At the Bedside: Innate immunity as an immunotherapeutic 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. γδ 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 γδ T cell function. As γδ 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: 000–000; 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 alloreactivity 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].

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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, DRB1, 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 γδ 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-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].
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 αβ+ 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).

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.
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 [29]). 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/α 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.

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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 \(<3 \times 10^6\)/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 \(8.5 \pm 0.5 \times 10^6\)/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>
<thead>
<tr>
<th>Reference</th>
<th>Approach</th>
<th>Disease</th>
<th>Number of patients</th>
<th>Source of NK cells</th>
<th>Dose</th>
<th>Response rate</th>
</tr>
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<tbody>
<tr>
<td>Miller et al. [63]</td>
<td>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</td>
<td>Poor-prognosis AML, metastatic melanoma, metastatic renal cell carcinoma</td>
<td>43</td>
<td>Haploidentical-related donors</td>
<td>NK activation overnight with 1000 UI/ml IL-2, infusion at increasing dose: from (10^6) to (2 \times 10^7)/kg recipient weight, subsequent administration of (1.75 \times 10^6) UI/m² IL-2 for 14 days</td>
<td>Five of 19 (26%) patients with poor-prognosis AML conditioned with hi-CY/FLU</td>
</tr>
<tr>
<td>Rubnitz et al. [64]</td>
<td>Infusion of fresh, isolated NK cells after preparative regimen with CY and FLU, followed by IL-2 administration</td>
<td>Pediatric AML (all but one with favorable risk disease)</td>
<td>10</td>
<td>Haploidentical-related donors</td>
<td>NK infusion at a median dose of (29 \times 10^6)/kg (range (5 \times 10^6)–(81 \times 10^6)/kg) recipient weight, subsequent administration of (1 \times 10^6) UI/m² IL-2 every other day for six doses (starting at Day −1)</td>
<td>All patients remained in remission</td>
</tr>
<tr>
<td>Yoon et al. [65]</td>
<td>Infusion of IL-15/21-activated NK cells (generated from CD34+ donor cells) after HLA-mismatched HSCT</td>
<td>Acute leukemias, MDS</td>
<td>14</td>
<td>HSC donors</td>
<td>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 \times 10^6)/kg recipient weight</td>
<td>Primary end-point was feasibility (no toxicity was reported, low-grade acute GvHD and chronic GvHD appeared), four of 14 (28%) alive and well</td>
</tr>
<tr>
<td>Curti et al. [36]</td>
<td>Infusion of NK cells after preparative regimen with CY and FLU, followed by IL-2 administration</td>
<td>AML</td>
<td>13</td>
<td>Haploidentical-related donors</td>
<td>Infusion of positive-selected NK cells at a median dose of (2.74 \times 10^7)/kg recipient weight, subsequent administration of 10 (\times 10^6) UI/day IL-2, three times weekly for 2 weeks (six total doses)</td>
<td>Six of 13 (46%) in CR</td>
</tr>
<tr>
<td>Stern et al. [66]</td>
<td>Infusion of positive-selected NK cells after haploidentical SCT</td>
<td>High-risk leukemias, Hodgkin lymphoma, Sarcomas</td>
<td>16</td>
<td>Haploidentical donors</td>
<td>One to three infusions of positive-selected NK cells at a median dose of (1.21 \times 10^7)/kg recipient weight</td>
<td>Four of 16 (25%) alive and well</td>
</tr>
</tbody>
</table>

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 chemotherapy regimen [36], five patients had active disease, two in molecular relapse of AML. The two 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 <1×10⁶/kg CD3⁺) graft with a high dose of CD34⁺ cells (target >10×10⁶/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 1.21×10⁷/kg and 0.03×10⁶/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 \( >0.5 \times 10^8 \) 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].

