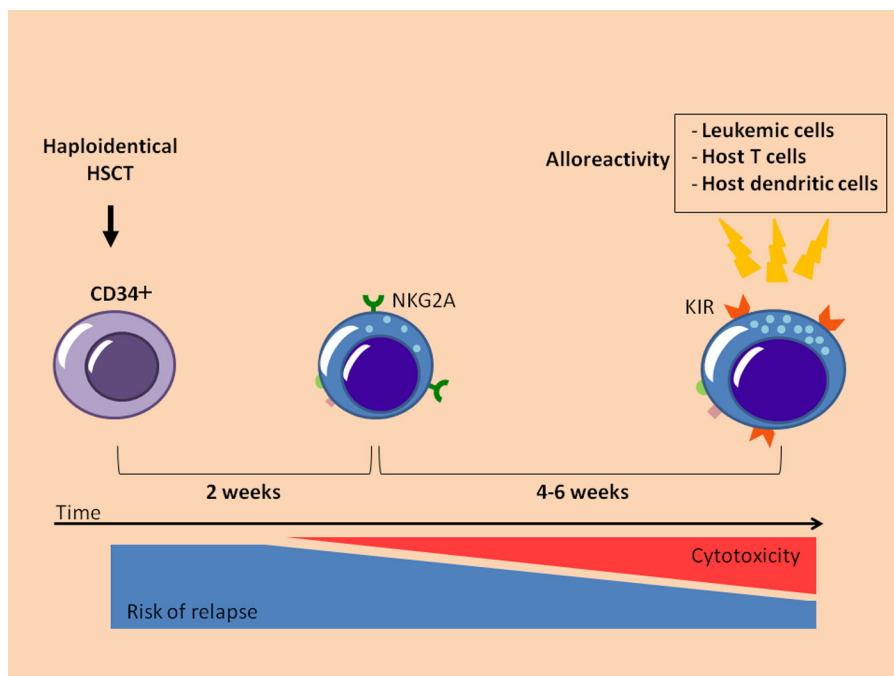


Figure 1. In haploidentical transplantation performed using CD34⁺ positively selected cells, differentiation/maturation of fully functioning NK cells requires 6–8 weeks to be completed. For this reason, in the case of alloreactive donor/recipient pairs, beneficial NK cell-mediated effects, in terms of prevention of risk of graft rejection, leukemia relapse, and GvHD, are relatively delayed.

Open basic research question: why do blast cells of pediatric patients with ALL seem to be more susceptible to NK cell lysis than leukemia cells of adult ALL?

As mentioned above, several studies have shown that protection against disease relapse occurs after T cell-depleted HLA haploidentical allo-HSCT in adult patients with AML and in children with ALL in the presence of alloreactivity mediated by NK cells [15, 16, 20, 21, 29, 34]. This reaction offers the peculiar advantage of inducing GvL without promoting GvHD occurrence, as nonhematopoietic tissues of the recipient are protected from donor NK cell-dependent alloreactivity, as they lack ligands for activating receptors (see above) [30, 31].

For being associated with a reasonable chance of success, transplantation must be performed in patients who have <5% leukemia cells in the BM, a condition conventionally referred to as morphological CR. In more recent years, several studies showed that the lower the tumor burden, the higher the chance of benefiting from HSCT. This observation led to the concept that patients should be transplanted with negative or very low MRD.

The donor NK-mediated GvL effect was particularly evident when patients with acute leukemia were transplanted in morphological CR [15, 20] and in children and young adults, when the donor was the mother [16].

Open basic research question: why is the mother a better donor than the father in terms of prevention of leukemia recurrence?

Serial monitoring of NK cell reconstitution following transplantation of CD34⁺-selected cells showed that mature, fully functioning NK cells, derived from the differentiation

of HSCs, emerge, persisting over time, in the recipient PB only several weeks after the allograft, whereas in the early, post-transplant period, immature, poorly functioning KIR[−]NKG2A⁺ NK cells predominate (see also Fig. 1) [34, 35]. This observation underlined the concept that patients given allo-HSCT from a NK-alloreactive relative cannot benefit from the NK-mediated GvL effect in the early post-transplant period and provides the rationale for strategies of adoptive infusion of ex vivo-activated NK cells [36] during this time window or for alternative approaches of graft manipulation, such as those based on the selective physical removal of $\alpha\beta$ ⁺ T lymphocytes [37, 38]. In fact, T lymphocytes carrying the α/β chains of TCR are the lymphocyte subset responsible for the occurrence of GvHD, and thus, their elimination allows preventing the occurrence of this life-threatening complication.

Open basic research question: are alloreactive NK cells recovering after cord blood transplantation capable of lysing leukemia cells as efficiently as NK cells deriving from BM or PB donors?

Less consistent data on the beneficial effect of NK cell alloreactivity have been reported in patients receiving unrelated UCB transplantation and unmanipulated adult stem-cell transplantation [39–42]. In the former group of patients, Willemze and colleagues [43] demonstrated that NK cell alloreactivity in the donor-recipient direction was associated with a better probability of LFS, mainly attributable to a decreased risk of disease recurrence. These findings were not confirmed by the Minneapolis group [44], which, however, analyzed a cohort of UCB transplant recipients highly heterogeneous in terms of underlying disease, preparative regimen, and number of UCB units infused (single vs. double).

Polyclonal antibodies, called ATG or antilymphocyte globulin, obtained immunizing horses or rabbits with thymocytes or the Jurkat cell line, are able to lyse donor and recipient T cells in vivo or to modulate their function, resulting in a reduced donor-versus-recipient and recipient-versus-donor alloreactivity (i.e., into lower incidence and severity of GvHD and of graft rejection).

In the setting of BM or PB transplantation, whereas a protective effect against leukemia recurrence displayed by donor NK-alloreactive cells was observed in a population of unrelated donor allo-HSCT recipients, given the high number of HSCs and ATG for GvHD prophylaxis, this finding was not confirmed by other studies, which evaluated patients given an unmanipulated allograft as well [39–41]. This discrepancy can be reconciled considering that patients with KIR ligand incompatibility are, by definition, at risk for donor T cell alloreactivity in unmanipulated transplantations and that in patients given a minimally T cell-depleted transplant, T cell alloreactivity dominates and outweighs the effect of NK cells [45]. This latter consideration also emphasizes the concept that proper studies have to be conducted and analyzed to dissect and unveil the role played by the different components of the immune system in terms of protection against malignant recurrence.

Open basic research question: do activating receptors play different roles in different types of leukemia?

