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Natural killer cells and engagers: Powerful weapons against cancer

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Summary

Natural killer (NK) cells are innate immune effectors whose functions rely on receptors binding cytokines, recognizing self-molecules, or detecting danger signals expressed by virus-infected or tumor cells. The potent cytotoxic potential makes NK cells promising candidates for cancer immunotherapy. To enhance their activity strategies include cytokine administration, blocking of immune checkpoints, and designing of antibody-based NK cell engagers (NKCEs). NKCEs represent a cutting-edge approach to cancer therapy: they strengthen the NK-to-target cell interactions and optimize tumor killing, possibly overcoming the immunosuppressive tumor microenvironment. NK cells belong to the innate lymphoid cells (ILCs) and are categorized into different subsets also including cells with a memory-like phenotype: this complexity needs to be explored in the context of cancer immunotherapy, particularly when designing NKCEs. Two strategies to enhance NK cell activity in cancer patients can be adopted: activating patients' own NK cells versus the adoptive transfer of ex vivo activated NK cells. Furthermore, the capability of NKCEs to activate γδ T cells could have a significant synergistic effect in immunotherapy.

KEYWORDS
cancer, human NK cells, immunotherapies, NK cell engagers

1 | INTRODUCTION

Natural killer (NK) cells are innate immune effectors that play a crucial role in physiological processes and various pathological conditions, including cancer. Unlike T and B cells, NK cells do not undergo functional gene rearrangement or require prior antigen priming to become functional. Their proliferation and activities are induced by cytokines and regulated by a wide repertoire of germline-encoded receptors, transducing inhibitory or activating signals, allowing them to discriminate between healthy self-cells and abnormal/stressed targets, such as those infected with viruses or undergoing tumor transformation. The potent cytotoxicity and the ability to secrete immunostimulatory cytokines make NK cells a key focus in the development of immunotherapies aimed at enhancing anti-tumor activity. Several strategies can harness NK cells, including systemic administration of cytokines, monoclonal antibodies blocking inhibitory axes, and engineered NK cell engagers (NKCEs). These approaches aim to exploit the

natural cytotoxic potential of NK cells and directly target tumor cells, overcoming the challenges posed by the immunosuppressive tumor microenvironment and immune evasion mechanisms. Furthermore, the recent advances made in the understanding of NK cell heterogeneity, which relies on the existence of distinct subsets with specific features in terms of phenotype, functionality, stimuli-sensitivity, and tissue distribution, as well as the discovery of NK cells with adaptive memory-like characteristics, have provided deeper insights into their potential therapeutic applications.

This review explores the multiple strategies to engage NK cells in cancer therapy, discusses the most effective NK cell subsets for therapeutic activation, and compares the benefits of enhancing patients' own NK cell activity or adoptively transferring in vitro-activated autologous or allogeneic, NK cells. We also briefly discuss the potential synergistic role of γδ T cell activation. Understanding these mechanisms may help to optimize cell-based interventions and develop innovative, effective immunotherapies to fight cancer.

2 | WHY NK CELLS?

NK cells are innate lymphocytes that play important roles in physiology and various pathological conditions.[1] The understanding of how innate immunity, particularly NK cells, works required a revolution in the way of thinking and the demolition of some dogma.[2] First, innate immunity is not truly "aspecific"; instead, it has receptors with broad specificity. This is a great advantage since it allows the more ancient, conserved part of our immune system to promptly recognize and kill a huge spectrum of enemies. NK cells constantly interact with the surrounding cells and monitor their pathogenic potential via a spectrum of activating receptors, which selectively recognize ligands expressed on the surface of "abnormal" cells. Among others, activating receptors/co-receptors include NKp46, NKp30, NKp44, members of the natural cytotoxicity receptor (NCR) family, NKG2D, DNAM-1, CD2, 2B4, and NTB-A.[3,4] Moreover, NK cells can acquire a "B cell-like" specificity thanks to the expression of CD16a (FcgRIIIa), a receptor that binds the Fc portion of IgG complexed with antigen and induces strong activation and antibody-dependent cellular cytotoxicity (ADCC). Second, innate immunity discriminates between self and non-self, a property allowing NK cells to attack dangerous cells while sparing healthy host cells. The revolution was to understand how this works. Indeed, NK cells recognize MHC class I molecules expressed at the surface of healthy cells but this interaction instead of alerting the cells, as occurs in T lymphocytes, transduces inhibitory signals impairing the NK cell activation and preventing the attack on healthy autologous tissues.[5] Self-recognizing receptors include the inhibitory killer Ig-like receptors (iKIRs) which are clonally distributed and recognize structural motifs shared by groups of classical MHC class I alleles (A, B, and C), the CD94/NKG2A heterodimer recognizing a non-classical MHC class I molecule (E), which presents conserved leader sequences peptides from most other MHC class I,[6-9] and LIR-1 (ILT-2) having a broad MHC class I specificity.[10] The discovery of KIRs demolished a third dogma related to the process of education. The stochastic expression of KIRs during maturation in the bone marrow is required to generate fully functional "educated" and "licensed" NK cells;[6,11] the activating form of KIRs (aKIR) and LIR-1 also contribute to the educational program.[12] Despite multiple developmental checkpoints, "uneducated" NK cells can still arise that, however, exhibit significant dysfunctionality.