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 MHG-independent manner through activating receptors (among others, \( \gamma^\delta \) TCR, NK2D, TLRs, DNAM-1; Fig. 2) [67]. These cells display a preactivated phenotype that allows rapid cytokine production (IFN-\( \gamma \), TNF-\( \alpha \)) 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 \( \gamma^\delta \) 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, \( \gamma^\delta \) 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 biological 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 \( \alpha \) and \( \beta \) chains of the TCR and of B lymphocytes. This approach leaves in the graft not only donor normal 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 \( \gamma^\delta \) V62 T lymphocytes that mimics the biological properties of natural PAgS, and the use of aminobiphosphonates. In fact, zolendronate 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-\( \gamma \) production [106]. Another approach refers to \( \gamma^\delta \) V62 T cell expansion obtained by coculturing PBMCs with autologous DCs pretreated with zole-dronate, BrHPP, or mevastatin [107].
<table>
<thead>
<tr>
<th>Trial</th>
<th>Approach</th>
<th>Adult/pediatric</th>
<th>Disease</th>
<th>Source of NK cells</th>
<th>NK selection/activation/dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00582816</td>
<td>Haploidentical HSCT, followed by post-transplant donor NK cell infusions</td>
<td>Pediatric</td>
<td>Relapsed ALL, AML, and high-risk solid tumors</td>
<td>Haploidentical donors</td>
<td>N.S.</td>
</tr>
<tr>
<td>NCT00640796</td>
<td>Infusion of NK cells after preparative regimen with CY and FLU, followed by IL-2 administration</td>
<td>Pediatric</td>
<td>Chemotherapy refractory or relapse malignancies, including AML, T cell ALL, T cell lymphoblastic lymphoma, CML, JMML, MDS, ESFT, rhabdomyosarcoma</td>
<td>Haploidentical donors</td>
<td>NK-positive selection</td>
</tr>
<tr>
<td>NCT00697671</td>
<td>Infusion of NK cells after preparative regimen with CY, clofarabine, and etoposide, followed by IL-2 administration</td>
<td>Pediatric</td>
<td>ALL, CML, JMML, MDS, NHL</td>
<td>Haploidentical donors</td>
<td>NK-positive selection, IL-2 administration three times/week after NK infusion for a minimum of 2 weeks</td>
</tr>
<tr>
<td>NCT01287104</td>
<td>Infusion of NK cells after T cell-depleted allogenic HSCT from matched family donors or matched unrelated donors</td>
<td>Pediatric</td>
<td>Acute leukemias and solid tumors</td>
<td>HSC donors</td>
<td>Ex vivo NK cell activation and expansion using KT64.4-BBL artificial APCs</td>
</tr>
<tr>
<td>NCT01337544</td>
<td>Haploidentical HSCT, followed by IL-15-stimulated NK cell infusion, 1 month after transplantation</td>
<td>Pediatric</td>
<td>Refractory solid tumors</td>
<td>Haploidentical donors</td>
<td>NK activation with IL-15 (dose and timing N.S.)</td>
</tr>
<tr>
<td>NCT01386619</td>
<td>Haploidentical HSCT, followed by NK cell infusion on Days +3, +40, and +100 after transplantation</td>
<td>Adult/pediatric</td>
<td>ALL, AML, MDS, lymphomas, neuroblastoma, rhabdomyosarcoma</td>
<td>Haploidentical donors</td>
<td>NK cell goal dose ≥1 × 10^7/kg body weight of recipient</td>
</tr>
<tr>
<td>NCT01700946</td>
<td>Immunotherapy regimen that includes chemotherapy, rituximab, and infusion of haploidentical NK cells</td>
<td>Pediatric</td>
<td>Relapsed ALL and lymphoblastic lymphoma</td>
<td>Haploidentical donors</td>
<td>N.S.</td>
</tr>
<tr>
<td>NCT00383994</td>
