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BASIC-TRANSLATIONAL REVIEW

At the Bench: Preclinical rationale for exploiting NK cells and γδ T lymphocytes for the treatment of high-risk leukemias

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ABSTRACT

NK cells and γδ T lymphocytes display potent cytolytic activity against leukemias and CMV-infected cells and are thus, promising immune effector cells in the context of allo-HSCT. NK cells express HLA class I-specific inhibitory receptors and preferentially kill HLA class I^{low} tumors or virus-infected cells. Killing occurs upon engagement of activating NKRs with ligands that are upregulated on tumors and infected cells. A similar activating receptor/ligand interaction strategy is used by γδ T cells, which in addition, use their TCRs for recognition of phosphorylated antigens and still largely undefined ligands on tumor cells. In the haploidentical allo-HSCT setting, alloreactive NK cells, derived from donor HSCs, can exert potent antileukemia activity and kill residual patient DCs and T cells, thus preventing GvHD and graft rejection. However, generation of KIR^+ alloreactive NK cells from HSCs requires many weeks, during which leukemia relapses, and life-threatening infections may occur. Importantly, mature NK cells and γδ T cells can control certain infectious agents efficiently, in particular, limit CMV reactivation, and infusion of such donor cells at the time of HSCT has been implemented. Development of novel, cell-based immunotherapies, allowing improved trafficking and better targeting, will endow NK cells and γδ T lymphocytes with enhanced antitumor activity, also making them key reagents for therapies against solid tumors. The clinical aspects of using NK cells and γδ T lymphocytes against hematological malignancies, including the allo-HSCT context, are reviewed in the related side-by-side paper by Locatelli and colleagues [1]. ***J. Leukoc. Biol.*** **94: 1123–1139; 2013.**

Introduction

Different effector cells are involved in immune responses against tumors. These include αβ TCR^+, γδ TCR^+, and NK cells [2]. The anti-tumor activity of these cells is primarily related to their ability to kill cancer cells or to secrete cytokines that may affect tumors directly or indirectly by the recruitment/activation of other effector cell types. In view of the proven capability of T and NK cells to kill tumor cells, various approaches have been adopted to increase their anti-tumor activity [3, 4]. In the case of αβ TCR^+ T cells, their ability to respond specifically to tumor antigens has been exploited for their selective in vitro and ex vivo expansion [5]. Thus, tumor-specific T cell populations or T cell clones have been generated and used for adoptive immunotherapy in cancer patients [6–8]. In addition, various protocols of vaccination have been attempted, particularly in melanoma, in which a number of relatively immunogenic tumor-associated antigens have been identified [9]. Whereas evidence of tumor-specific responses has been clearly obtained, in most instances, the clinical outcomes have been less positive than expected. The partial clinical failure of tumor vaccines may be related, at least in part to an ineffective homing of specific T cells to tumor lesions, to the high tumor burden (resulting in low effector:tumor-target ratios), and to the inhibitory effect exerted by the tumor microenvironment on T cell function [9, 10]. Several of these inhibitory mechanisms, mediated by tumor cells themselves and/or by

Abbreviations: ADCC=antibody dependent cellular cytotoxicity, ALL=acute lymphoblastic leukemia, allo-HSCT=allogeneic hematopoietic stem cell transplantation, AML=acute myeloid leukemia, B-CLL=B cell chronic lymphocytic leukemia, BM=bone marrow, BTN3A1=butyrophilin 3A1, CAR=chimeric antigen receptor, CCR=chemokine receptor, CMV=cytomegalovirus, CTL=cytolytic T lymphocyte, DC=dendritic cell, DNAM-1=DNAX accessory molecule-1, GvHD=graft-versus-host disease, GvHR=graft-versus-host response, GvL=graft-versus-leukemia, HLA=human leukocyte antigen, HMBPP=(E)-4-hydroxy-3-methyl-butenyl pyrophosphate, HSC=hematopoietic stem cell, ID2=inhibitor of DNA-binding 2, IDO=indoleamine 2,3-dioxygenase, IL=interleukin, ILC=innate lymphoid cell, IPP=isopenthenyl pyrophosphate, KIR=killer Ig-like receptor, KIR2DL=long-tailed KIR2D, KIR2DS=short-tailed KIR2D, LGL=large granular lymphocyte, LTi=lymphoid tissue inducers, MDSC=myeloid-derived suppressor cell, MHC=major histocompatibility complex, MICA/B=MHC class I chain-related A/B, MIUR=Ministero dell'Istruzione, Università e Ricerca, NCR=natural cytotoxicity receptor, NK=natural killer, NKR=NK cell receptor, NTBA=NK-T-B-cell antigen, PB=peripheral blood, PGE2=prostaglandin e2, PVR=poliovirus receptor, Rae=retinoic acid early-inducible, ROR=retinoic acid receptor-related orphan receptor, TCR=T cell receptor, TGF-β=transforming growth factor β, TLR=toll-like receptor, ULBP=UL16-binding protein

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tumor-induced immunosuppressive cells (e.g., regulatory T cells, M2-macrophages, MDSCs, tumor-associated fibroblasts), have been molecularly identified (e.g., PGE2, IDO-induced kynurenine, TGF-β, programmed death ligand 1) [11–17]. Notably, metronomic use of standard chemotherapeutic agents may help revert tumor-induced immunosuppression. 5-Fluorouracil was, e.g., shown to selectively kill MDSCs and thereby, lead to enhancement of anti-tumor immune responses [18]. Nevertheless, the genetic instability of many cancers ensures considerable intratumor heterogeneity, which makes each population of malignant cells a mosaic of different biological targets [19]. This facilitates the development and selection of immune escape and/or therapy-resistant subclonal tumor variants [20].

In the case of NK cells or $\gamma\delta$ T lymphocytes—two cell types sharing properties of innate and adaptive effector cells—their anti-tumor activity is based on the capability of recognizing ligands that are primarily, if not exclusively, expressed by tumor cells [21–25]. These include an array of ligands recognized by NK cells via their activating receptors or coreceptors (some of which are also recognized by $\gamma\delta$ T cells, primarily via the NKG2D receptor) and phosphoantigens recognized by $\gamma\delta$ T cells via their TCRs (see below). Both cell types are capable of cytokine-dependent (primarily IL-2 and IL-15) proliferation, which may allow the selective expansion of subsets displaying a more pronounced anti-tumor activity [26]. Tumor cell killing is also regulated by HLA class I-specific inhibitory receptors, particularly in the case of NK cells, where KIR-mediated recognition of allotypic HLA class I antigens is a major determinant for target-cell recognition [27, 28]. Tumor cells may frequently display down-regulation of HLA class I molecules, involving all class I surface molecules or given alleles. This results in the lack of inhibitory interactions and may greatly favor tumor cell lysis, which in the case of allelic losses by tumor cells, is mediated preferentially by given KIR^+ subsets. To exploit better the anti-tumor activity of $\gamma\delta$ T lymphocytes and NK cells, in vivo stimulation with appropriate ligands and/or cytokines (e.g., IL-2 or IL-15) and adoptive transfer of ex vivo-expanded populations have been applied in tumor immunotherapy [29]. Perhaps more importantly, adoptive transfer of HSCs also gives rise to $\gamma\delta$ T cells and NK cells, including mature KIR^+ alloreactive NK cells, as in the case of haploidentical allo-HSCT to cure potentially high-risk leukemias [3, 6, 30]. In this therapeutic context, innate immune effectors will also play critical roles against infections, in particular, CMV reactivation [31–33]. Specifically, as donor $\alpha\beta$ T cell responses must be minimized after haploidentical allo-HSCT, to prevent harmful GvHD, NK cells and $\gamma\delta$ T lymphocytes may be the major antiviral effector cell populations post-transplantation [31, 33]. Consequently, they are crucial to limit CMV reactivation until a new, functional recipient adaptive-immune system eventually develops from the transferred HSCs [31, 33].

In this review, we will illustrate the main phenotypic and functional characteristics of NK cells and $\gamma\delta$ T lymphocytes, with particular focus on aspects/properties that are relevant to their optimal clinical exploitation. Clues and suggestions will be provided for possible future implementations and improvements of clinical protocols. In this context, it is worth noting that whereas remarkable clinical results have been obtained in the therapy of high-risk leukemias, results with solid tumors have been limited.