Firth, recent studies demonstrated that innate immune cells remember, a process identified as "trained," "adaptive-like," or "memory-like" immunity.[13,14] Upon antigen recognition, adaptive NK cells undergo clonal-like expansions, persisting in the body for years as effectors more prone and rapid to respond to a second challenge. The better-characterized stimulus inducing adaptive-NK cells in both humans and mice is cytomegalovirus (CMV) infection (see below).

Among immune effectors, NK cells are characterized by the highest cytolytic potential. When appropriately stimulated, they efficiently kill tumor cells through multiple receptors-ligands interactions, the release of preformed perforin- and granzymes-containing cytotoxic granules, and the death receptor/ligand interactions (i.e. FAS/FAS-L and TRAIL-R/TRAIL). Moreover, activated NK cells through both cell-to-cell contacts and the release of soluble factors, recruit and support the survival and functions of other immune cells including dendritic cells, macrophages, and T cells,[15,16] increasing the antigen presentation capability of myeloid cells and shaping adaptive immune responses. A therapeutic option is the adoptive transfer, in cancer patients, of in vitro-expanded autologous or allogeneic NK cells. The use of allogeneic NK cells from healthy donors is feasible and advantageous since they are not MHC-restricted and do not display a graft versus host effect. The iKIR-KIR-ligand-mismatch between donor and recipient can even improve the effectiveness of the therapy by circumventing inhibitory signals on host cells while engaging on allogeneic NK cells MHC class I-specific activating receptors such as the activating form of KIRs (aKIR).[17]

Overall, enhancing the anti-tumor activities of autologous NK cells in cancer patients represents an appealing possibility to develop effective immunotherapies and improve clinical outcomes.

3 | HOW CAN NK CELLS BE ENGAGED?

Different approaches were designed to activate and/or restore NK cell-mediated anti-tumor activity in patients. The systemic administration of recombinant cytokines such as IL-2 or IL-15 either caused unwanted side effects, including cytokine release (CRS) and vascular leak syndromes (VLS), or results were far from expectations. Strategies for limiting cytokines' side effects and increasing effectiveness are needed.[18] Along this line, to improve the cytokines' half-life and bioactivity, muteins have been developed with promising results.[19] A promising strategy is to restore the NK cell activity by using monoclonal antibodies (mAb) blocking the "off" signals mediated by MHC class I inhibitory receptors such as CD94/NKG2A or immune checkpoints (IC).[20] NK cells express at steady state or

upon activation different IC receptors including PD-1, TIGIT, LAG-3, and B7-H3R recognizing ligands either constitutively expressed by tumor cells or upregulated by cytokines such as IFN-γ or TNFα released during the immune responses.[21]

MAbs are extraordinarily powerful tools and different chimeric or humanized mAbs have been generated that are currently used in therapy or tested in innovative clinical trials. The Fc portion is selected according to the function required. Therapeutic mAbs designed for blocking IC are IgG4, an isotype that has a reduced ability to activate Fcγ receptors (FcgRs) and complement; they often contain mutations such as S228P, which prevent Fab-arm exchange in vivo. In this context, therapeutic IgG4 mAbs were designed also to block the "off" signals of inhibitory NK receptors. MAbs of the IgG1 isotype are used instead to engage FcγRs such as CD16 on NK cells and target a tumor-associated antigen (TAA) through their Fabs portion thus triggering ADCC. CD16 polymorphisms exist that can modify the binding affinity to the IgG1 Fc portion lowering the efficacy of therapeutic mAbs. The most studied variant is at position 158; the presence of a valine residue in this position increases the Fc binding affinity with the highest and lowest binding strength in 158 V/V and 158F/F homozygous donors, respectively.[22] To improve the efficacy in patients, engineered mAbs have been generated that contain amino acid substitutions in the Fc portion that increase the affinity for CD16 or decrease the binding with the inhibitory FcgRIIB.[23] Several IgG mAbs have been produced that bind different TAA, and those targeting CD19, CD20, CD38, SLAMF7, and KIR2DL3 in hematopoietic cancers, or HER2, EGFR, and GD2 in solid tumors (breast, gastric, colon, lung, head and neck cancers, and neuroblastoma) are currently used in clinics.[24] More recently, this approach has evolved with the development of new generations of antibodies with multiple specificities, recognizing more than one TAA, a TAA and a T or NK receptor, and eventually containing cytokine receptor agonists.