<td>Nonmyeloablative allogenic HSCT, followed by infusion of rituximab rhu-GM-CSF and NK cells</td>
<td>Adult</td>
<td>Persistent or recurrent CD20+ B cell CLL and NHL</td>
<td>HSC donors</td>
<td>GM-CSF, 250 μg, s.c., three times/week for 4 weeks starting 1 day before the administration of rituximab, 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</td>
</tr>
<tr>
<td>NCT00402558</td>
<td>Allogenic 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)</td>
<td>Adult</td>
<td>AML, MDS, CML</td>
<td>Haploidentical alloreactive-related donors</td>
<td>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</td>
</tr>
<tr>
<td>Trial</td>
<td>Approach</td>
<td>Adult/pediatric</td>
<td>Disease</td>
<td>Source of NK cells</td>
<td>NK selection/activation/dose</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------------</td>
<td>----------------------------------</td>
<td>-------------------</td>
<td>--------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NCT00586703</td>
<td>Nonmyeloablative HSCT from mismatched donors, followed by NK cells infusion</td>
<td>Adult</td>
<td>Lymphomas</td>
<td>HSC donors</td>
<td>Target cell dose for NK cell infusion will be up to $1 \times 10^7$ cells/kg patient body weight</td>
</tr>
<tr>
<td>NCT00941928</td>
<td>Infusion of NK cells after preparative regimen with CY and FLU with the adjunction of epratuzumab, followed by low-dose IL-2 administration</td>
<td>Adult/pediatric</td>
<td>Relapsed ALL</td>
<td>Haploidentical donors</td>
<td>Epratuzumab, 300 mg/m², 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</td>
</tr>
<tr>
<td>NCT01106950</td>
<td>Infusion of NK cells after preparative regimen with CY, FLU, and denileukin difitox, followed by low-dose IL-2 administration</td>
<td>Adult/pediatric</td>
<td>Refractory or relapsed AML</td>
<td>Haploidentical donors</td>
<td>IL-2 administered after NK cell infusion, 10 million units every-other day for a total of six doses</td>
</tr>
<tr>
<td>NCT01370213</td>
<td>RIC haploidentical HSCT with IL-2-activated NK cell infusion (NK cells from donor will be infused during the conditioning regimen before thymoglobulin infusion)</td>
<td>Adult</td>
<td>AML, MDS</td>
<td>Haploidentical donors</td>
<td>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</td>
</tr>
<tr>
<td>NCT01795378</td>
<td>Haploidentical HSCT, followed by two NK cell infusions (Days 6–9 and Days 13–20 after transplantation)</td>
<td>Adult</td>
<td>A.LI., AML</td>
<td>Haploidentical donors</td>
<td>In vitro NK generation (protocol N.S.), the first infusion of NK cell will be done at an escalating dose of $2 \times 10^7$/kg, $5 \times 10^7$/kg, $1 \times 10^8$/kg, and $1–4 \times 10^9$/kg/kg, the second infusion of NK will be done at a dose of $1–4 \times 10^9$/kg/kg</td>
</tr>
<tr>
<td>NCT01853358</td>
<td>RIC allogenic HSCT, followed by infusion of IL-2-activated NK cells</td>
<td>Adult</td>
<td>High-risk hematologic malignancies</td>
<td>HSC donors</td>
<td>Ex vivo NK activation with 1000 U/ml IL-2 for 7 days</td>
</tr>
<tr>
<td>NCT01404702</td>
<td>Combination of zoledronic acid and IL-2 to enhance γδ T cell expansion and cytotoxicity</td>
<td>Pediatric</td>
<td>Neuroblastoma</td>
<td>–</td>
<td>N.S.</td>
</tr>
<tr>
<td>NCT00562666</td>
<td>Single hepatic intra-arterial injection of γδ T cells</td>
<td>Adult</td>
<td>Hepatocellular carcinoma</td>
<td>N.S. (autologous?)</td>
<td>Dose escalating from $5 \times 10^8$ γδ T cells to $4 \times 10^9$ γδ T cells</td>
</tr>
</tbody>
</table>