BIOLOGY AND ANTI-TUMOR ACTIVITY OF NK CELLS AND $\gamma\delta$ T CELLS

NK cells

NK cells are an important component of the innate immune system and play a key role in host defense by virtue of their cytolytic activity against tumor cells and virus-infected cells and their ability to release a number of chemokines mediating inflammatory responses [34–36]. NK cells were described originally four decades ago on a functional basis; the ability to kill various tumors in a "natural fashion", i.e., without deliberate activation in vitro or in vivo [37]. Further studies revealed that this anti-tumor activity was associated with a lymphoid population displaying a typical morphology, characterized by cytoplasmic azurophilic granules. Accordingly, they were named LGLs. Only in the 1980s were mAb available that recognized antigens selectively expressed by a fraction of LGL. One antigen targeted by these mAb was CD16, a low-affinity receptor allowing NK cells to mediate ADCC, which is an important effector function of NK cells [38–40]. As CD16 is acquired at late stages of NK cell maturation (**Fig. 1**), ADCC is mainly confined to the most mature NK cells with high cytolytic potential. Notably, mature NK cells also acquire KIRs. Thus, $CD16^+KIR^+$ NK cells may play an important role, not only because of their potential "alloreactivity" in the haploidentical allo-HSCT setting (see below) but also for their ADCC activity in cancer patients treated with anti-tumor mAb. The clinical importance of CD16-dependent, NK cell-mediated ADCC for the treatment of cancer has indeed been demonstrated, e.g., in the context of rituximab administration to lymphoma patients with different CD16 polymorphisms [41].

Another relevant human NK cell marker, also useful to identify NK cell subsets endowed with different functional properties, is CD56 (Fig. 1). Indeed, the levels of CD56 surface expression correlate with stages of human peripheral NK cell maturation and function [42, 43]. Although several NK cell markers had been identified, the molecular mechanism by which NK cells kill tumor cells, while sparing normal cells, remained mysterious. In the late 1980s, the "missing self-hypothesis" provided an important conceptual clue for subsequent studies in human and mice that revealed the existence of MHC class I-specific inhibitory receptors [44]. The missing self-hypothesis was based on the observation that murine NK cells killed MHC class I-deficient lymphoma cells, while sparing the parental MHC class I^+ cells [45]. The prototypes of human HLA class I-specific receptors were identified, thanks to the production of mAb specific for two homologous surface molecules expressed by NK cell subsets and capable of modulating NK cell function [46, 47].

Missing self hypothesis: NK cells preferentially kill target cells missing (self-) MHC class I expression.

KIRs are HLA class I-specific receptors recognizing determinants shared by groups of HLA class I alleles. Inhibitory and activating KIRs exist. KIRs are expressed at late stages of NK cell differentiation.

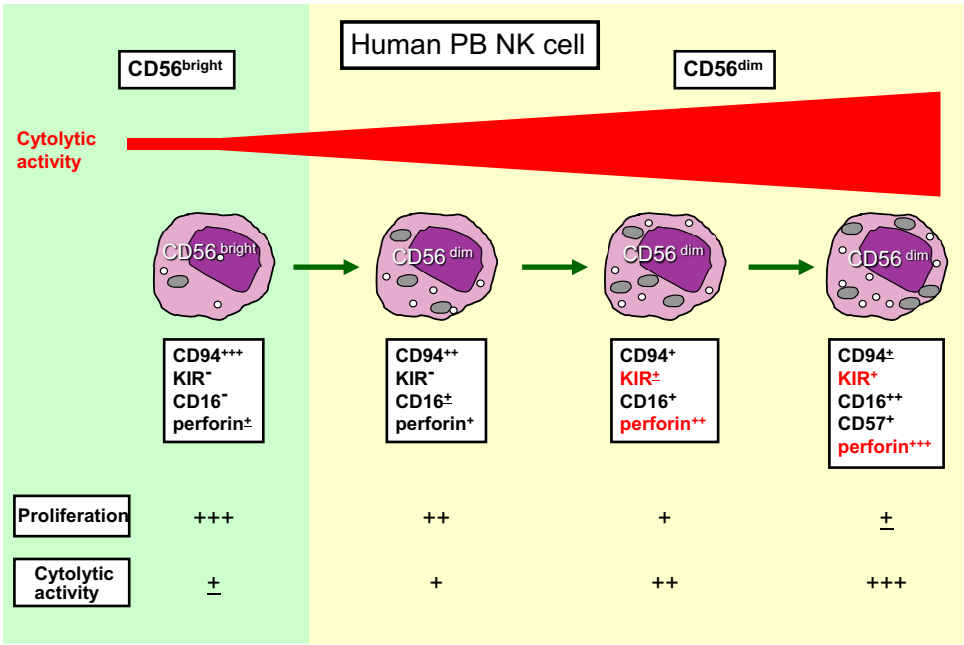


Figure 1. NK cell subsets present in human PB. The level of CD56 surface molecules allows for distinguishing two major, developmentally related NK cell populations: $CD56^{bright}$ and $CD56^{dim}$. There is now evidence that the $CD56^{dim}$ subset derives from $CD56^{bright}$ NK cells and that further maturation of $CD56^{dim}$ cells can be traced on the basis of the expression of several additional markers/receptors, as depicted in this figure. Notably, the acquisition of CD16 and CD57 antigens corresponds to the most mature stage(s) of differentiation, characterized by a potent cytolytic activity but low capability of proliferation. Note that also, KIR expression occurs late during NK cell maturation. Accordingly, "alloreactive" (KIR^+) NK cells are confined to the most mature NK cell fraction.

The human NK cell subsets, identified on the basis of the expression of these molecules, termed p58.1 and p58.2, differed in their capability of killing normal allogeneic cells (e.g., PHA-induced lymphoblasts) from certain individuals [46, 47]. p58 Receptors were the prototypes of the KIRs and found to be specific for two groups of allotypic determinants expressed by HLA-C alleles, delivering inhibitory signals upon engagement with their specific HLA class I ligands [48–51]. Additional members of the KIR family were identified, including KIRs with two or three extracellular domains (KIR2D and KIR3D, respectively) [52, 53] and activating KIRs [54]. The activating KIRs are highly homologous to the corresponding inhibitory form in the extracellular portion but are characterized by relevant differences in their transmembrane and cytoplasmic domains [55]. Specifically, the cytoplasmic domain is short and linked to signaling polypeptides mediating NK cell activation upon KIR cross-linking with mAb or interaction with relevant HLA class I alleles. Another important HLA class I-specific inhibitory receptor is the heterodimer NKG2A/CD94, related to C-type lectins and specific for the class Ib molecule HLA-E [56, 57]. In an autologous setting, virtually all NK cells express at least one inhibitory receptor for self-MHC class I alleles. The lack of expression of appropriate HLA ligands renders cells susceptible to lysis. Whereas in an autologous setting, the defective surface expression of HLA molecules often reflects tumor transformation or viral infection, in an allogeneic setting, some NK cells may express KIRs that do not recognize the normally expressed HLA class I alleles. As they can kill normal allogeneic cells (e.g., PHA-induced lymphoblasts), they were defined alloreactive [27, 46–48, 58]. KIR2DL and CD94/NKG2A were also detected in a subset of $\alpha\beta$ TCR^+ CTLs of some individuals, in which they could exert an inhibitory effect on TCR-induced cytolytic activity or lymphokine production [59, 60]. CTLs expressing KIRs were found to be mono- or oligoclonally expanded populations [61, 62]. More recent studies revealed that the TCRs of these KIR^+ CTLs could be HLA-E-specific, with only moderate peptide dependence [63–65], or display highly selective, specific reactivity against certain HLA-E-restricted CMV-derived peptides [66].

NK cell triggering is mediated by an array of non-MHC-specific activating receptors (**Fig. 2**). The prototypes of such activating NKRs in humans are NKp46 [67, 68], NKp44 [69, 70], and NKp30 [71], and these were collectively named NCRs [72]. NKp46 is also expressed in mice and represents a useful marker for human and murine NK cell identification. Whereas NKp46 and NKp30 are expressed on resting and activated NK cells, NKp44 is expressed only by cytokine-activated effector cells.

Activating NKRs are involved in NK cell and γδ T lymphocyte recognition of target cells, primarily "stressed" cells, such as tumors or virus-infected cells. NCRs refer to the prototypes of human-activating receptors: NKp46, NKp30, and NKp44. Together with NKG2D, DNAM-1, and activating KIRs, NCRs play a central role in tumor cell killing by NK cells and γδ T lymphocytes.