At present, all the tools, including "classical" IgG mAbs, engaging NK cells and tumor targets and facilitating immune synapse formation, are termed "NK cell engagers" (NKCE)[25] (Figure 1). NKCEs can bind to receptors other than CD16; these include NKp46, NKp30, NKG2D, DNAM-1, SLAMF7, and 4-1BB, which are expressed by NK cells but can be also present in other effectors such as γδ T cells (see below). Innovative NKCEs are formed by the H and L variable regions of IgG linked to form single chain variable fragments (scFv); the better tools should be characterized by small size, well-fitting into the synaptic cleft, hydrophilicity, flexibility, proteolytic stability, and low immunogenicity. In this regard, the presence of the Fc portion instead of an anti-CD16 scFv portion is preferable; Fc extends the NKCE life span being recognized by the neonatal Fc receptor (FcRn), which functions as a recycling receptor responsible for protecting IgG in the circulation[26] and has tolerogenic properties.[27]

NKCEs can also include cytokines such as IL-15 and IL-2 that stimulate and prolong the survival of NK cells and increase their anti-tumor potential. For example, a three-specific NKCE (TriKE) has been generated formed by an anti-CD16 heavy chain camelid single-domain antibody (sdAb), IL-15, and a scFv recognizing CLEC12A, a myeloid lineage antigen highly expressed by acute myeloid leukemia (AML) cells. CLEC12A TriKE induced proliferation

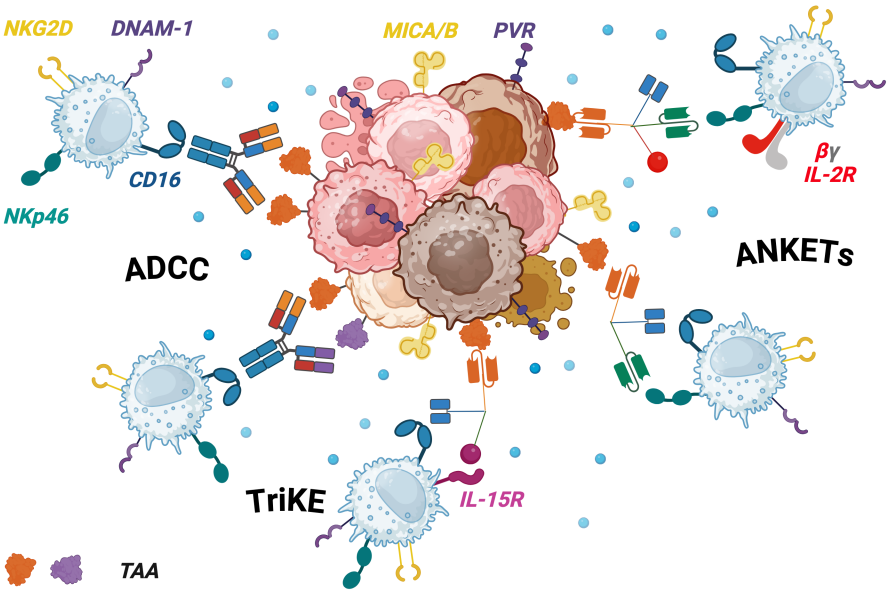

FIGURE 1 Natural Killer (NK) cells can be activated by NK cell engagers (NKCE) that interact with one or more tumor-associated antigens (TAA) and activating receptors, including CD16, which induces antibody-dependent cell-mediated cytotoxicity (ADCC), and NKp46. The effect is enhanced by the inclusion of immunostimulatory cytokines (such as IL-15 or vIL-2) in the NKCE. Additionally, receptor-ligand interactions that naturally occur between other NK receptors (NKG2D and DNAM-1) and their ligands expressed by tumor cells further potentiate this effect. TriKE, three specific NK cell engagers; ANKET®, Antibody-based NK cell Engager Therapeutics. Created with BioRender.com.

and activation of NK cells in vitro and in AML preclinical mouse models.[28] Camelid antibodies (cAb) are interesting tools formed by two heavy chains (VHH) with high affinity. They have superior physicochemical properties and low production cost since they can be generated using available naive or immune libraries from llamas and alpacas.[29] The small size of cAb has positive effects facilitating genetic manipulation, access in the antigen clefts, and diffusion in tissues, but may result in reduced life span due to renal filtration; the latter effect, however, can be overcome by conjugating VHH with IgG Fc, implementing the FcRn-mediate recycling. cAb can be used to generate "nanobodies" formed by the single VH regions that have the typical IgV fold with three CDRs. In constructing NKCEs, nanobodies represent an appealing alternative to scFv.[30]