N.S., Not specified; JMML, juvenile myelomonocytic leukemia; ESFT, Ewing sarcoma family of tumors; CLL, chronic lymphoblastic leukemia; RIC, reduced intensity conditioning.
Two main strategies can be envisaged to exploit γδ T lymphocytes for immunotherapy: in vivo administration of compounds stimulating γδ 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-γδ/CD19-depleted haploidentical HSCT (thus enriched with TCR-γδ 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 γδ 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 γδ Th17 cells (i.e., IL-17-producing γδ Vγ4ε and Vγ6ε 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 γδ T cells express CD16 (i.e., FcγRIII), ADCC can be exploited to improve the efficacy of cancer immunotherapy; rituximab, alemtuzumab, and trastuzumab were shown to enhance γδ 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].

**ANTI-TUMOR IMMUNOTHERAPEUTIC APPROACHES WITH γδ T CELLS**

To date, apart from the transplantation setting, only few Phase I/II clinical studies have investigated the role of immunotherapy with γδ 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...
Adoptive transfer of Vγ9Vδ2 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]. Vγ9Vδ2 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 Vγ9Vδ2 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).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The last decades have witnessed a growing interest in the antitumor 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 γδ 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 γδ 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⁺ αβ⁺ T cells. As Vγ9Vδ2 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 γδ TCRs [129] and of their cognate ligands on target cells [150], as well as how they are modified by current or future protocols of expansion [131]. A recent study demonstrated a potential role of Vγ9Vδ2 T cells for the therapy of AML, by showing that γδ 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

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 γδ 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 γδ 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 γδ T cell response and showed progressive clinical deterioration. These data suggest that addition of IL-2 to ABP improves γδ T cell-mediated anti-tumor responses.

A robust expansion of peripheral Vγ9Vδ2 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 γδ 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

BASIC-TRANSLATIONAL REVIEW Locatelli et al. NK cells and γδ T cells in leukemia
same receptor was implicated in recognition of hepatocarcinoma cells expressing Ncl-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 γδ 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 “γδ-susceptible” or “γδ-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 γδ T cells. On the tumor side, it will be interesting to explore the possibility to enhance the expression of ligands for γδ TCRs on malignant cells; for example, in vitro treatment of tumor cells with TLR3 and TLR7 agonists resulted in enhanced cytotoxicity of γδ T cells [131].

An important question that deserves further investigation is the optimal cytokine stimulation able to obtain the best expansion of γδ T cells with TLR3 and TLR7 agonists resulted in enhanced cytotoxicity of γδ T cells [131].

TABLE 3. Overview of the Current Studies Addressing the Immunotherapeutic Effect of γδ T Lymphocytes in Cancer