NK cell function is also influenced by activating signals, and among receptors that trigger NK cell function, the main non-MHC-specific activating receptors crucial for anti-tumor activity are NKp46, NKp30, NKp44 (collectively termed natural cytotoxicity receptors), NKG2D, and DNAM-1 [46] (see also the companion review by Norell and coworkers [23]). Analysis of the ligands for triggering NKRs revealed the consistent expression of PVR and nectin-2 (i.e., the ligands for DNAM-1) on AML cells and, in the same study, the crucial role played by NKp46 and NKp30 was also confirmed [47]. In addition, human NK cells can express HLA class I-specific activating receptors including KIR2DS1 and KIR2DS4 and CD94/NKG2C. The activating KIRs show a high homology with the corresponding inhibitory form in the extracellular portion but are characterized by relevant differences in their transmembrane and cytoplasmic domains [48]. Recently, mAb capable of discriminating between activating and inhibitory KIRs have become available, and through their use, the identification of KIR2DS1⁺ cells as well as of the magnitude of the alloreactive subset are now possible [49].

Several clinical studies have also focused on the influence of NK cell activating receptors on the outcome of allo-HSCT recipients [50–52]. Patients affected by AML and transplanted from an unrelated volunteer showed a significantly improved outcome when B/x haplotype donors were used compared with A/A donors [50]. The relevance of selected activating KIR receptors in terms of protection against leukemia recurrence and infections has been confirmed recently by Venstrom and colleagues [53], analyzing a large cohort of patients with AML. In this study, patients with AML, receiving the allograft from donors who were positive for KIR2DS1—33% of the whole

population of donors—had a lower relapse rate than those transplanted from donors negative for KIR2DS1. Importantly, this benefit disappeared in transplants from donors with HLA-C2/C2, as high levels of HLA-C2 in HLA-C2/C2 donors reduce NK cell reactivity. This was backed by in vitro data showing that NK clones from KIR2DS1-positive donors with HLA-C1/C1 or -C1/C2 genotypes exhibited higher cytotoxic activity against leukemia targets, at variance with NK clones derived from donors with HLA-C2/C2 [54]. Finally, the use of donors carrying KIR3DS1, a gene coding for another activating receptor, in positive genetic linkage disequilibrium with KIR2DS1, was associated with decreased nonrelapse mortality [53]. Support to the findings of this study is provided by experimental evidences showing that KIR2DS1 expression in HSC donors may represent a remarkable advantage in terms of alloreactive NK responses [55]. This expression results into a substantial increase in the NK-mediated capability to kill not only recipients' leukemic cells [34, 55, 56] but upon CCR7 chemokine receptor acquisition, also DCs and T lymphocytes, potentially resulting into a better prevention of GvHD and graft rejection, as recipient DCs play a crucial role in triggering the T cell-mediated donor alloreactivity, and recipient T lymphocytes can kill donor HSCs [57, 58]. This experimental evidence also suggests that KIR2DS1 expression may significantly amplify the size of the alloreactive NK cell subset by switching a subset of “nonalloreactive” NK cells into potent alloreactive cells.

A further and more recent advancement in the analysis of the role of activating receptors has been provided by an algorithm based on donor KIR-B gene content [59]. More specifically, KIR-A and -B haplotypes have distinctive *Cen* and *Tel* gene-content motifs. In patients with AML, compared with A haplotype motifs, *Cen*- and *Tel*-B motifs contributed to protection against recurrence and to improved survival; *Cen*-B homozygosity had the strongest effect [52].

Open clinical question: which is the hierarchy for the factors related to the NK cell-mediated GvL effect to be considered in the choice of the best donor for a haploidentical allo-HSCT?

Altogether, these data indicate that an accurate choice of the best-available donor can improve the outcome of patients given T cell-depleted, HLA disparate, and perhaps, unmanipulated, HLA-matched allo-HSCT. In the future, biological and clinical studies will further elucidate the contribution of different activating receptors to patient outcome in the context of each transplantation setting.

IMMUNOTHERAPY TRIALS WITH ACTIVATED NK CELLS

It is well-known for many years that human NK cells expressing FcγRIII (CD16) are the main mediators of ADCC and that NK cells efficiently kill target cells opsonized with mAb directed against tumor targets. A clear example of the synergistic action of NK cells and mAb is represented by the enhanced NK-mediated lysis of B cells exposed to rituximab [60–62]. This effect of NK cells mediated by an antibody can be exploited to kill tumor cells coated with mAb, as demonstrated in patients with CD20⁺ B cell lymphoproliferative disorders.

By contrast, the concept of exploiting the anti-tumor effect of NK cells, independently of the use of mAb, is more recent. Indeed, in the last decade, approaches of adoptive immunotherapy based on the infusion of ex vivo-activated NK cells have been explored (see also **Table 1**). A landmark study by Miller and coworkers [63] evaluated the efficacy of haploidentical NK cells in patients with cancer. This study enrolled 43 patients with solid tumors and hematological malignancies.

Leukapheresis products were initially depleted of CD3⁺ T cells, which were kept at $<3 \times 10^5$ /kg recipient's body weight and then cultured with IL-2 until the day of infusion. The final IL-2-activated product contained a NK cell number of $8.5 \pm 0.5 \times 10^6$ cells/kg. Three preparative regimens of different intensity were used for preventing killing of infused NK cells and for favoring their homeostatic expansion. No adverse event during or after NK cell transfer, including GvHD, was

TABLE 1. Overview of the Current Studies Addressing the Immunotherapeutic Effect of NK Cells in Hematologic Malignancies