More recently a platform has been created to design tetraspecific engagers, termed Antibody-based NK cell Engager Therapeutics (ANKET®). It represents the evolution of the trifunctional NKCE targeting NKp46, CD16, and a TAA; the NKp46/Fc/CD123 ANKET displayed potent antitumor activity against AML cells, both in vitro and in mouse tumor models, and is now tested in a clinical trial.[31] Tetraspecific ANKETs are composed of the IgG1 Fc domain engaging CD16, two scFv targeting the NKp46 activating receptor and a given TAA, and an IL-2 variant (IL-2v). The IL-2v contains a point mutation allowing its binding to the low-affinity βγIL-2 receptor, expressed by NK cells at steady state, but not the high-affinity αβγIL-2 receptor constitutively expressed by Tregs; this avoids the undesired stimulation of cells with great immunomodulatory properties. The IgG1-Fc/aNKp46/aCD20/IL-2v ANKET induced preferential NK cell proliferation, chemokine and cytokine release, and anti-tumor in vitro and in a mouse tumor model. Moreover, it depleted B cells without sign of toxicity in non-human primates.[32] Recently, an ANKET recognizing B7-H3 has been developed (IgG1-Fc/aNKp46/aB7-H3/IL-2v), which may offer the opportunity to enhance the NK cell-mediated activity against various malignancies including Neuroblastoma. Neuroblastoma (NB) is the most prevalent extracranial solid tumor in early childhood, affecting over 500 European patients annually; half of these patients, classified as high-risk (HR-NB), present metastatic disease with bone marrow (BM) involvement, a common and fatal site of tumor infiltration and relapse. B7-H3 (CD276), is a TAA highly expressed by neuroblastoma, including the tumor variants lacking or expressing low amounts of GD2,[33,34] the TAA targeted in the maintenance phase of the standard therapeutic protocol for high-risk patients approved by the International Society of Pediatric Oncology European Neuroblastoma Research Network (SIOPEN-NET).[35] B7-H3 is an immune checkpoint ligand that dampens the NK and T cell activity, promotes tumor migration/invasiveness, and limits sensitivity to chemotherapy-induced apoptosis, thus holding a negative prognostic value.[36] B7-H3 protein is absent in most normal tissues while it marks tumor-associated vasculature as well as a variety of adult and pediatric hematologic and solid tumors,[37] expanding the therapeutic application of NKCE targeting B7-H3 far beyond neuroblastoma.

4 | WHICH NK CELL SUBSET COULD BE THE BEST?

NK cells represent a promising platform for designing therapeutic NKCEs that enhance anti-tumor cytolytic activity. Understanding NK cell biology and function has required decades of groundbreaking discoveries by research groups worldwide.[2] Initially, NK cells were identified as a distinct "non-T and non-B" lymphocyte subset with unknown functions. The interest burst when it was discovered that these cells were able to kill a wide range of autologous and allogeneic transformed cells without prior sensitization, becoming appealing effectors termed "natural killers" due to their inherent non-specific cytotoxicity. Since then, the basis of their function has been largely elucidated with the discovery of a huge number of germline-encoded surface receptors specifically recognizing ligands expressed by potential targets and transducing either inhibitory or activating signals (see above).

Now we know that NK cells are specific, educated lymphocytes that have crucial roles in physiology and pathology. Through cell-to-cell contacts, cytotoxicity, and the release of cytokines, chemokines, and growth factors, NK cells control viral infections, and tumor transformation, also supporting adaptive immunity. Studies from the last decades demonstrated that NK cells represent highly heterogeneous populations with different transcriptomic, epigenetic, and phenotypic features, functional potentials, and reactivity/sensitivity to external stimuli. Three major NK cell subsets have been described, NK1, NK2, and NK3, which can be further differentiated into six distinct subgroups.[38] [Correction added on 30 Aug 2024, after first online publication: the in-text citation was changed to 38 from 33 in the preceding sentence.] This scenario is becoming more and more complicated since the discovery that NK cells represent a member of a larger family of innate lymphoid cells (ILCs)[38,39] that are divided into three major subgroups. Group 1 (G1) consists of IFNγ-producing cells that include "classical" NK cells expressing Eomes and T-bet transcription factors, and ILC1s expressing T-bet. G1 ILCs have been described in most organs, either resident or recruited from peripheral blood, where they mostly exhibit a $CD56^{bright}$ phenotype. Interestingly, recent data suggest that NK cells can also develop from early progenitors distinct from the common precursors of ILCs.[38,40] G1 ILCs are hardly distinguishable; in humans, it has been proposed that some surface markers (CD200R, CXCR3, CXCR6, TRAIL) could distinguish ILC1 from NK cells. However, ILCs are plastic cells whose phenotype may vary depending on the tissue in which they reside, and upon the influence of the tumor microenvironment, particularly during inflammation or tumor growth. TGF-β is a master regulator of immune responses maintaining immune homeostasis and resolving inflammation.[41] It also represents a potent tumor escape mechanism being produced/activated by tumor cells and immune cells populating the tumor microenvironment such as macrophages and Tregs. In G1 ILCs, TGF-β dampens the anti-tumor functions reducing the expression of activating receptors (NCRs, NKG2D, and DNAM-1), modifying the chemokine receptor repertoire affecting their recruitment in peripheral tissues, inducing up-regulation of