Ligands of activating NKRs: structures expressed de novo or up-regulated in stressed cells, e.g., virus-infected or transformed cells. Their expression represents an important checkpoint in NK cell activation and also governs γδ T cell tumor targeting.

Other activating NKRs include NKG2D, DNAM-1 (CD226), 2B4 (CD244), and NKp80, which are also present on certain T cell subsets, whereas the NTBA receptor is expressed on T and B cells. Various ligands of activating receptors have been identified and include B7H6 [73], HLA-B-associated transcript 3 [74], and the human CMV pp65 tegument protein [75] for NKp30 [73, 74]; ULBPs 1–6 and MICA/MICB for human NKG2D [76]; PVR (nectin-like-5, CD155) and nectin-2

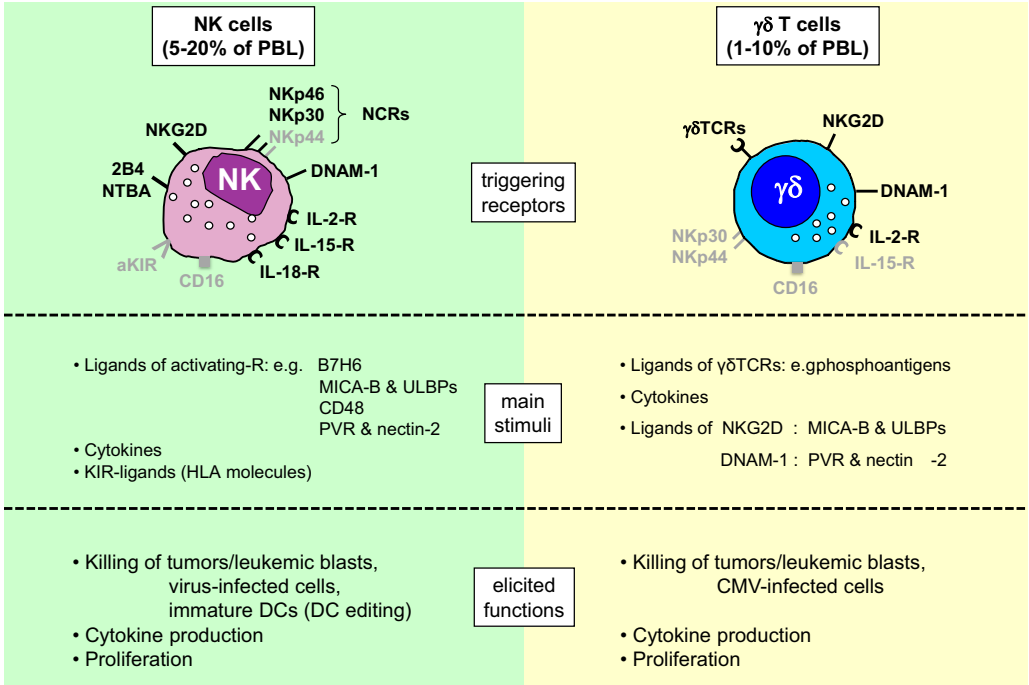


Figure 2. Human NK cells and γδ T lymphocytes. This figure highlights the main receptors involved in the activation of these effector cells and lists the corresponding ligands. Some of these ligands are expressed preferentially in tumor/leukemia cells, and their recognition by NK cells or γδ T lymphocytes leads to immune cell activation and/or target cell lysis. These mechanisms of recognition/activation are all important for the antileukemic activity of these effector cells. Gray represents receptors that are expressed (or selectively induced) on only a limited subset of the cells, whereas black represents receptors that are constitutively expressed on most of the cells. aKIR, activating KIR.

(CD112) for DNAM-1 [23]; and CD48 for 2B4 [77]. NK cells also express various TLRs, including TLR2, -3, -7, -8, and -9, which can amplify responses mediated by activating NKRs [78–80]. The expression of TLRs implies that NK cells can sense and be activated rapidly by different microbial products. In addition to receptor-mediated activation, NK cell effector function may be induced (or amplified) by cytokines, including IL-2, IL-15, IL-12, and IL-18. Thus, NK cell activation and target cell lysis depend on complex interactions between inhibitory and activating signals delivered by surface receptors and cytokines [81]. On the other hand, other cytokines, such as TGF-β [82, 83] and IL-10, soluble factors, such as the IDO metabolite kynurenine [84], and prostaglandins (PGE2) may exert an inhibitory effect [12, 14, 82, 83, 85].

Another issue of the NK cell biology relevant to their anti-tumor activity in the HSC transplantation setting is their differentiation from HSCs. NK cells originate from $CD34^+$ HSCs through discrete stages characterized by the progressive acquisition of markers/receptors and functional characteristics [86–89]. NK cells derive from $ID2^+$ precursors, and their progression is regulated by transcription factors, such as E4 promoter-binding protein 4 and thymocyte selection-associated 3-hydroxy-3-methylglutaryl box factor [90–92]. Interestingly, it has been shown that common granulocyte and monocyte precursors maintain the ability to differentiate in vitro toward NK cells, for example, in the presence of steroids [93–95]. BM was originally considered the only site of NK cell differentiation. However, recent studies revealed that NK cell development can occur also in other tissues, and $CD34^+$ cells isolated from several sites, including PB, umbilical cord blood, LNs, tonsils, thymus, decidua, liver, and intestinal lamina propria, can undergo in vitro differentiation toward NK cells in the presence of appropriate cytokines [96–103]. Moreover, NK cells at different stages of differentiation have been identified in some of these tissues (e.g., tonsils, LNs, and decidua) [86–89, 99]. Thus, it is likely that HSCs or committed precursors may migrate from BM to the periphery, where they can differentiate toward NK cells. Notably, $CD34^+$ cells from different sites can also undergo differentiation toward other ILCs, including the poorly cytotoxic "NK22", LTi, and natural helper cells [104]; whether these cells arise from precursors common to NK cells or rather from still-undefined committed precursors existing in the $CD34^+$ cell fraction remains to be defined and is presently under investigation (E. Montaldo and C. Romagnani, personal communication). In addition, it would be interesting to investigate whether and how these ILCs are generated from HSCs after transplantation. This information will be particularly important for LTi in view of their role in lymphoid tissue generation from donor $CD34^+$ cells, as well as for the NK22 cells, in view of their role in the innate defenses against intestinal pathogens/maintenance of mucosal integrity [105].

ILCs are an emerging family of NK cell-related innate cells derived from HSCs under the influence of specific transcription factors, such as RORα, GATA3, ID2, or RORγt. ILCs appear to play different functions, including LTi, defense against extracellular parasites (natural helper), epithelial homeostasis, and intestinal immunity (NK22).

Streoids induce preferential and rapid differentiation of $CD34^+$ precursors toward NK cells in vitro. Clinical question: is there any clinical evidence that steroid treatment accelerates the appearance (and the maturation) of NK cells in the PB of patients receiving allo-HSCT?

γδ T cells

The evolutionary advent of somatic recombination generated three types of variable region molecules: the BCR and two types of TCRs—αβ and γδ. Despite being endowed with great potential for diversification, the γδ TCR repertoires tend to be restricted and are often oligoclonal in various murine tissues [106]. Human circulating γδ T cells, which account for 1–10% of PBLs, are polyclonal but frequently express TCRs composed of Vγ9 and Vδ2 chains. By contrast, the majority of tissue-associated human γδ T cells expresses Vδ1$^+$ TCRs, which are also found in 5–30% of circulating γδ T cells [106]. The functional parallel between γδ T cells and NK cells was evident in genome-wide expression analyses, comparing the transcriptional profiles of activated human Vγ9Vδ2 T cell lines and NK cells [107].

On the genomic level, γδ T cells have potential for an extraordinary degree of γδ TCR diversity based on somatic recombination. This notwithstanding, the selected γδ TCR repertoires tend to be quite restricted and are often oligoclonal in specific tissues.

Human γδ T cells account for 1–10% of the PBLs, where the majority expresses TCRs composed of Vγ9 and Vδ2 chains. By contrast, most of tissue-associated human γδ T cells express Vδ1$^+$ TCRs.