Reference	Approach	Disease	Number of patients	Source of NK cells	Dose	Response rate
Miller et al. [63]	Infusion of IL-2-activated NK cells after preparative regimen with CY and methylprednisolone, FLU or CY, and FLU (hi-CY/FLU), followed by IL-2 administration	Poor-prognosis AML, metastatic melanoma, metastatic renal cell carcinoma	43	Haploidentical-related donors	NK activation overnight with 1000 UI/ml IL-2, infusion at increasing dose: from 10^5 to 2×10^7 /kg recipient weight, subsequent administration of 1.75×10^6 UI/m ² IL-2 for 14 days	Five of 19 (26%) patients with poor-prognosis AML conditioned with hi-CY/FLU
Rubnitz et al. [64]	Infusion of fresh, isolated NK cells after preparative regimen with CY and FLU, followed by IL-2 administration	Pediatric AML (all but one with favorable risk disease)	10	Haploidentical-related donors	NK infusion at a median dose of 29×10^6 /kg (range 5×10^6 – 81×10^6 /kg) recipient weight, subsequent administration of 1×10^6 UI/m ² IL-2 every other day for six doses (starting at Day -1)	All patients remained in remission
Yoon et al. [65]	Infusion of IL-15/21-activated NK cells (generated from CD34 ⁺ donor cells) after HLA-mismatched HSCT	Acute leukemias, MDS	14	HSC donors	Generation of NK cells from CD34 ⁺ donor cells (HSC factor, human Flt3, and hydrocortisone), NK activation with 30 ng/ml IL-15 and 30 ng/ml IL-21, infusion at a median dose of 9.28×10^6 /kg recipient weight	Primary end-point was feasibility (no toxicity was reported, low-grade acute GvHD and chronic GvHD appeared), four of 14 (28%) alive and well
Curti et al. [36]	Infusion of NK cells after preparative regimen with CY and FLU, followed by IL-2 administration	AML	13	Haploidentical-related donors	Infusion of positive-selected NK cells at a median dose of 2.74×10^6 /kg recipient weight, subsequent administration of 10×10^6 UI/day IL-2, three times weekly for 2 weeks (six total doses)	Six of 13 (46%) in CR
Stern et al. [66]	Infusion of positive-selected NK cells after haploidentical SCT	High-risk leukemias Hodgkin lymphoma Sarcomas	16	Haploidentical donors	One to three infusions of positive-selected NK cells at a median dose of 1.21×10^7 /kg recipient weight	Four of 16 (25%) alive and well

MDS, Myelodysplastic syndrome.

observed. Two patients with renal cell carcinoma had stable disease at 20 and 21 months after NK cell infusion; four patients with melanoma and stable disease after adoptive cell transfer received a second course of NK cells, 4–9 months after the initial NK cell administration. However, disease progressed 4–6 weeks after the second infusion [63]. Five of 19 patients with overt AML disease achieved CR after haploidentical NK cell therapy, with a significantly higher response rate when KIR ligand-mismatched haploidentical relatives were used as donors. For six patients, unique RT-PCR primers for donor-specific MHC class I alleles were generated. In five of them, donor NK cells persisted for only 5 days. However, one patient had persistence of donor cells for 138 days. In vitro assays with patient/donor-derived PBMCs suggested that the inability to expand haploidentical NK cells in vivo may be attributed to low-intensity immune suppression, leading to insufficient clearing of recipient lymphoid cells. In support of this hypothesis, eight of 15 evaluable patients with AML, given a higher-intensity conditioning regimen, i.e., 60 mg/kg CY and 25 mg/m² FLU for 5 consecutive days, showed at least 1% engraftment of donor cells. In one patient, sorting experiments showed that circulating blood and marrow NK cells, but not T or B cells, were of donor origin. The CY/FLU therapy per se increased IL-15 plasma levels, which likely promoted in vivo NK cell expansion in patients with AML [63].

A subsequent trial (NKAML), to determine safety, feasibility, and engraftment of haploidentical NK cells in children with AML, was run at St. Jude Children's Research Hospital in 2010 [64]. Ten children (0.7–21 years old), in first CR of AML after four or five courses of chemotherapy on the AML02 trial, were enrolled. All patients had favorable or intermediate-risk leukemia disease. They received lymphodepleting immune-suppressive therapy, consisting of CY (60 mg/kg on Day -7) and FLU (25 mg/m²/day on Days -6 through -2), followed, in nine out of 10 patients, by KIR-HLA-mismatched NK cells and six doses of IL-2 (1×10^6 U/m²) on alternate days, starting on Day -1 to activate/expand donor NK cells, which were infused immediately after cell enrichment and without in vitro exposure to cytokine stimuli [64]. Patients received a median of 29×10^6 NK cells/kg. Although T cells were detected occasionally in cell-therapy products, their number was more than tenfold lower (1×10^3 /kg) than the threshold of T cells known to promote GvHD occurrence. Nonhematologic toxicity was tolerable, with no GvHD. Transient donor cell engraftment was documented in all patients for a median of 10 days, associated with a significant expansion of KIR-mismatched NK cells (peaking at 5800/mL blood on Day 14) [64]. One patient had prolonged NK cell engraftment (namely, 2% donor NK cells at Day+189), with delayed neutrophil and platelet recovery. With a median follow-up time of 964 days, all patients in the NKAML study remained in remission and were MRD-negative at 1, 2, and 4 months after NK infusion. Despite the favorable prognostic characteristics of this cohort, these data suggest that lymphodepleting therapy, followed by donor-recipient inhibitory KIR-HLA-mismatched NK cells, may reduce the risk of relapse in childhood AML.

Fourteen adult patients, 12 with advanced acute leukemia (mostly AML) and two with myelodysplastic syndrome, under-

went haploidentical HSCT, followed on Days +40/+50 by the infusion of donor NK cells [65]. At time of transplant, among the 12 patients with acute leukemia, one was in first CR after salvage chemotherapy, five were in second or subsequent CR, and six had refractory disease. Cells for the adoptive NK infusion were obtained from the last-day leukapheresis collection and were CD34-selected by MACS, yielding a median of 2.22×10^6 cells/kg. NK cells were ex vivo-generated through a 6-week period culture. In particular, cells were expanded in culture with stem cell factor, Flt3 ligand, IL-7, and hydrocortisone for the first 3 weeks, followed by incubation with IL-15, IL-21, and hydrocortisone for an additional 3 weeks to obtain mature NK cells. No signs of acute toxicity were observed in patients infused with NK cells, 6–7 weeks post-transplant. The median follow-up of surviving patients after donor NK cell infusion was 18.7 months. Overall, one and five patients developed acute and chronic GvHD during the post-transplant period, respectively. Among six patients with refractory acute leukemia and six patients with acute leukemia in CR at the time of HSCT, four in each group achieved CR after HSCT. Unfortunately, three of six patients with refractory disease and four of six patients in CR eventually relapsed after HSCT and NK cell therapy.

Thirteen adult patients with AML were treated, combining IL-2 with highly purified NK cells from HLA haploidentical, KIR ligand-mismatched donors, following a lymphodepleting chemotherapy regimen [36]. Five patients had active disease, two were in molecular relapse, and six were in morphological CR. The number of infused T cells was consistently $<1 \times 10^5$ /kg, whereas the median number of infused NK cells was 2.74×10^6 /kg. Also in this study, the administration of NK cells was safe and not associated with untoward clinical events. Among the five patients with active disease, one transient CR was documented, with no clear clinical benefit for the remaining four cases. The two patients with molecular relapse of AML achieved CR that lasted for 9 and 14 months. Finally, 50% of patients given NK cells in CR were alive and disease-free at up to 34 months. Also in this study, peak levels of serum IL-15 were detected following lymphodepleting therapy and NK cell infusion. Chimerism assay indicated that donor-derived cells expanded and were detectable after NK cell infusion, with peaks on Days 10 and 5 in PB and BM, respectively. The magnitude of patients' alloreactive NK cell subset shortly after NK cell infusions mirrored that detected originally in the donor.