IC and exhaustion markers (TIGIT, LAG3, NKG2A), and promoting the expression of CD39 and CD73 with sequestration of extracellular pro-inflammatory ATP and production of anti-inflammatory adenosine.[39] Moreover, TGF-β promotes the conversion of NK cells into ILC1-like cells with a tissue-resident profile, as evidenced by the strong expression of markers such as CD103 and CD49a.[42] While some studies suggest a pro-tumoral function of ILC1, others indicate an anti-tumor potential; for example, tumor infiltration by ILCs positively associated with patient survival and IL-15 supported their expansion and anti-tumor functionality.[43]

Considering the possible engagement by NKCEs, while G1 ILC subsets can lack or express low amounts of CD16, all are responsive to immunostimulatory cytokines such as IL-15 and IL-2 and maintain significant expression levels of receptors such as NKG2D, NKp30, NKp46, and DNAM-1. NKG2D recognizes the stress-inducible molecules MICA/B and ULBPs, NKp30 binds B7-H6 expressed in many tumors, and, very recently, NKp46 was shown to recognize, in mice, calreticulin which translocates from the endoplasmic reticulum to the cell membrane (ecto-CRT) in stressed cells during virus infections, senescence process and immunogenic death.[44] The NKp30 expression can be low in G1 ILC and, in a tumor context, its surface expression can be downmodulated by the soluble B7-H6 ligand. Moreover, in cancer microenvironments, TGF-β reduces the expression of both NKp30 and NKG2D[24] limiting the efficacy of NKCEs targeting these receptors. Conversely, NKp46 is stably expressed by all G1 ILCs, including those infiltrating tumors; its engagement in combination with stimulatory cytokine/s might be sufficient to activate the cytotoxicity also in tissues, possibly inducing upregulation of CD16 expression, and dampening the immunomodulatory properties of tumor microenvironment.

The NK3 subset is composed by adaptive NK cells that could be suitable for therapeutic activation through NKCEs. In mice and humans, NK3 are cells engaged by cytomegalovirus (CMV), although some evidence suggests that adaptive-like cells can also emerge following other viral infections.[45-47] A seminal study showed that CMV-infected individuals presented a persistent expansion of NK cells expressing high surface levels of CD94/NKG2C.[48] Later it was demonstrated that NKG2C binds UL40 CMV peptides presented by MHC class I-E, a recognition supported by immunostimulatory/pro-inflammatory cytokines (i.e., IL-12, IL-18, IL-33, and IFN-I) that are essential, especially during the expansion phase, and the co-receptor activity of CD2. During CMV infection, these signals collectively induce memory NK cell differentiation, activation, and expansion[49] Interestingly, a peculiar "trained" NK cell population emerging during pregnancy has been described; it is equipped with unique transcriptome and epigenetic signature, high expression of NKG2C and LIR-1, and characterized by secretion of IFN-γ and VEGFα.[50]

CD94/NKG2C positive cells represent large clonal expansions of NK cells with memory characteristics such as long-term persistence and increased reactivity against CMV-infected cells, and, thereafter, were termed "adaptive-NK" or "memory NK."[13,46] In CMV+ individuals CD94/NKG2C positive cells, lack NKG2A, express the CD57 differentiation marker, iKIRs, ILT2, CD16, NKG2D, and CD2, and have reduced expression of NKp30, NKp46, Siglec7, and CD161. Adaptive NK cells can be distinguished from conventional NK cells being characterized by transcriptional and epigenetic remodeling, induced by antigen recognition and cytokine (IL-12, IL-18, and IL-15) stimulations. Epigenetic modifications, such as those acting on methylation, downregulate or upregulate signaling molecules and transcription factors.[51] For example, memory NK cells have reduced expression of the FcεRIγ molecule; CD16, however, can transduce optimal activating signals through the recruitment of the CD3ζ chain. Adaptive NKs are strong producers of IFNγ and demethylation of the CNS1 regulatory region of the IFNG gene is a mechanism enabling such potent cytokine production. Consistently, the metabolic machinery is intensified and cells become resistant to the immunosuppressive effects mediated by Treg and MDSC, particularly thanks to a low TIGIT expression and a reduced IL-37 sensitivity.[52,53] Moreover, they show enrichment in IRF4, NFAT, AP-1, NF-κB, and STAT4 that act cooperatively inducing the chromatin remodeling required for adaptive NK cell differentiation. The use of NKCEs to harness adaptive NK cells against tumor cells in immunotherapeutic protocols could be interesting considering their molecular and functional profiles, the increased lifespan, hyper-responsiveness to cytokine stimulation and receptor engagement, increased cytotoxicity and secretory properties, characteristics that can be maintained in daughter cells.