The functional properties of γδ T cells make them promising players in cancer immunotherapy; they rapidly produce large amounts of IFN-γ and TNF-α, while being strongly cytotoxic as a result of the expression of perforin, granzymes, and death receptor ligands (Fas ligand, tumor necrosis factor-related apoptosis-inducing ligand) [106]. Like NK cells, but unlike αβ T cells, γδ T cells do not recognize antigens in the context of MHC molecules, thus bypassing tumor immune evasion mechanisms based on MHC class I down-regulation.

It is known, since the mid-1990s, that the major γδ T cell subset in the human PB—Vγ9Vδ2 T cells—is uniquely activated by nonpeptidic prenylpyrophosphate moieties ("phosphoantigens"), such as IPP (reviewed in ref. [108]. Given that the production of IPP, as an intermediate of the mevalonate pathway of isoprenoid biosynthesis, can increase significantly upon malignant transformation, it could potentially act as a "tumor-associated antigen" for Vγ9Vδ2 T cells [109]. Furthermore, as the reactivity to phosphoantigens could be abolished with anti-TCR-blocking antibodies and conferred by Vγ9Vδ2 TCR gene transfer [110], they were postulated to act as Vγ9Vδ2 TCR ligands. However, no direct binding could be demonstrated between the Vγ9Vδ2 TCRs and phophoantigens, including those of microbial origin, such as HMBPP, which possess much higher bioactivity than IPP [111]. This issue was resolved recently with the identification of BTN3A1 as the phosphoantigen-presenting molecule [112]. IPP and HMBPP were shown to bind in a small cleft in the distal V-like domain of BTN3A1, which established cognate interactions with soluble Vγ9Vδ2 TCRs [112]. These results contrasted with those of Scotet and collaborators [113], who failed to detect cognate interactions between BTN3A1 and Vγ9Vδ2 TCRs and instead proposed that phosphoantigens interacted with the cytoplasmic domain of BTN3A1 to induce conformational changes and/or clustering of BTN3A1 in target cells.

Phosphoantigens (prenylpyrophosphates) are well-known to activate specifically the major γδ T cell subset in the human PB, which expresses Vγ9Vδ2 TCRs. These have been shown recently to bind phosphoantigens presented by BTN3A1, a ubiquitously expressed, nonpolymorphic, Ig-like protein. Various ligands recognized by individual Vδ2$^-$ T cell clones have also been described, but these have not represented frequent TCR specificities that are readily identifiable in multiple individuals. The cognate ligands for the TCRs of most Vδ2$^-$ T cells thus remain elusive.

Beyond the γδ TCRs, the recognition of target cells by γδ T cells is (as for NK cells) often dependent on multimolecular stress signatures involving diverse costimulatory ligands (Fig. 2). Many typical NKRs, e.g., NKG2D, CD94/NKG2A, CD94/NKG2C, DNAM-1, Ig-like transcript 2, CD161, KIR2DL1–3, NKp30, and NKp44, can be expressed by γδ T cells and can regulate their activities [26, 111, 114–117].

Various studies have emphasized the unique contribution of NKG2D to γδ T cell-mediated tumor immunosurveillance in mice and humans. NKG2D ligands are not expressed by most normal tissues but are up-regulated in many tumor cell types, as well as virus- or bacteria-infected cells [114, 118–120]. In mice, Rae, murine ULBP-like transcript, and H-60 proteins are indeed critical, stress-induced molecules acting as ligands for NKG2D [120]. Mice lacking γδ T cells were highly susceptible to multiple regimens of cutaneous carcinogenesis, and this susceptibility was regulated by NKG2D expressed on γδ T cells [121]. Murine γδ T cells responded rapidly in vivo to the NKG2D ligand Rae-1 [122], which is strongly up-regulated upon carcinogenesis [121]. Moreover, the sustained expression of transgenic Rae-1 impaired T cell cytotoxicity and strongly interfered with tumor immunosurveillance [123]. Of note, NKG2D-deficient mice showed impaired immunosurveillance of epithelial and lymphoid malignancies in two transgenic models of de novo carcinogenesis [124].

In humans, the NKG2D ligands ULBP1, ULBP2, ULBP3, and ULBP4 have all been implicated in tumor cell recognition by γδ T cells. ULBP1 was abundant in susceptible hematological tumor cell lines [125] and was required for leukemia/lymphoma cell recognition by Vγ9Vδ2 T cells [126]. Furthermore, ectopic expression of ULBP4 in ovarian and colon carcinoma cells triggered Vγ9Vδ2 T cell cytotoxicity against these solid tumors [127]. On the other hand, patient-derived Vδ1$^+$ T cells recognized ULBP2 and ULBP3, expressed on B-CLL and non-Hodgkin lymphoma tumor cells [128, 129]. These data make a strong case for NKG2D as a major determinant of tumor cell recognition by human γδ T cells, not least in the context of leukemias [117].

Another activating receptor implicated in tumor cell recognition by NK cells and γδ T cells is DNAM-1 [23, 115, 130–135]. DNAM-1 ligands PVR and nectin-2 are expressed on various types of carcinomas, and the interactions between DNAM-1 and PVR regulated γδ T cell recognition of hepatocellular carcinoma cell lines [115]. Among hematological cancers, DNAM-1 ligands are expressed by blasts from patients with, e.g., myelodysplastic syndrome, multiple myeloma, and AML

and regulated tumor killing by NK cells and γδ T cells [130, 133, 136, 137]. Specifically, Vγ9Vδ2 T cells efficiently killed AML blasts in vitro, triggered by a combination of TCR and DNAM-1 signals, which translated into enhanced survival of leukemic mice in a xenotransplantation model of AML [130]. Recently, DNAM-1 signaling in murine NK and T cells was also shown to govern their ability to induce regression of spontaneous early-stage B cell tumors [138].

Correia et al. [26] have recently characterized a $V\delta1^+$ T cell population capable of targeting hematological tumor cell lines and primary B-CLL cells that were resistant to fully activated Vγ9Vδ2 T cells. It was shown that this $V\delta1^+$ subset owes its specialized killer function to the induced expression of the NCRs NKp30 and NKp44, which had been regarded previously as NK cell-specific markers [139]. Although neither $V\delta1^+$ nor $V\delta2^+$ T cells express NCRs constitutively, these can be up-regulated selectively in $V\delta1^+$ T cells by AKT-dependent signals provided synergistically by γ-chain cytokines (IL-2 or IL-15) and TCR stimulation [26]. This observation provides a link between the two types of receptor signals, with NCR induction in $V\delta1^+$ T cells induced "downstream" of TCR activation. NKp30 and NKp44 are functional in NCR^+ $V\delta1^+$ T cells and contribute nonredundantly to enhanced targeting of lymphocytic leukemia cells, with NKp30 playing the major role in this process [26].

Several different subsets of γδ T cells also express CD16 and mediate ADCC [140–142], which provides a solid rationale for treatment regimens combining distribution of anti-tumor mAb with agents activating γδ T cells in vivo (e.g., phosphoantigens) or adoptive transfer of ex vivo-expanded γδ T cells in cancer patients [143, 144].

γδ T cells express multiple activating NKRs and can recognize various types of tumor cells via these and their TCRs. γδ T cells can also express functionally inhibitory NKRs, such as CD94/NKG2A and KIRs.

The evidence for human γδ T cell targeting of a variety of tumor types in vitro (and in vivo in xenograft models) has been upheld by seminal studies with animal models that have clearly demonstrated that the genetic absence of γδ T cells renders mice significantly more susceptible to transgenic [145], chemically induced [121], transplantable [146], and spontaneous [147] tumors in vivo. This global knowledge of the anti-tumor properties of γδ T cells has fueled various clinical efforts. Phase I and II studies have thus been performed to test the safety and efficacy of γδ T cell-based immunotherapy in patients with advanced malignant disease, refractory to conventional treatments, as reviewed elsewhere [148, 149].

The expression of CD94/NKG2A receptors, functionally inhibiting cells upon interaction with HLA-E, may prevent NK cell alloreactivity. Clinical question: is there any evidence that increased expression levels of CD94/NKG2A (or any other inhibitory NKR) on γδ T cells may negatively correlate with the clinical outcome in cancer patients?

allo-HSCT FOR LEUKEMIA TREATMENT

Allogeneic HSCT is the preferred treatment for a variety of high-risk or relapsed leukemias [150–153]. However, most (~75%) patients lack a HLA-matched sibling and thus, require an alternative donor [154]. Although a HLA-matched, unrelated donor can be found in ~40% of patients, the use of readily available, haploidentical-related donors, i.e., first-degree relatives who share one haplotype with the patient, while being fully or largely mismatched for the other HLA alleles, has been implemented successfully [30, 154–156].