In a prospective Phase II study, conducted in two centers, purified NK cells were given pre-emptively to high-risk pediatric and adult patients treated with haploidentical T cell-depleted allo-HSCT for high-risk solid or hematological malignancies [66]. The transplant procedure was performed, as per institutional protocols, after myeloablative conditioning, including ATG or OKT3, G-CSF-mobilized PB stem cells, a T cell-depleted (target $<1 \times 10^5$ /kg CD3⁺) graft with a high dose of CD34⁺ cells (target $>10 \times 10^6$ /kg), post-transplant immunosuppression with OKT3, and short-course cyclosporine. Sixteen patients received a total of 29 NK-DLI on Days +3, +40, and +100 after HSCT. Median doses of infused NK and T cells/product were 1.21×10^7 /kg and 0.03×10^5 /kg, respectively. With a median follow-up of 5.8 years, four out of 16 patients

were alive. Causes of death were relapse in five, GvHD in four, graft failure in three, and transplant-related neurotoxicity in one patient. Four patients, who had received $>0.5 \times 10^5$ T cells/kg, developed more than Grade II acute GvHD. NK-DLIs did not affect graft failure or relapse rates apparently, compared with historical controls treated with HLA haploidentical allo-HSCT without NK-DLI. Furthermore, no differences in favor of NK-DLI in recipients—KIR ligand-mismatched with their respective donors compared with KIR ligand-matched patients—could be found [66].

Open basic research question: which is the best cytokine combination and duration of ex vivo culture to optimize the effect of NK cells against malignant cells?

Altogether, these data indicate that infusion of allogeneic NK cells, immediately after collection or after ex vivo activation, is largely safe, provided that the number of T lymphocytes contaminating the product is below the threshold known to cause GvHD. Although some responses are encouraging, the real clinical benefit of this procedure remains to be proven definitively. Many clinical studies aimed at exploring the role of NK cell adoptive immunotherapy in patients with cancer are ongoing (see also **Table 2** for details).

$\gamma\delta$ T CELLS

$\gamma\delta$ T cells (also termed “innate-like” T cells or “transitional” T cells) are lymphocytes capable of recognizing their targets in a MHC-independent manner through activating receptors (among others, $\gamma\delta$ TCR, NKG2D, TLRs, DNAM-1; **Fig. 2**) [67]. These cells display a preactivated phenotype that allows rapid cytokine production (IFN- γ , TNF- α) and strong cytotoxic response upon activation. $\gamma\delta$ T cell functions are heterogeneous, ranging from protection against intra- and extracellular pathogens [68–70] to tumor surveillance [71–73], immune response modulation [74], and maintenance of tissue homeostasis [75].

A number of preclinical and clinical observations point to their potentially beneficial role against cancer. $\gamma\delta$ T cells recognize and kill malignant cells of hematological [76–80] and solid tumors [81–84]; it has also been demonstrated that absolute numbers of V γ 9V δ 2 T cells (the main population of circulating $\gamma\delta$ T cells), as well as their effector functions, are decreased in patients with melanoma [85] and nasopharyngeal carcinoma [86]. Moreover, V γ 9V δ 2 T cells, derived from patients with AML, are dysfunctional, having reduced expansion potential [87]. Altogether, these features render $\gamma\delta$ T cells attractive for adoptive immune therapy in cancer and particularly, in the allo-HSCT setting.

TCR- $\alpha\beta$ /CD19-DEPLETED HAPLOIDENTICAL HSCT

A better recovery of $\gamma\delta$ T lymphocytes after unmanipulated allogeneic BM transplantation has been associated with an increased probability of LFS, without any augmented risk of GvHD [88, 89]. These clinical data support the concept that $\gamma\delta$ T lymphocytes do not cause GvHD [88, 89], and the bio-

logical explanation for this observation lies on the fact that they target molecules apparently not involved in GvHD pathophysiology. As mentioned above, a new method of graft manipulation has been tested recently in HLA haploidentical HSCT recipients. This method relies on the negative selection of T lymphocytes carrying the α and β chains of the TCR and of B lymphocytes. This approach leaves in the graft not only donor mature NK cells and committed hematopoietic progenitors but also $\gamma\delta$ T lymphocytes [37]. The preliminary clinical data of the new method of T cell depletion are promising [90, 91], as patients receiving an $\alpha\beta$ T lymphocyte/B lymphocyte-depleted allograft have a negligible risk of developing severe acute GvHD and chronic GvHD, while being protected from life-threatening infections [90, 91]. Interestingly, the early T cell recovery is mainly contributed to by $\gamma\delta$ T lymphocytes, which represent the predominating T cell subset until 45–60 days after the allograft (unpublished results).

CD27 is expressed by ~80% of human $\gamma\delta$ T lymphocytes, particularly those with naive or central memory phenotypes.

EX VIVO AND IN VIVO STIMULATION AND EXPANSION OF $\gamma\delta$ T CELLS

A major issue in immunotherapy is activation and expansion of effector cells. In the case of $\gamma\delta$ T cells, the most-studied stimuli are small nonpeptidic-phosphorylated metabolites (also known as PAgS, such as IPP) [92–94]; they are produced by several pathogens through the cholesterol pathway [95, 96] (**Fig. 3**). Moreover, these metabolites can be produced in large amounts by malignant cells through abnormal metabolic pathways, such as the mevalonate and nonmevalonate 1-deoxy-d-xylulose-5-phosphate route [97]. Following these observations, two main strategies were conceived to stimulate $\gamma\delta$ T cells: the use of phosphorylated bromohydrin (Phosphostim; Innate Pharma, Marseille, France), a specific, synthetic agonist of V γ 9V δ 2 T lymphocytes that mimics the biological properties of natural PAgS, and the use of aminobiphosphonates. In fact, zoledronate and pamidronate, two of the most used drugs of this class, and alkylamines, such as isobutylamine and sec-butylamine, inhibit farnesyl diphosphate synthase, a key enzyme of the cholesterol/mevalonate pathway, thus leading to intracellular accumulation of PAgS and consequently, to indirect activation of $\gamma\delta$ T cells [97–101]. Moreover, stimulation with these compounds can be coupled with cytokines, such as IL-2 and IL-15, to improve expansion and cytotoxicity [102, 103]. Other compounds used to enhance direct or indirect anti-tumor effects of $\gamma\delta$ T cells are: polysaccharide K [104], a mushroom extract that has been demonstrated to activate $\gamma\delta$ T cells through TLR2; bacillus Calmette-Guerin, which may stimulate $\gamma\delta$ T lymphocytes to recruit neutrophils in bladder cancer [105]; and soluble CD70, a CD27 agonist, which promotes $\gamma\delta$ T cell survival, proliferation, and IFN- γ production [106]. Another approach refers to V γ 9V δ 2 T cell expansion obtained by coculturing PBMCs with autologous DCs pretreated with zoledronate, BrHPP, or mevastatin [107].