5 | WHICH IS BETTER: ACTIVATION OF PATIENTS' EFFECTORS OR ADOPTIVE TRANSFER OF ACTIVATED NK CELLS?

To improve the anti-tumor activity of NK cells in patients we have two options; potentiate the function of patients' own NK cells or infuse in patients autologous or allogeneic NK cells expanded in vitro; both are feasible and, eventually, can be combined. Brilliant results have been obtained by re-activating the function of patients' anti-tumor activity of lymphocytes using IC inhibitors. In pioneering studies, ICIs such as the anti-PD-1 were used in high-risk melanoma patients with refractory, metastatic, and relapsing diseases; despite the unfavorable conditions, ICIs showed exciting results with a great improvement in overall survival (OS) and quality of life (QoL).[54] The application of PD-1 blockade has become a milestone of immunotherapy and, currently, it is applied in a large panel of tumors; in some cases, it has been proposed as a frontline therapy as proposed in patients with lung tumors. The ICI function relies on the reactivation of both T and NK cells, the latter being crucial when treating MHC class I-negative cancers such as neuroblastoma.[24,55] Despite unprecedented results, however, a high percentage of patients do not respond to treatment (primary/innate resistance) or become resistant after an initial period of response (secondary/acquired resistance).[56] The expression and function of additional inhibitory immune checkpoint receptors (LAG3, TIM3, TIGIT, and NKG2A) and downregulation, on tumors, of ligands for activating receptors are among the mechanisms implicated in both types of resistance.

NKCEs can be infused in cancer patients and different clinical trials are ongoing to demonstrate their safety profile and capability

to improve the clinical outcome.[20] Also, in NKCE-based therapy, however, there are important challenges. One is related to the therapeutics received by the patient before NKCE infusion. The combination of aggressive radio and chemotherapies can profoundly alter the immunological status of the patient reducing the number, vitality, and fitness of immune effectors. There are also crucial differences in treating hematological malignancies and solid tumors; most immunotherapeutics including NKCEs are tested in hematological malignancies, and few are now considered in patients with solid tumors. The inherent characteristics of solid tumors, such as increased stiffness, the presence of regulatory/immunosuppressive agents, increasing hypoxia, and metabolic changes create a complex microenvironment that severely limits the survival, proliferation, activation, and infiltration of immune effectors, as underscored by the fact that these cells often remain in the surrounding stroma. Yet, the administration of NKCEs in vivo requires the presence of sufficient amount of NK cells. Therefore, to increase the probability of success of NKCE-based immunotherapies, these molecules might be able to reinvigorate the anti-tumor function of circulating and peri-tumoral NK cells despite the hostile microenvironment. Other points need to be analyzed in preclinical and clinical studies; these include whether a given NKCE induces prolonged activation of NK cells without cell exhaustion, which subset is expanded/activated upon treatment with a given NKCE, particularly considering that NK2 cells lacking or having reduced CD16 expression often populates the tumor microenvironment, and which chemokine receptors are expressed by NK cells that may facilitate their recruitment into tumors.

It is mandatory also to study the capacity of NKCE and NKCE-engaged NK cells to cross the blood–brain barrier (BBB).[57] Tumors of the central nervous system (CNS) are frequent, difficult to treat, and among the most invalidating and deadly tumors in both adults and children. We know now that BBB is not as impenetrable as we thought, and chronic inflammatory processes such as cancer allow the recruitment of immune effectors into the brain and even the formation of tertiary lymphoid structures (TLS), transiently formed aggregates of lymphoid and stromal cells.[58] Resident G1 ILCs have been identified in CNS that can be replenished by circulating blood precursors[59]; in this regard, neurons produce the CX3CL1 chemokine, and NK cells in the CNS express high levels of CX3CR1. Importantly, the presence in CNS of NK cell correlated with better prognosis in patients with gliomas.[60]

As mentioned above, an appealing possibility is "adoptive immunotherapy," for example, infusing cancer patients with in vitro-activated NK cells either in an autologous or allogeneic setting, utilizing NK cells from healthy donors (Figure 2). An innovative technique to cure cancer patients, particularly those affected by leukemia, is the αβ T and CD19-depleted haploidentical hematopoietic stem cell transplants (haplo-HSCT). HSCTs are mobilized in the peripheral blood of the donor through the administration of G-CSF followed, if necessary, by AMD3100, an antagonist of CXCR4, the CXCL12 receptor. After the depletion of αβ T and B cells, the HSCT-enriched product is administered to patients upon adequate myeloablative preparative regimen. This HSCT methodology was successful, particularly in pediatric patients affected by aggressive acute lymphoblastic leukemias who needed an urgent transplant to survive and were rapidly engrafted using as donor close relatives, in most cases parents.[61] Haplo-HSCT is now applied in different tumors including neuroblastoma.[35,62] Compared to transplants performed