T cell-mediated GvHRs, following allo-HSCT, results in potent therapeutic opportunities as well as potentially severe side-effects. GvHRs exert multiple beneficial functions in the haploidentical allo-HSCT setting. They reduce the risk of graft rejection/improve engraftment by (1) promoting cytokine production by donor T cells and (2) underlying killing of residual host T cells. Moreover, GvHRs efficiently eliminate residual leukemia blasts directly. The later activity, i.e., the GvL effect, is considered the most important type of GvHR for the cure of high-risk blood cancers. However, as most αβ T cells responsible for the beneficial GvHRs are not tumor-specific but are activated by ubiquitous αβ TCR-HLA interactions, they also react against normal tissues and may thereby cause life-threatening GvHD.

On the contrary, γδ T cells and alloreactive NK cells, via their triggering receptors, recognize and are activated by ligands that are preferentially expressed by tumor cells but not by normal tissues (with the exception of hematopoietic cells). Thus, these innate effector cells do not cause GvHD, whereas they kill the patient's leukemia cells, as well as DCs and T cells and thereby, further prevent graft rejection and GvHD mediated by the few contaminating donor αβ T cells.

Haploidentical allo-HSCT using T cell-replete grafts presents major risks associated with GvHD, which may be counteracted partially by post-transplantation prophylactic treatment with, e.g., mycophenolate mofetil plus a calcineurin inhibitor (i.e., tacrolimus or cyclosporine) and cyclophosphamide [157, 158] or methotrexate [159]. Rapamycin and various cell-based therapies are also being tested as novel strategies for GvHD prevention. Regrettably, the administration of immunosuppressive agents post-transplantation was associated with an increased risk of leukemia relapse [160].

The principal mediators of GvHD after haploidentical allo-HSCT are donor-derived alloreactive αβ T cells targeting the mismatched HLA molecules. To avoid GvHD, transplants were thus based on purified $CD34^+$ HSCs. The poor engraftment obtained initially was tackled by transferring megadoses of purified $CD34^+$ cells [155, 156, 161]. Under this transplantation setting (that did not require subsequent pharmacological GvHD prophylaxis), the engraftment rate was >90%, whereas the incidence of Grade II–IV GvHD was <10% [30, 155, 156]. Subsequent clinical trials using megadoses of purified $CD34^+$ cells confirmed a high engraftment rate and a low occurrence of GvHD in adult patients with AML or myelodysplastic syndromes.

As the same αβ T cells that cause GvHD also play a major role in the GvL effect, leukemia relapses were expected in most patients receiving purified $CD34^+$ cells. On the contrary, a subgroup of AML patients was characterized by long-term survival and very low risk of relapses. A strong correlation was found between lack of leukemia relapses and the presence, in the donor, of NK cells alloreactive toward patient target cells [30].

Alloreactive NK cells were described over 20 years ago [46, 47, 58, 162], when killing of allogeneic lymphoblasts was associated with subsets characterized by the expression or lack of expression of novel surface molecules [46, 47], subsequently identified as HLA class I-specific KIRs [27, 48, 49, 163, 164]. Although transplantation of megadoses of $CD34^+$ cells in patients with high-risk AML was undoubtedly a major breakthrough, it suffered from relevant limitations, such as the slow immune reconstitution and the risk of infections and early relapses. A likely explanation for the latter problem is the considerable time period required for generation of KIR^+ alloreactive NK cells (6–8 weeks). In an attempt to overcome some of these limitations, CD3-depleted grafts, rather than purified $CD34^+$ cells, were used [165]. To limit viral-driven malignant growth of B cells and/or B cell-driven GvHD under the T cell-depleted conditions, $CD19^+$ cells were also routinely eliminated from the graft before transplantation [166, 167]. These protocols allowed retention of NK cells as well as monocytes and DCs in the graft and also supported engraftment after reduced-intensity conditioning regimens [168]. This notwithstanding, these transplants were still associated with slow immune reconstitution and infectious complications [169]. A potential factor contributing to this could be that the recipients, in addition to conditioning fludarabine, thiotepa, and melphalan, received daily injections of the anti-CD3 mAb OKT-3, from 5 days before the transplant until 2 weeks post-transplantation, to deplete remaining T cells in vivo [170]. In addition, mycophenolate mofetil was used as post-transplantation immunosuppression when the T cell content in the graft was $\geq 5 \times 10^4$ $CD3^+$ cells/kg [170]. Selective depletion of or induction of anergy in the alloreactive T lymphocytes, as well as adoptive transfer of pathogen-specific T cell populations, has been developed to offer refined ways to target infections, without increasing the risk of GvHD [171–174].

A valuable strategy to improve further the clinical outcome in haploidentical allo-HSCT is to use protocols that preserve not only NK cells but also γδ T cells in grafts. This can be achieved by depleting αβ TCR^+ T cells (and $CD19^+$ B cells) specifically [175–177]. The rationale for this "TCR-αβ/CD19-depletion protocol" comes from early clinical studies that reported better disease-free and overall survival, in the absence of exacerbated GvHD, in acute leukemia patients developing unusually high counts of γδ T cells after receiving partial HLA-mismatched allo-HSCT [178]. Notably, such γδ T cell expansions were observed preferentially when the γδ T cells had been left in the graft, i.e., when anti-αβ TCR antibodies (rather than anti-CD3 antibodies) were used for T cell-depletion [178]. In this transplantation setting, $CD34^+$ HSCs are given, together with fresh, mature NK cells and γδ TCR^+ T cells. It is important to stress that patients given such allografts, depleted of αβ T cells and B lymphocytes, normally do not receive any type of pharmacological immunosuppression after the HSCT. This protocol thereby makes it possible to provide functional γδ T cells and alloreactive NK cells that may exert their antileukemia activity soon after transplantation, thus lowering the risk of rapid leukemia relapses occurring in cases of partial responses to the conditioning regimen and/or in rapidly proliferating leukemias. The subsequent generation of alloreactive NK cells from grafted HSCs assures a late and more-durable protection, as suggested by the detection of donor-derived alloreactive NK cell subsets, months or even years after transplantation in patients transplanted with $CD34^+$ cells alone [179].

Allogeneic HSCT is a potentially curative treatment for various hematological malignancies, but the slow immune reconstitution after haploidentical allo-HSCT has led to high risk of detrimental infections. Notably, acute leukemia patients that spontaneously developed atypically high levels of Vδ1$^+$ T cells after allo-HSCT exhibited enhanced disease-free and overall survival.

Preliminary clinical data available from HSCT patients that received TCR-αβ/CD19-depleted haploidentical grafts clearly show that this approach leads to low incidence of GvHD that furthermore, is restricted to the skin and thus, does not affect the gut or liver. It is conceivable that the immediate availability of donor-derived, mature NK cells and γδ T lymphocytes in the graft mediate prompts elimination of the recipient's hematopoietic cells (including DCs) and thus, further limits hyperacute and acute GvHD, which is of course already largely prevented by the physical removal of the αβ T cells, the principal effectors driving this complication. Importantly, this translates into a prevention of transplantation-related mortality. Longer follow-up is needed to fully understand the impact of this type of graft manipulation on the risk of leukemia recurrence and patient survival (F. Locatelli, personal communication).