TABLE 2. Overview of Ongoing Studies Addressing the Immunotherapeutic Effect of NK and $\gamma\delta$ T Cells in Hematologic and Solid Malignancies

Trial	Approach	Adult/pediatric	Disease	Source of NK cells	NK selection/activation/dose
NCT00582816	Haploidentical HSCT, followed by post-transplant donor NK cell infusions	Pediatric	Relapsed ALL, AML, and high-risk solid tumors	Haploidentical donors	N.S.
NCT00640796	Infusion of NK cells after preparative regimen with CY and FLU, followed by IL-2 administration	Pediatric	Chemotherapy refractory or relapse malignancies, including AML, T cell ALL, T cell lymphoblastic lymphoma, CML, JMML, MDS, ESFT, rhabdomyosarcoma	Haploidentical donors	NK-positive selection
NCT00697671	Infusion of NK cells after preparative regimen with CY, clofarabine, and etoposide, followed by IL-2 administration	Pediatric	ALL, CML, JMML, MDS, NHL	Haploidentical donors	NK-positive selection, IL-2 administration three times/week after NK infusion for a minimum of 2 weeks
NCT01287104	Infusion of NK cells after T cell-depleted allogeneic HSCT from matched family donors or matched unrelated donors	Pediatric	Acute leukemias and solid tumors	HSC donors	Ex vivo NK cell activation and expansion using KT64.4-BBL artificial APCs
NCT01337544	Haploidentical HSCT, followed by IL-15-stimulated NK cell infusion, 1 month after transplantation	Pediatric	Refractory solid tumors	Haploidentical donors	NK activation with IL-15 (dose and timing N.S.)
NCT01386619	Haploidentical HSCT, followed by NK cell infusion on Days +3, +40, and +100 after transplantation	Adult/pediatric	ALL, AML, MDS, lymphomas, neuroblastoma, rhabdomyosarcoma	Haploidentical donors	NK cell goal dose $\geq 1 \times 10^7$ /kg body weight of recipient
NCT01700946	Immunotherapy regimen that includes chemotherapy, rituximab, and infusion of haploidentical NK cells	Pediatric	Relapsed ALL and lymphoblastic lymphoma	Haploidentical donors	N.S.
NCT00383994	Nonmyeloablative allogeneic HSCT, followed by infusion of rituximab rhu-GM-CSF and NK cells	Adult	Persistent or recurrent CD20+ B cell CLL and NHL	HSC donors	GM-CSF, 250 μ g, s.c., three times/week for 4 weeks starting 1 day before the administration of rituximab, 375 mg/m ² , followed by 1000 mg/m ² weekly for 3 weeks for a total of four doses, NK cells will be infused 1 week after the fourth dose of rituximab
NCT00402558	Allogeneic HSCT with IL-2-activated NK cell infusion (NK cells from an alloreactive-related donor will be infused during the conditioning regimen, before thymoglobulin infusion)	Adult	AML, MDS, CML	Haploidentical alloreactive-related donors	Alloreactive NK activation with IL-2 (dose N.S.), NK infusion at one of four dose levels 10^6 , 5×10^6 , 3×10^7 cells/kg, and three 10^7 NK cells plus systemic IL-2 treatment on Day -8 from transplantation

Trial	Approach	Adult/pediatric	Disease	Source of NK cells	NK selection/activation/dose
NCT00586703	Nonmyeloablative HSCT from mismatched donors, followed by NK cells infusion	Adult	Lymphomas	HSC donors	Target cell dose for NK cell infusion will be up to 1×10^7 cells/kg patient body weight
NCT00941928	Infusion of NK cells after preparative regimen with CY and FLU with the adjunction of epratuzumab, followed by low-dose IL-2 administration	Adult/pediatric	Relapsed ALL	Haploidentical donors	Epratuzumab, 360 mg/m ² , once/day, i.v., on Day -4, Day -1, and Days 3, 6, 10, 13, and 17 and low-dose IL-2 s.c. injections, three times/week for nine doses on Days 0–21
NCT01106950	Infusion of NK cells after preparative regimen with CY, FLU, and denileukin difitox, followed by low-dose IL-2 administration	Adult/pediatric	Refractory or relapsed AML	Haploidentical donors	IL-2 administered after NK cell infusion, 10 million units every-other day for a total of six doses
NCT01370213	RIC haploidentical HSCT with IL-2-activated NK cell infusion (NK cells from donor will be infused during the conditioning regimen before thymoglobulin infusion)	Adult	AML, MDS	Haploidentical donors	CD3 ⁺ /CD19 ⁺ selected, IL-2-activated, haploidentical donor NK cells infused on Day -12, IL-2, 6 million units s.c. every-other day for six doses beginning the evening of NK cell infusion
NCT01795378	Haploidentical HSCT, followed by two NK cell infusions (Days 6–9 and Days 13–20 after transplantation)	Adult	ALL, AML	Haploidentical donors	In vitro NK generation (protocol N.S.), the first infusion of NK cell will be done at an escalating dose of 2×10^7 /kg, 5×10^7 /kg, 1×10^8 /kg, and $1-4 \times 10^8$ /kg cells, the second infusion of NK will be done at a dose of $1-4 \times 10^8$ /kg.
NCT01853358	RIC allogeneic HSCT, followed by infusion of IL-2-activated NK cells	Adult	High-risk hematologic malignancies	HSC donors	Ex vivo NK activation with 1000 U/ml IL-2 for 7 days
NCT01404702	Combination of zoledronic acid and IL-2 to enhance $\gamma\delta$ T cell expansion and cytotoxicity	Pediatric	Neuroblastoma	–	N.S.
NCT00562666	Single hepatic intra-arterial injection of $\gamma\delta$ T cells	Adult	Hepatocellular carcinoma	N.S. (autologous?)	Dose escalating from 5×10^8 $\gamma\delta$ T cells to 4×10^9 $\gamma\delta$ T cells

N.S., Not specified; JMML, juvenile myelomonocytic leukemia; ESFT, Ewing sarcoma family of tumors; CLL, chronic lymphoblastic leukemia; RIC, reduced intensity conditioning.