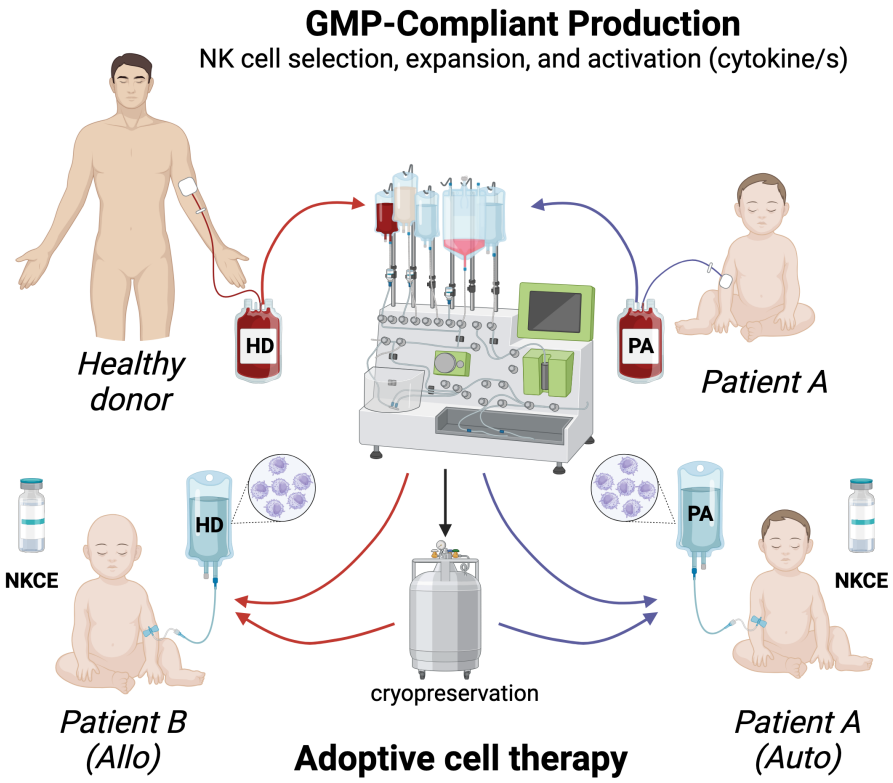

FIGURE 2 In Adoptive Cell Therapy (ACT), blood is collected through apheresis, and natural killer (NK) cells are selected and expanded under Good Manufacturing Practice (GMP) conditions using immunostimulatory cytokine/s.[64] The resulting cellular product is then infused into cancer patients possibly followed by the administration of NKCEs. This treatment can utilize either the patient's own NK cells (autologous ACT) or NK cells from a healthy donor (allogeneic ACT). An alternative approach is ACT with expanded γδ T cell populations. Created with BioRender.com.

using megadoses of purified HSC, αβ T and B-cell-depleted transplants have the advantage of containing the donor's mature NK, as well as γδ T and NKT cells. This type of transplant is not associated with significant graft-versus-host disease (GvHD) and has decreased graft rejection, partly due to the capability of the donor's NK cells to kill the recipient's dendritic cells (DC) and activated T cells.[63] During the early phase after transplantation, donors' effectors play a role in controlling virus infections/reactivations and tumor growth/relapse. This effect, however, is limited in time and the complete reconstitution of the immune system in the patient requires approximately 1 year; in particular, in the first trimester, after HSCT, NK cells developing in patients are immature and express high levels of CD94/NKG2A. A possibility to extend the monitoring of infections and tumor relapses is to infuse NK cells purified from a selected donor and in vitro expanded/activated with stimulatory cytokines under Good Manufacturing Practice (GMP) conditions.[64] NK cells are easy to purify and nicely expand upon treatment with cytokines such as IL-2. Interestingly, NK cells with long-term life span, adaptive-like characteristics, and capability to control tumor growth were obtained upon activation with IL-12 + IL-15 + IL-18 cytokine combination (termed cytokine-induced memory-like, CIML).[65] NKCEs could represent an alternative method to expand NK cell populations and, depending on their composition, could selectively promote the proliferation of NK cells with optimal phenotypic and functional characteristics, including memory. NKCEs could be used in combination with adoptive therapy to maintain NK cell activation in vivo, and further promote the properties of infused NK cells. The infusion in humans of engineered NK cell lines such as NK92 or NK cells derived from induced pluripotent stem cells (iPSC) has been proposed and some clinical trials are ongoing: however, the use of genetically altered or manipulated cells can be risky for patients. The adoptive therapy with allogeneic NK cells from healthy donors is feasible and safe and represents a promising off-the-shell therapy for cancer patients, either transplanted or not, overcoming the technical limitation related to the expansion of autologous cells, particularly when treating infants.