Clinical questions: *were the high levels of γδ T cells, established long after allo-HSCT, the cause and/or an effect of the beneficial clinical antileukemia response in the patients? By what mechanism does the presence of γδ T cells in the graft promote the development of high levels of Vδ1$^+$ T cells long after allo-HSCT? Will certain subsets of unmanipulated γδ T cells preferentially undergo homeostatic proliferation when infused as part of a graft devoid of αβ T cells and B cells?*

NK CELL ALLOREACTIVITY AND DONOR SELECTION IN ALLOGENEIC HSCT

Alloreactive NK cells have been shown to play a fundamental role in the favorable clinical outcome of patients receiving haploidentical allo-HSCT to treat high-risk leukemias, primarily in adults with AML and in pediatric ALL patients. Therefore, phenotypic identification of the alloreactive NK cell subset and assessment of the NK cytotoxicity against leukemic cells represent important criteria in donor selection. Certain inhibitory or activating KIRs have been shown to play a central role in determining NK cell alloreactivity. Particularly important are the inhibitory KIR2DL1 and -2/3, specific for two distinct allelic determinants expressed by two groups of HLA-C alleles, and also, the three Ig-like domain receptors (KIR3D), recognizing a group of HLA-B and HLA-A alleles sharing the HLA-Bw4 supertypic specificity. Among the activating KIRs, the specificity for HLA class I molecules has been clearly established only for KIR2DS1 and KIR2DS4. KIR2DS1 has been shown to play an important role in mediating alloreactivity [179, 180]. KIR2DS1 recognizes the C2 specificity, and thanks

to the potent activating signal delivered, it may also overcome the KIR2DL2/3 or the CD94/NKG2A-mediated inhibition [80] and result in efficient killing of C2/C2 leukemic cells [179]. Therefore, the detection of KIR2DS1 may reveal NK cells with potent alloreactivity and enable a more accurate definition of the size of the alloreactive NK cell subset [28, 179].

A major obstacle in the discrimination between KIR2DL and the corresponding KIR2DS is their high degree of homology. However, recently, mAb capable of discriminating between activating and inhibitory KIRs became available, thus allowing identification of KIR2DS1$^+$ NK cells [181]. This possibility is particularly relevant in the case of pediatric ALL, which express higher levels of HLA class I molecules than AML blasts. Remarkably, the presence of activating KIRs can also be revealed by the analysis of the KIR genotype. In addition, the cytolytic activity of alternative donors' NK cells should be assessed against the patient's leukemic blasts or selected EBV B cell lines to select the donor with the best alloreactivity. It is of note that a direct correlation generally exists between the degree of cytolytic activity and the size of the alloreactive populations.

As alloreactive NK cells derive from haploidentical donor HSCs, it is important to verify whether they are generated in the patient and whether they acquire the expected effector function. It is now well established, in adults and in pediatric patients, that functional alloreactive NK cell subsets consistently develop and can be detected even years after transplantation [179]. Moreover, a correlation could be clearly established between the size of the alloreactive subset and the clinical outcome.

Clinical question: *why do not alloreactive NK cells cause GvHD in spite of the KIR/HLA mismatch?*

An interesting question is why alloreactive NK cells attack leukemic cells but not normal tissues of the recipient. In this context, early evidence suggested that NK cells predominantly kill hematopoietic cells, while sparing tissues that are common targets of T cell-mediated GvHD [182–184]. More recent animal studies unequivocally confirmed that alloreactive NK cells can mediate GvL effects but not GvHD [30]. In this murine model, the authors also showed that alloreactive NK cells kill host DCs, thus reducing the risk of GvHD, as well as residual host T cells, thus preventing graft rejection. As the ligands recognized by activating NKRs are expressed or up-regulated in tissues of different histotypes only upon cell stress, tumor transformation, or viral infection, this may provide the molecular basis to explain the inability of NK cells to attack normal tissues (with the exception of the hematopoietic cells that constitutively express such ligands).

Recent reports have suggested a novel approach for donor selection only based on the KIR genotypic analysis. In particular, it was found that the selection of donors with the B haplotype (containing various genes coding for activating KIRs) correlates with a better overall and relapse-free survival, suggesting that the presence of genes for activating KIRs (particularly those located in the centromeric portion) is, per se, a favorable prognostic element [185]. The main criteria adopted by our group in the selection of haploidentical donors are summarized in **Table 1**. Whereas in our experience, the presence and the size of the alloreactive NK cell population remain the most relevant for donor selection, additional criteria may be of help for further improvement. Such criteria may be crucial when no alloreactive NK cells can be identified.

TABLE 1. Donor Selection Criteria in Haploidentical allo-HSCT

Presence of NK cell alloreactivity
Larger size of the alloreactive NK cell subset
Presence of KIR2DS1 in the case of the C2 patient
KIR genotype: B/x better than A/A
Mother better than father
Higher B content score
Higher percentage of NK cells

When alternative haploidentical donors are available, the features listed correlate with a better clinical outcome and may allow optimal donor selection.

Clinical question: *why have some studies failed to confirm the clinical impact of the presence of donor NK cell alloreactivity in the context of haploidentical allo-HSCT?*

The advent of CD3/CD19-depletion HSCT protocols were largely based on the retention of mature NK cells in the graft. As discussed above, the novel TCR-$\alpha\beta$/CD19-depletion protocol, also preserving $\gamma\delta$ T lymphocytes in the graft, may improve immune reconstitution further and provide early protection against pathogens and leukemia relapse.

NATURAL AND THERAPEUTIC ROLES OF $\gamma\delta$ T CELLS IN THE CONTEXT OF ALLOGENEIC HSCT

The fact that $\gamma\delta$ T cells, in contrast to $\alpha\beta$ T cells, are not restricted by MHC molecules makes them unlikely to elicit GvHD, based on HLA alloreactivity [186]. This feature, in combination with the potent antiviral, by targeting, e.g., CMV-infected cells, and anti-tumor functions, makes $\gamma\delta$ T cells valuable, potential immune effector cells during the lymphopenic post-transplantation phase. Cell-based immunotherapy for lymphoid malignancies has also gained increasing attention, as patients develop resistance to conventional treatments. Notably, similar to NK cells, the spontaneous and cytokine-induced anti-tumor activities of $\gamma\delta$ T cells appear more prominent against hematological cancers than other types of malignancies [24, 147, 148, 187]. There may be common reasons for why these two types of innate effector cells are particularly reactive against leukemia. First, myeloid and B cell lineage cancers may exhibit features of professional APCs, ensuring strong interactions with cells of innate and adaptive immunity. In addition, different from solid tumors, the single-cell nature of leukemias may greatly facilitate their elimination by immune cells. Indeed, the fact that effector cells do not need to infiltrate a solid tumor facilitates target-cell accessibility in blood cancers.

As underlined in Introduction, the tumor microenvironment in solid tumors may contain high concentrations of factors inhibiting lymphocyte proliferation and effector function. This inhibitory effect may be less relevant in leukemia as a result of a more disperse distribution of cancer cells, with consequent lower concentration of inhibitory factors. The response to chemo- and radiotherapy of the conditioning regimen also results frequently in low tumor burden, which greatly facilitates the efficient intervention of alloreactive NK cells against blood cancers. In line with this, the strong benefit of KIR ligand-mismatched allo-HSCT, seen in high-risk leukemias, has not translated to equally successful results with solid tumors [30, 188].

Among the γδ T cells, there is a unique and direct explanation for the preferential targeting of hematological cancers for the Vγ9Vδ2 subset [189, 190]. The phosphoantigens triggering the Vγ9Vδ2 T cells can be induced to accumulate in various types of tumors by targeting of the mevalonate pathway [109, 191]. However, it is primarily hematological tumor cells, in particular, malignant B cells, that naturally produce sufficient endogenous amounts of phosphoantigens to activate resting Vγ9Vδ2 T cells [24]. As the major γδ T cell subset in circulation, further readily expandable in vitro and in vivo, Vγ9Vδ2 T cells have, so far, been the focus of clinical trials exploiting γδ T cells for the treatment of cancer. These cells indeed showed most-promising results in the treatment of hematological cancers [24, 148, 187].

Notably, most hematopoietic cancer cells also express various stress-induced molecules, acting as ligands for activating receptors, present not only in NK cells but also in γδ T cells [126, 192–194]. The resulting uncertainty around the relative contributions of the γδ TCRs and NKRs to tumor cell targeting by γδ T cells has been reviewed recently in detail [117]. Also, Vδ1$^+$ T cells are highly promising as an effector population for cancer immunotherapy, particularly in adoptive cell therapy protocols. Vδ1$^+$ T cells are usually the most abundant γδ T cell subset in tumor infiltrates [194–198]; for example, Vδ1$^+$ T cells were the dominant tumor-infiltrating T cell population in a collection of 74 cutaneous melanomas and displayed enhanced anti-tumor cytotoxicity compared with their Vγ9Vδ2 counterparts [197]. Moreover, the capacity to express NCRs that increase their capacity to kill hematological [26] and solid (unpublished data) tumor cells is unique to the Vδ1$^+$ subset. This feature made Vδ1$^+$ T cells superior to the corresponding autologous Vγ9Vδ2 T cells in killing primary leukemia blasts [26].