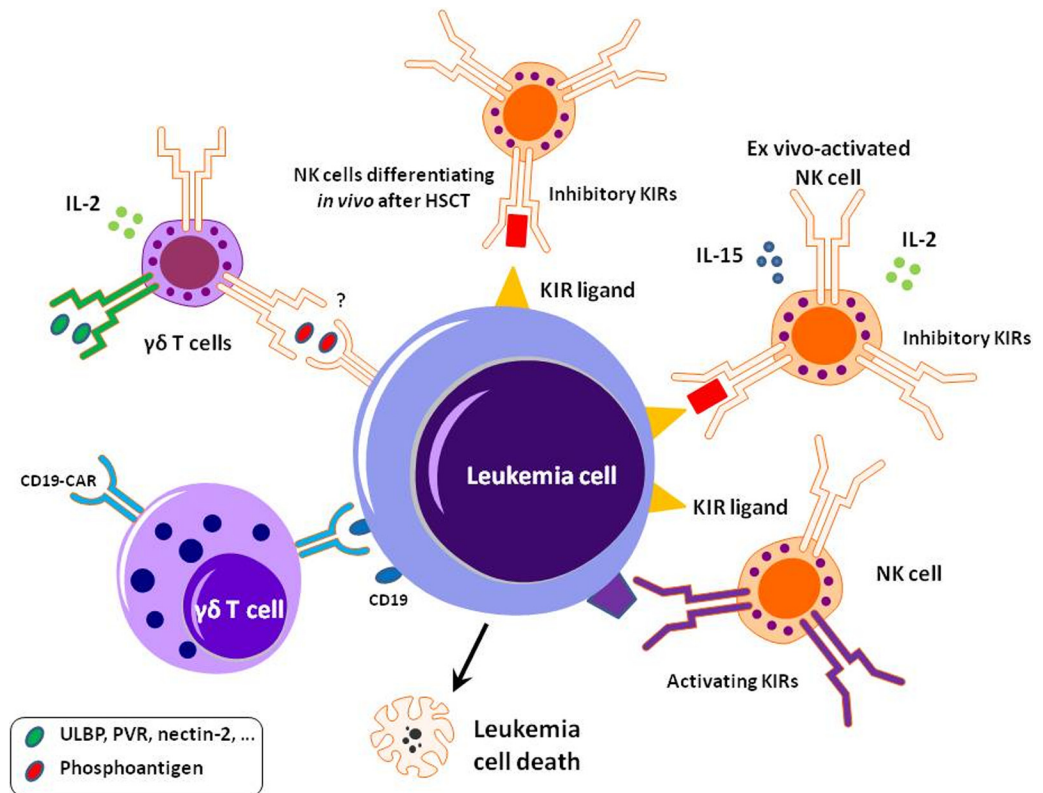


Figure 2. Schematic representation of the antileukemia effect displayed by NK and $\gamma\delta$ T cells, with the relative contribution of inhibitory KIRs and activating receptors.

Two main strategies can be envisaged to exploit $\gamma\delta$ T lymphocytes for immunotherapy: in vivo administration of compounds stimulating $\gamma\delta$ T cells or infusion of ex vivo-activated and expanded cells. Obviously, the first approach can find a greater applicability, because of its easier feasibility; in fact, no GLP and GMP factories are required. We are currently exploring, in a Phase I study, the safety and efficacy of administering zoledronic acid in patients undergoing TCR- $\alpha\beta$ /CD19-depleted haploidentical HSCT (thus enriched with TCR- $\gamma\delta$ lymphocytes; see above). Nonetheless, several clinical-grade protocols of purification/expansion/stimulation have been developed already and wait to be tested in a clinical setting [108–111].

SYNERGISTIC USE OF CHEMOTHERAPY AND mAb

Improvement of anti-tumor activity of $\gamma\delta$ T cells can be achieved also with synchronous/ metachronous administration of chemotherapy and/or mAb. In fact, some chemotherapeutic agents, such as anthracyclines and platinum derivatives, can trigger a tumor-specific, T cell-mediated response. Ma and colleagues demonstrated in a murine xenograft model that chemotherapy induces a rapid and prominent infiltration of $\gamma\delta$ Th17 cells (i.e., IL-17-producing $\gamma\delta$ V γ 4⁺ and V γ 6⁺ T lymphocytes) with a consequent accumulation of CTLs within the tumor; abrogation of such a response significantly reduced tumor-specific T cell responses elicited by chemotherapy [112].

Furthermore, as activated $\gamma\delta$ T cells express CD16 (i.e., Fc γ RIII), ADCC can be exploited to improve the efficacy of

cancer immunotherapy; rituximab, alemtuzumab, and trastuzumab were shown to enhance $\gamma\delta$ T cell cytotoxicity against CD20-positive B lineage lymphoma or chronic lymphocytic leukemia, CD52⁺, and human epidermal growth factor receptor 2⁺ cells, respectively, in vitro and in animal models [113, 114].

Clinical open question: what protocols can be designed to exploit these properties and optimize cancer immunotherapy?

ANTI-TUMOR IMMUNOTHERAPEUTIC APPROACHES WITH $\gamma\delta$ T CELLS

To date, apart from the transplantation setting, only few Phase I/II clinical studies have investigated the role of immunotherapy with $\gamma\delta$ T cells; moreover, most of the studies available investigated such a therapy for solid tumors (Table 3). Wilhelm and colleagues [115], in a pilot study conducted on patients with relapsed/refractory low-grade NHL or MM, tested in vivo pamidronate administration, followed by increasing doses of i.v. IL-2. Although treatment was well-tolerated, among the first 10 patients, only one achieved stable disease; by contrast, in the second cohort (including patients treated with a modified protocol consisting of IL-2 from Day 1 to Day 6, directly after the pamidronate infusion in the form of increasing dose levels), three out of nine patients achieved an objective response. Subsequently, Dieli and colleagues [116] studied the administration of zoledronic acid alone or in combination with low-dose IL-2 in patients with hormone-refractory metastatic prostate cancer. Treatment was well-tolerated