6 | BEYOND NK CELLS: γδ T CELLS

Besides NK cells, other lymphocyte subtypes are emerging as suitable effectors in cancer immunotherapy, particularly, γδ T cells that combine characteristics of innate and adaptive immunity.[66] Like αβ T cells, they develop in the thymus and express a surface receptor composed of two chains generated through V(D)J recombination; however, like NK cells, they recognize stress-induced antigens in a non-MHC-restricted manner and do not attack healthy allogeneic tissues. This occurs through a plethora of activating innate receptors that are constitutively expressed or induced by cytokines (IL-2, IL-15), placing these cells at the boundary between innate and adaptive immunity. Receptors include NCRs, NKG2D, DNAM-1, and CD16, the latter giving them the ability to perform efficient ADCC.[66,67] In humans, two major γδ T cell subsets are detectable, characterized by the predominant usage of Vδ1 and Vδ2 gene segments. Vδ2 virtually exclusively pairs with Vγ9 forming a TCR recognizing phosphoantigens (PAs), which, in infected and malignant cells, are over-produced through the mevalonate pathway. PAs induce a conformational change in butyrophilin 3 A1 (BTN3A1) and its association with BTN2A1, which directly binds to the Vγ9 chain inducing cell activation. Vγ9/Vδ2 T cells represent the major subset in peripheral blood and lymphoid organs, while Vδ1 T cells are preferentially located in other tissues, especially mucosal ones. In adults, Vδ1 T cells with specific TCR rearrangements can be detected possibly resulting from clonal expansions; these are long-life cells with memory-like characteristics. Vδ1 T cells recognize different ligands including CD1c and CD1d, lipid-presenting, MHC-like proteins belonging to the CD1 family. Interestingly, Vδ1 T cells expressing high levels of NKp46 were detected in the gut that exhibited strong antitumor activity against colorectal cancer.[68] Like NK cells, γδ T cells have potent anti-tumor cytolytic activity and stimulate other immune effectors such as NK cells through the release of immunostimulatory cytokines (i.e. IFNγ, TNFα) and the expression of ligands such as 4-1BBL.[69] Moreover, there is evidence of a correlation between γδ T cell infiltration in the tumor microenvironment and favorable clinical outcomes. The characteristics described above make γδ T cells suitable candidates for therapies, either by enhancing their function in patients or through adoptive therapy following in vitro expansion.[70] Studies and, in some cases, clinical trials, are ongoing that focus on the use of γδ T cells, and different approaches are used to expand in vitro the Vδ2 or Vδ1 subset.[71] The γδ T cells features strongly suggest that IgG1-Fc/aNKp46/aTAA/IL-2v ANKETs could induce the expansion and activation not only of NK cells but also of circulating and tumor resident γδ T cell subsets, further increasing their in vivo efficacy.

Finally, another potential target of NKCEs could be NKT cells. They express activating receptors including NKp46, NKp44, NKG2D, and DNAM1, are responsive to pro-inflammatory cytokines (IL-2, IL-12, IL-15, IL-18, and IFN-I), can infiltrate tumors, are cytotoxic, and secrete pro-inflammatory cytokines/chemokines.[72]

7 | CONCLUDING REMARKS

Fighting cancer is challenging and preclinical and clinical data show that we need to combine therapies to increase the probability of succeeding. NK cell engagers offer a novel approach to treating cancer. NKCEs are designed to bridge NK and cancer cells facilitating the formation of the immunological synapse, harnessing NK cells, and precisely directing their cytotoxic activity toward the cancer cells. Moreover, they may act also on other important effectors such as γδ T cells that share with NK cells many important characteristics. Data show that NKCEs are highly specific, targeting cancer cells with minimal damage to healthy tissues, potent, effectively activating the killing capabilities of crucial immune effectors, and versatile; by altering their composition, NKCEs can be designed to target different types of cancer and immune effectors. Additionally, by activating

effectors that express many innate activating receptors recognizing tumors, NKCEs may overcome immune evasion mechanisms based on the downregulation of a given TAA.

In conclusion, NKCEs, or more properly killer cell engagers (KCEs), represent an exciting frontier in cancer therapy, offering hope for more effective and targeted therapeutic options for patients with various types of cancer. It is notable, however, that most immunotherapies, including those based on NKCEs, face a common challenge: the treatment of solid tumors.

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CONFLICT OF INTEREST STATEMENT

E. Vivier is a co-founder, Senior Vice President, and Chief Scientific Officer of INNATE Pharma (Marseille, France). V. Picant is an employee of INNATE Pharma (Marseille, France). The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing does not apply to this article as no new data were created or analyzed in this study.

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