In the specific case of allo-HSCT for leukemia treatment, the use of allogeneic donor-derived effector cells greatly facilitates implementation of protocols based on Vδ1$^+$ T cells. Importantly, the predominant subpopulation that expanded and correlated with enhanced survival of leukemia patients developing high levels of γδ T cells after receiving allo-HSCT was Vδ1$^+$ T cells [178]. Further studies showed that the underlying mechanism for this selective expansion was likely that donor-derived Vδ1$^+$CD4$^-$CD8$^-$ T cells proliferated spontaneously in response to recipient primary ALL blasts that they then lysed [199].

The receptor expression and functional capacity of ex vivo-expanded Vδ1$^+$ T cells are highly plastic. NCR$^+$ Vδ1$^+$ effector cells, which exhibit enhanced anti-tumor activity against primary B-CLL blasts, require AKT-dependent signals, provided synergistically by Vδ1$^+$ TCR stimulation and IL-2/IL-15 signals.

Clinical questions: *will unmanipulated Vδ1$^+$ T cells, infused as part of TCR-αβ/CD19-depleted grafts, gain NCR expression in vivo? Can we make more HSCT patients establish high levels of Vδ1$^+$ T cells long-term by specifically depleting only the αβ TCR$^+$ T cells (and CD19$^+$ B cells) from the grafts and/or use adoptive immunotherapy with specific subtypes of fresh or ex vivo expanded γδT cells? Will high Vδ1$^+$ T cell frequencies generated under such circumstances still correlate with/translate to an enhanced survival?*

NK CELLS IN CMV INFECTION

It is well-known that CMV infections, by different mechanisms, inhibit the expression of MHC class I molecules in infected cells [200]. Whereas this is viewed as an escape mechanism from CTL-mediated control, infected cells become potentially susceptible to NK cells. Thus, in CMV infections, NK cells may represent the most relevant effectors for host defenses. In addition, other features suggest a primary role of NK cells in defense against CMV, both in mice and in humans.

In mice, during the course of CMV infection, a population of Ly49H$^+$ NK cells expands and is responsible for disease clearance through the induction of a "memory-like NK cell response" [201, 202]. NK cells expressing the activating receptor Ly49H persist in high numbers after CMV infection. More importantly, this response is driven by the interaction of Ly49H with the viral protein m157 [201, 203]. Similarly, human CMV has been reported to induce, in healthy individuals and in patients with different pathological conditions, the expansion of a NK cell subset displaying high surface levels of the HLA-E-specific CD94/NKG2C-activating receptor [204–207]. NK cells expressing the NKG2C-activating receptor undergo preferential expansion when cocultured with CMV-infected fibroblasts. Moreover, NKG2C$^+$ NK cells are present in high percentages in seropositive but not in seronegative individuals. Remarkably, the expanded NKG2C$^+$ NK cell subset in CMV$^+$ individuals has the features of a mature NK cell phenotype, being mostly KIR$^+$NKG2A$^-$ [204–207] (Fig. 1). Their phenotypic signature is also characterized by the down-regulation of the non-HLA-specific inhibitory receptor p75/adhesion receptor molecule 1 (or Siglec-7) [208, 209]. These cells appear to be fully competent in terms of cytolytic activity and cytokine production. Thus, an emerging concept is that CMV infection may also shape the NKR repertoire, favoring the preferential expansion of memory-like KIR$^+$NKG2C$^+$Siglec-7$^-$ NK cells and their persistence over time.

It is conceivable that similar to murine Ly49H$^+$ NK cells, human KIR$^+$NKG2C$^+$ NK cells may also be responsible for virus clearance. In this context, a NK cell subpopulation with this phenotype was detected recently in a SCID patient with a JAK3 deficiency displaying an unusual T$^-$ NK$^+$ B$^+$ phenotype [210]. The absence of detectable CMV DNA in this CMV$^+$ pa-

tient could be the result of a successful control of CMV infection by NK cells. A role for NKG2C$^+$ NK cells in the resolution of CMV infection has also been suggested in another T cell-deficient patient (carrying an IL-7R α-chain mutation) [32]. These case reports further suggest that NKG2C$^+$ NK cells can control CMV infection in the absence of T cells [32].

In patients undergoing HSCT, two different studies showed that CMV reactivation was accompanied by the appearance of a mature NK cell phenotype characterized by a rapid increase in CD56^{dim} NK cells expressing the KIR$^+$NKG2C$^+$NKG2A$^-$Siglec-7$^-$CD57$^+$ signature (Fig. 1). Increased proportions of these NK cells persisted and even increased 1 year after HSCT in patients who reactivated CMV but not in those with no CMV reactivation [208, 211]. Thus, CMV appears to sharply accelerate the process of NK cell maturation from HSCs. Notably, in the case of KIR-mismatched haploidentical allo-HSCT, this phenomenon may be of particular benefit, as it results in rapid expansion of alloreactive NK cells (characterized by the KIR$^+$NKG2A$^-$ phenotype). Thus, CMV-induced NK cells with a memory-like surface phenotype may contribute not only to the control of virus infection but also to the protection from leukemia relapses after HSCT.

It remains unclear whether the expansion of NKG2C$^+$ NK cells is driven through interaction with HLA-E. Notably, the expression of HLA-E is stabilized by the signal peptide of the CMV UL40 protein, accompanied by the CMV-induced down-regulation of classical HLA class I molecules.

NKG2C might be the receptor for recognition of CMV-infected cells. Clinical questions: what happens in the cured individuals that have homozygous mutations of the NKG2C gene, causing loss of function? Is the NKR repertoire and NK cell maturation as in the case of NKG2C$^+$ individuals?

Vδ1$^+$ T CELLS DISPLAY POTENT ANTI-CMV ACTIVITY

Therapies focused on providing anti-tumor Vδ1$^+$ T cells are likely to have added benefits, by tackling CMV reactivation and associated post-HSCT mortality, given the strong antiviral responses mounted by Vδ1$^+$ T cells. Seminal work by Déchanet and colleagues [212] demonstrated that Vδ1$^+$ T cells, as well as the minor Vδ3$^+$ T cell population, are endowed with potent anti-CMV activity [213]. This work was performed largely on renal allograft recipients suffering from CMV infection. Vδ2$^-$ γδ T cells showed a specific, dramatic, and long-lasting (>1 year) expansion in the PB of CMV-infected individuals [212, 214]. Moreover, γδ T cell expansion was concomitant with the resolution of CMV infection/disease, regardless of the CMV serologic status of donor and recipient before transplantation [215]. Critically, Vδ1$^+$ T cells were able to kill CMV-infected target cells and limit CMV propagation in vitro [213]. Although pathogen-derived antigens or stress-induced self-molecules (up-regulated on, e.g., infection or malignant transformation) are likely involved in the recognition of CMV-infected cells by Vδ1$^+$ T lymphocytes, the molecular determinants governing this reactivity vary between effector populations, and the overall surface receptor profile that mediates CMV recognition remains quite elusive [33, 213, 216]. The CMV-induced Vδ2$^-$ γδ T cells were also reactive against epithelial tumor cells and shown to be protective against cancer in xenograft mouse models and also associated with reduced cancer risk in kidney transplant patients [217–219].

These findings were extended recently to the allo-HSCT setting. Significant long-term expansions of Vδ1$^+$ T cells (but not Vδ2$^+$ T cells) were observed during CMV reactivation early after HSCT, and Vδ1$^+$ T cell lines generated from CMV$^+$ donors lysed CMV-infected targets in all cases analyzed [31]. Several studies have also suggested that CMV pp65 antigenemia early after allo-HSCT can help to prevent relapse of AML [220, 221]. The mechanism for this was proposed recently to be a CMV-driven expansion of CD8αα$^+$ Vδ2$^-$ γδ T cell clones that also cross-recognize the autologous tumor cells [33]. The recognition of primary leukemic blasts but notably, not the CMV-infected cells, was shown to be mediated often by the Vδ1$^+$ TCRs specifically [33].

Interestingly, a specific fetal Vδ1$^+$ T cell population, expressing a public Vγ8Vδ1 TCR, was found to expand following CMV infection in utero [216]. These cells expressed high levels of IFN-γ, NKRs, and cytotoxic mediators and showed antiviral activity in vitro. These findings suggest that Vδ1$^+$ T cells may be efficient in fighting CMV as very early life (from 21 weeks of gestation onward).