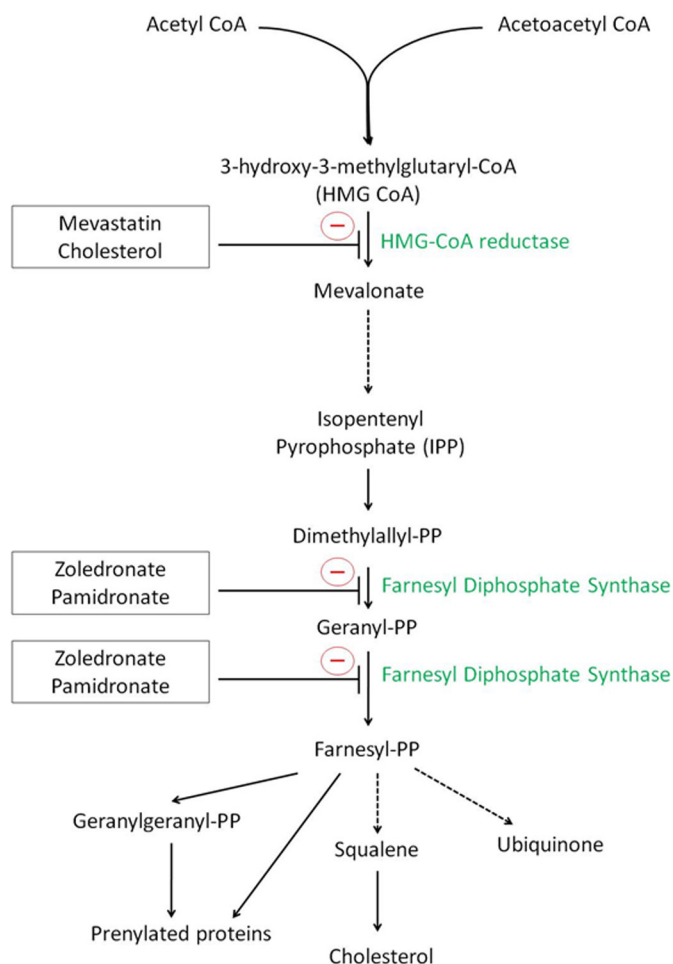


Figure 3. Cholesterol/mevalonate pathway. Key enzymes susceptible to be inhibited by pharmacological stimulation are depicted in green. The “minus” symbol encircled in red denotes inhibition.

with no significant side-effects; moreover, an increase in the number of circulating $\gamma\delta$ T cells was observed. Most importantly, a decrease of prostate-specific antigen levels associated with objective clinical responses was recorded, including three partial remissions and five cases of stable disease. However, whereas 25% of patients in the arm treated with ABP and IL-2 showed a long-term shift of peripheral $\gamma\delta$ T cells toward an activated effector-memory-like state, with production of IFN- γ and perforin, the majority of patients ($n=53$) treated with zoledronate alone did not have a sustained $\gamma\delta$ T cell response and showed progressive clinical deterioration. These data suggest that addition of IL-2 to ABP improves $\gamma\delta$ T cell-mediated anti-tumor responses.

A robust expansion of peripheral V γ 9V δ 2 T cells was also demonstrated in patients with advanced metastatic breast cancer given zoledronate and IL-2 [117]. The demonstration that large-scale ex vivo expansion of functional $\gamma\delta$ T cells from cancer patients was feasible [123, 124] led to initial clinical studies addressing their adoptive transfer. Several protocols of stimulation in different types of malignancies have been tested, including incubation with 2M3B1-PP and IL-2 [82] and

adoptive transfer of V γ 9V δ 2 T cells amplified by using 2M3B1-PP, followed by patient treatment with zoledronic acid and human rIL-2 [118]. A decrease of tumor burden or stable disease was observed in a remarkable proportion of patients, although reversible and manageable toxicities were recorded frequently [122]. V γ 9V δ 2 T cells expanded with zoledronic acid and IL-2 were used in six patients with MM; in four of them, stable M-protein serum levels were observed after treatment [119]. Sakamoto and colleagues [121] treated 15 patients with zoledronate and IL-2-expanded V γ 9V δ 2 T cells without further in vivo IL-2 administration; no objective response was observed, but six out of 12 evaluable patients experienced stable disease. Two clinical trials (NCT00562666 and NCT01404702) are currently under way for patients with hepatocellular carcinoma and refractory neuroblastoma, respectively (see Table 2 for details).

Open basic research question: which tests can be used to monitor the $\gamma\delta$ T cell response to stimulation (besides evaluation of tumor burden) and its in vivo-activated state?

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The last decades have witnessed a growing interest in the anti-tumor role played by cells belonging to the innate immunity. In particular, it has become evident that donor-derived alloreactive NK cells can protect the recipient from the regrowth of leukemia cells escaping the conditioning regimen. A similar beneficial effect seems to be associated also to the $\gamma\delta$ T cell subset. Although immunotherapy with cells of the innate immune system is in its infancy, some promising results are already available for solid and for hematological malignancies. Laboratory observations have led to the development of expansion and activation protocols; however, other biological features of this cell population have not been addressed yet. For instance, it has been noticed that $\gamma\delta$ T cells may act as APCs [125, 126]; in fact, they are able to process soluble proteins, up-regulate costimulatory molecules, induce proliferation, and target cell killing and cytokine production responses in antigen-experienced and naive CD8 $^{+}$ $\alpha\beta^{+}$ T cells. As V γ 9V δ 2 T cells can induce DC maturation through cytokine production and CD1d molecule [127], these two immunological interactions could also be exploited to optimize other strategies of immunotherapy (i.e., antigen-specific immunotherapy or cancer vaccines). Such a strategy has just been investigated in tuberculosis vaccination in monkeys [128], thus delineating a basis for similar approaches for immunotherapy.

Another important field of research is the molecular characterization of $\gamma\delta$ TCRs [129] and of their cognate ligands on target cells [130], as well as how they are modified by current or future protocols of expansion [131]. A recent study demonstrated a potential role of V γ 9V δ 2 T cells for the therapy of AML, by showing that $\gamma\delta$ T cells recognize myeloid blasts through the TCR and DNAM-1 (its ligands, PVR, and nectin-2 were expressed on the surface of AML blasts) and kill them through a perforin/granzyme-mediated mechanism [87]. The

TABLE 3. Overview of the Current Studies Addressing the Immunotherapeutic Effect of $\gamma\delta$ T Lymphocytes in Cancer