<table>
<thead>
<tr>
<th>Reference</th>
<th>Approach</th>
<th>Disease</th>
<th>Number of patients</th>
<th>Dose</th>
<th>Response rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilhelm et al. [115]</td>
<td>In vivo administration of pamidronate and IL-2</td>
<td>NHL or MM</td>
<td>19</td>
<td>Pamidronate (90 mg) and 0.25–3 × 10⁶ IU/m² IL-2, Days 3–8 or 1–6</td>
<td>One of 10 (10%) and three of nine (33%)</td>
</tr>
<tr>
<td>Dieli et al. [116]</td>
<td>In vivo administration of zoledronic acid ± IL-2</td>
<td>Metastatic hormone-refractory prostate cancer</td>
<td>18</td>
<td>Zoledronate (4 mg), every 21 days, 0.6 × 10⁴ IU IL-2, s.c., immediately after each zoledronate administration</td>
<td>25%</td>
</tr>
<tr>
<td>Meraviglia et al. [117]</td>
<td>In vivo administration of zoledronic acid and IL-2</td>
<td>Metastatic breast cancer</td>
<td>10</td>
<td>Zoledronate (4 mg), every 21 days, 10⁶ IU IL-2, s.c., immediately after each zoledronate administration</td>
<td>30%</td>
</tr>
<tr>
<td>Kobayashi et al. [82]</td>
<td>Adoptive transfer of Vγ9Vδ2 T cell amplified by using 2M3B1-PP and IL-2</td>
<td>Advanced renal cell carcinoma</td>
<td>7</td>
<td>5.0–3400 × 10⁶, every week or every-other week up to six to 12 times for 12 weeks</td>
<td>43%</td>
</tr>
<tr>
<td>Kobayashi et al. [118]</td>
<td>Adoptive transfer of Vγ9Vδ2 T cell amplified by using 2M3B1-PP, followed by infusion of zoledronic acid and human rIL-2</td>
<td>Advanced renal cell carcinoma</td>
<td>11</td>
<td>Cell quantity not specified, 4 mg zoledronate, 1.4 × 10⁶ IU rIL-2, Days 0–4, all of the procedure was repeated every 4 weeks for six times</td>
<td>one complete remission, five stable disease, and five progressive disease</td>
</tr>
<tr>
<td>Abe et al. [119]</td>
<td>Adoptive transfer of Vγ9Vδ2 T cells expanded with zoledronic acid and IL-2</td>
<td>MM</td>
<td>6</td>
<td>9.9 × 10⁸/Infusion, four infusions at 2-week intervals</td>
<td>66%</td>
</tr>
<tr>
<td>Bennouna et al. [120]</td>
<td>Adoptive transfer of Vγ9Vδ2 T cells expanded with BrHPP and IL-2, followed by s.c. injections of IL-2</td>
<td>Metastatic renal cell carcinoma</td>
<td>10</td>
<td>Cells (1–8×10⁶)/infusion, followed by s.c. IL-2 (2×10⁶ IU/m²/day), three infusions at 21-day intervals</td>
<td>60%</td>
</tr>
<tr>
<td>Sakamoto et al. [121]</td>
<td>Adoptive transfer of Vγ9Vδ2 T cells expanded with zoledronic acid and IL-2</td>
<td>Advanced nonsmall cell lung cancer</td>
<td>15 (12 evaluable)</td>
<td>Cumulative number of γδ T cells infused 2.6–45.1 × 10⁶, divided in six to 12 infusions every 2 weeks</td>
<td>No objective responses, six patients had stable disease</td>
</tr>
<tr>
<td>Noguchi et al. [122]</td>
<td>Adoptive transfer of Vγ9Vδ2 T cells expanded with zoledronic acid and IL-2</td>
<td>Various advanced solid tumors</td>
<td>25</td>
<td>4 ± 2 × 10⁹ cells/infusion</td>
<td>Primary end-point was feasibility; out of four patients given Vγ9Vδ2 T cells alone, two had stable disease, and two progressive disease</td>
</tr>
</tbody>
</table>
TABLE 4. Summary of “Open Basic Research and Clinical Questions”

<table>
<thead>
<tr>
<th>Summary: basic research questions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Which are the molecular and cellular mechanisms underlying the differential susceptibility of ALL blasts from pediatric and adult patients to NK cell lysis?</td>
</tr>
<tr>
<td>Why is the mother a better donor than the father in terms of prevention of leukemia recurrence?</td>
</tr>
<tr>
<td>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?</td>
</tr>
<tr>
<td>Do activating receptors play different roles in different types of leukemia?</td>
</tr>
<tr>
<td>Which is the best cytokine combination and duration of ex vivo culture to optimize the effect of NK cells against malignant cells?</td>
</tr>
<tr>
<td>Which tests can be used to monitor the γδ T cell response to stimulation (besides evaluation of tumor burden) and its in vivo-activated state?</td>
</tr>
<tr>
<td>Summary: clinical questions</td>
</tr>
<tr>
<td>Which is the hierarchy for the factors related to the NK cell-</td>
</tr>
<tr>
<td>What protocols can be designed to exploit these properties and optimize cancer immunotherapy?</td>
</tr>
<tr>
<td>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 γδ T cells infused in patients with colorectal carcinoma, demonstrated that they express IL-2Rβγc, 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 γδ T cells. In this respect, ex vivo-expanded Vγ9Vδ2 T cells maintained with IL-15 have increased effector functions [154]. A further layer of complexity is a result of the recent observation that V81+ 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 Vγ9Vδ2 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].</td>
</tr>
<tr>
<td>Finally, studies are currently under way to evaluate the possibility of transducing γδ 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 αβ chains of the TCR, involved in GvHD pathophysiology.</td>
</tr>
</tbody>
</table>

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