In addition to anti-tumor activity, γδ T cells possess potent antiviral functions. Specifically, Vδ1$^+$ and Vδ3$^+$ T cells have been shown to constitute important effector populations against CMV and thus, represent valuable effector populations during the lymphopenic post-transplantation period.

Clinical questions: *given the reported anti-CMV and anti-tumor cross-reactivity of Vδ2$^-$ γδ T cells, how will the retention of γδ T cells in the grafts translate in terms of tackling CMV reactivation versus preventing leukemia relapse? Can CMV reactivation in the TCR-αβ/CD19-depleted allo-HSCT setting remain controlled, whereas specifically inducing expansion of large numbers of cross-reactive antileukemia Vδ2$^-$ γδ T cells and thus, associating with better prognosis?*

CONCLUSIONS AND FUTURE PERSPECTIVES

It is evident that major advances in the therapy of high-risk leukemias have been possible, thanks to the discovery and the functional characterization of inhibitory and activating NKRs that are expressed on NK cells and γδ T cells. In particular, the precise definition of the specificity of the various receptors represented a solid basis for building therapies exploiting NK cell and γδ T lymphocyte intervention. These innate effector cell types may be particularly valuable in the context of haploidentical allo-HSCT, where any beneficial GvHRs of αβ T cells is often accompanied with undesirable GvHD. Despite important achievements, further progress may be expected from the use of donor-alloreactive NK cells as a tool for improving the clinical outcome of high-risk leukemias and for preventing GvHD. For example, effective prevention of GvHD

may be achieved in patients at risk on the basis of various experiments performed in mice [222, 223]. Based on the knowledge that host DCs initiate GvHD through presentation of host alloantigens to donor αβ T cells, these studies analyzed the effect of infusion of alloreactive NK cells prior to transplantation and showed that it could eliminate DCs to such an extent that mice could receive mismatched BM grafts containing up to 30 times the lethal dose of allogeneic αβ T cells in the absence of any GvHD. Translation of this approach to humans appears promising to prevent or even treat GvHD. In general, infusion of NK cells and/or γδ T lymphocytes around the time of allo-HSCT may also protect the patient from early relapse, viral infection (in particular, CMV reactivation), and graft rejection. This is of great importance during the time window when alloreactive NK cells have not yet been generated from HSCs. Initial efforts are already under way in the form of haploidentical allo-HSCT protocols, using TCR-αβ/CD19-depleted cell populations, which contain $CD34^+$ progenitors, as well as mature NK cells and γδ T lymphocytes, as discussed above and in the paired clinical review by Locatelli and colleagues [1].

Suitable clinical protocols may be designed to exploit innate immune effector cells further, by adoptively transferring higher numbers of more effective NK cells and γδ T cells that have been expanded and/or potentiated ex vivo. The possibility to enhance the anti-tumor activity of primary NK cells and γδ T lymphocytes by equipping them with additional activating receptors through genetic engineering has been demonstrated [224, 225]. Specifically, γδ T cells have been redirected successfully with αβ TCRs and CARs [225–230]. Such strategies will be particularly valuable in the posthaploidentical allo-HSCT setting, where CAR-expressing donor αβ T cells could drive GvHD through their TCRs. In contrast, the corresponding CAR^+ γδ T cells should not promote GvHD development, even in this strongly HLA-mismatched context. The MHC class I-independent TCRs and many innate tumor reactive receptors expressed intrinsically by γδ T cells further make these cells very broadly tumor-reactive. We therefore suggest that γδ T cells, especially the NCR^+ $V\delta1^+$ subset, will be ideal CAR-therapy effectors, able to limit the development of tumor-immune escape variants driving leukemia relapse after allo-HSCT (in the absence of GvHD). Our advanced knowledge on receptor specificity, cell function, and trafficking to tumor sites also opens up new possibilities. The trafficking to LNs and thus, the effectiveness of alloreactive NK cells may, e.g., be improved by translating recent, basic experimental studies. KIR^+ alloreactive NK cells gained the ability to migrate to LNs by acquiring chemokine receptors (CCRs) from $CCR7^+$ cells (e.g., DCs or lymphoblastoid B cells) by the mechanism of trogocytosis [231, 232]. In addition, the appreciation that different tumor types, including ALL and AML, inhibit lymphocyte proliferation and effector function, offers an important clue for improvement of future clinical protocols. For example, in high-risk leukemias with high tumor burden (as well as in solid tumors), the adoptive immunotherapy with NK cells and γδ (or αβ) T lymphocytes could be combined with drugs (including antibodies) interfering with the inhibitory signals generated in the tumor microenvironment. The possibility of enhancing the effector cell function by in vitro treatment with TLR ligands before cell infusion should also be considered in future protocols [78]. The potent cytotoxicity and independence from MHC-mediated antigen presentation from NK cells and γδ T lymphocytes make them unique cellular tools that are complementary to immunotherapeutic strategies mobilizing adaptive immunity, in particular, for tackling tumor-immune escape. We, therefore, believe that these cells will be key reagents in future successful immune-based therapies against advanced cancer (**Table 2**).

TABLE 2. Summary of Outstanding Clinical Questions

Is there any clinical evidence that steroid treatment accelerates the appearance (and the maturation) of NK cells in the PB of patients receiving allo-HSCT?
Is there any evidence that increased expression levels of CD94/NKG2A (or any other inhibitory NKR) on γδ T cells may negatively correlate with the clinical outcome in cancer patients?
Were the high levels of γδ T cells, established long after allo-HSCT, the cause and/or an effect of the beneficial clinical antileukemia response in the patients?
By what mechanism does the presence of γδ T cells in the graft promote the development of high levels of $V\delta1^+$ T cells long after allo-HSCT?
Will certain subsets of unmanipulated γδ T cells preferentially undergo homeostatic proliferation when infused as part of a graft devoid of αβ T cells and B cells?
Why do not alloreactive NK cells cause GvHD in spite of the KIR/HLA mismatch?
Why have some studies failed to confirm the clinical impact of the presence of donor NK cell alloreactivity in the context of haploidentical allo-HSCT?
Will unmanipulated $V\delta1^+$ T cells, infused as part of TCR-αβ/CD19-depleted grafts, gain NCR expression in vivo?
Can we make more HSCT patients establish high levels of $V\delta1^+$ T cells long-term by specifically depleting only the αβ TCR^+ T cells (and $CD19^+$ B cells) from the grafts and/or use adoptive immunotherapy with specific subtypes of fresh or ex vivo expanded γδ T cells?
Will high $V\delta1^+$ T cell frequencies generated under such circumstances still correlate with/translate to an enhanced survival?
What happens in the cured individuals that have homozygous mutations of the NKG2C gene, causing loss of function?
Is the NKR repertoire and NK cell maturation as in the case of $NKG2C^+$ individuals?
Given the reported anti-CMV and anti-tumor cross-reactivity of $V\delta2^-$ γδ T cells, how will the retention of γδ T cells in the grafts translate in terms of tackling CMV reactivation versus preventing leukemia relapse?
Can CMV reactivation in the TCR-αβ/CD19-depleted allo-HSCT setting remain controlled, whereas specifically inducing expansion of large numbers of cross-reactive antileukemia $V\delta2^-$ γδ T cells and thus, associating with better prognosis?

KIR^+ alloreactive NK cells can be infused prior to HSCT to reduce the risk of GvHD, as predicted by murine models.

Adoptively transferred KIR$^+$ alloreactive NK cells (CCR7$^-$) can be redirected to LNs upon acquisition of CCR7 in vitro by trogocytosis. This allows more efficient removal of host mature DCs, primarily located in secondary lymphoid organs, thus preventing GvHD.

The tissue tropism and distinct chemokine receptor profile of Vδ1$^+$ T cells, as compared with that of Vγ9Vδ2 T cells, may allow them to infiltrate tumors more efficiently.

AUTHORSHIP

H.N., A.M, B.S-S., and L.M wrote the review.

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DISCLOSURES

H.N. and L.M declare no competing financial interests. A.M. is a founder and shareholder of Innate-Pharma (Marseille, France). B.S-S. is a scientific consultant and shareholder of LymphAct/Delta One Therapeutics.

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