Reference	Approach	Disease	Number of patients	Dose	Response rate
Wilhelm et al. [115]	In vivo administration of pamidronate and IL-2	NHL or MM	19	Pamidronate (90 mg) and $0.25\text{--}3 \times 10^6$ IU/m ² IL-2, Days 3–8 or 1–6	One of 10 (10%) and three of nine (33%)
Dieli et al. [116]	In vivo administration of zoledronic acid \pm IL-2	Metastatic hormone-refractory prostate cancer	18	Zoledronate (4 mg), every 21 days, 0.6×10^6 IU IL-2, s.c., immediately after each zoledronate administration	25%
Meraviglia et al. [117]	In vivo administration of zoledronic acid and IL-2	Metastatic breast cancer	10	Zoledronate (4 mg), every 21 days, 10^6 IU IL-2, s.c., immediately after each zoledronate administration	30%
Kobayashi et al. [82]	Adoptive transfer of V γ 9V δ 2 T cell amplified by using 2M3B1-PP and IL-2	Advanced renal cell carcinoma	7	$5.0\text{--}3400 \times 10^6$, every week or every-other week up to six to 12 times for 12 weeks	43%
Kobayashi et al. [118]	Adoptive transfer of V γ 9V δ 2 T cell amplified by using 2M3B1-PP, followed by infusion of zoledronic acid and human rIL-2	Advanced renal cell carcinoma	11	Cell quantity not specified, 4 mg zoledronate, 1.4×10^6 IU rIL-2, Days 0–4, all of the procedure was repeated every 4 weeks for six times	one complete remission, five stable disease, and five progressive disease
Abe et al. [119]	Adoptive transfer of V γ 9V δ 2 T cells expanded with zoledronic acid and IL-2	MM	6	9.9×10^8 /Infusion, four infusions at 2-week intervals	66%
Bennouna et al. [120]	Adoptive transfer of V γ 9V δ 2 T cells expanded with BrHPP and IL-2, followed by s.c. injections of IL-2	Metastatic renal cell carcinoma	10	Cells ($1\text{--}8 \times 10^9$)/infusion, followed by s.c. IL-2 (2×10^6 IU/m ² /day), three infusions at 21-day intervals	60%
Sakamoto et al. [121]	Adoptive transfer of V γ 9V δ 2 T cells expanded with zoledronic acid and IL-2	Advanced nonsmall cell lung cancer	15 (12 evaluable)	Cumulative number of $\gamma\delta$ T cells infused $2.6\text{--}45.1 \times 10^9$, divided in six to 12 infusions every 2 weeks	No objective responses, six patients had stable disease
Noguchi et al. [122]	Adoptive transfer of V γ 9V δ 2 T cells expanded with zoledronic acid and IL-2	Various advanced solid tumors	25	$4 \pm 2 \times 10^9$ cells/infusion	Primary end-point was feasibility, out of four patients given V γ 9V δ 2 T cells alone, two had stable disease, and two progressive disease

same receptor was implicated in recognition of hepatocarcinoma cells expressing Necl-5 [132]. Also, NKG2D, which recognizes nonclassical MHC proteins of the MHC class I chain-related molecule and ULBP family, seems critical for tumor cell recognition; in fact, ULBP1 and ULBP4 have been proposed recently as determinants for leukemia and lymphoma cell recognition [133]. Thus, it is of crucial relevance to obtain a comprehensive characterization of $\gamma\delta$ T cell phenotypes [87] with the aim at optimizing and “standardizing” cellular products for adoptive immunotherapy. Moreover, studying the expression of ligands on target cells could be useful to identify

patients with “ $\gamma\delta$ -susceptible” or “ $\gamma\delta$ -resistant” cancers. In this regard, Gomes and colleagues [130] identified recently 10 cell-surface protein antigens on ALL and NHL cells, associated with increased or decreased probability of response to $\gamma\delta$ T cells. On the tumor side, it will be interesting to explore the possibility to enhance the expression of ligands for $\gamma\delta$ TCRs on malignant cells; for example, in vitro treatment of tumor cells with TLR3 and TLR7 agonists resulted in enhanced cytotoxicity of $\gamma\delta$ T cells [131].

An important question that deserves further investigation is the optimal cytokine stimulation able to obtain the best expan-

TABLE 4. Summary of “Open Basic Research and Clinical Questions”

Summary: basic research questions
Which are the molecular and cellular mechanisms underlying the differential susceptibility of ALL blasts from pediatric and adult patients to NK cell lysis?
Why is the mother a better donor than the father in terms of prevention of leukemia recurrence?
Are alloreactive NK cells recovering after cord blood transplantation capable of lysing leukemia cells as efficiently as NK cells deriving from BM or PB donors?
Do activating receptors play different roles in different types of leukemia?
Which is the best cytokine combination and duration of ex vivo culture to optimize the effect of NK cells against malignant cells?
Which tests can be used to monitor the $\gamma\delta$ T cell response to stimulation (besides evaluation of tumor burden) and its in vivo-activated state?
Summary: clinical questions
Which is the hierarchy for the factors related to the NK cell-mediated GvL effect to be considered in the choice of the best donor for a haploidentical allo-HSCT?
What protocols can be designed to exploit these properties and optimize cancer immunotherapy?

sion and activation of these cells (Table 4). Very recently, Izumi and colleagues [134], examining the in vivo dynamics of zoledronate and IL-2-expanded $\gamma\delta$ T cells infused in patients with colorectal carcinoma, demonstrated that they express IL-2R $\beta\gamma_c$, thus supporting the concept that endogenous IL-15 promotes the expansion/survival of these cells in vivo. As IL-15 has different effects on regulatory T cells than those exerted by IL-2 and as this cytokine is now available in a clinical-grade formulation [135], it will be interesting to test its role in adoptive immunotherapy protocols involving NK cells and $\gamma\delta$ T cells. In this respect, ex vivo-expanded V γ 9V δ 2 T cells maintained with IL-15 have increased effector functions [134]. A further layer of complexity is a result of the recent observation that V δ 1⁺ T cells can also be induced to express activating receptors, such as NKp30 and NKp44, thus rendering them able to recognize lymphoid leukemia cells resistant to fully activated V γ 9V δ 2 T cells [136]. Likewise, the optimal culture conditions for activating ex vivo NK cells to be adoptively infused remain to be addressed [137, 138]. Therefore, focused clinical protocols are needed to further evaluate the anti-tumor effects of these cells in everyday clinical practice [64].

Finally, studies are currently under way to evaluate the possibility of transducing $\gamma\delta$ T cells with chimeric antigen receptors [139]. In fact, available evidence supports the concept that this lymphocyte subset does not promote and/or sustain GvHD development [88, 89]. Thus, these cells could represent the ideal target for being transduced with tumor-associated chimeric antigen receptors. Preliminary reports on the anti-tumor efficacy of T lymphocytes genetically modified with chimeric antigen receptors in patients with ALL and chronic lymphocytic leukemia have been published over the last few years [140–143]. However, concerns about the safety of genetically modified T lymphocytes exist [144, 145] and the possibility of

using—especially in the allo-HSCT setting—cells unable to expand in response to alloantigens could represent an advantage compared with T lymphocytes, such as those carrying the $\alpha\beta$ chains of the TCR, involved in GvHD pathophysiology.

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KEY WORDS:

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