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Review Article

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Current Developments in NK Cell Engagers for Cancer Immunotherapy: Focus on CD16A and NKp46

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
ADCC, Ab-dependent cellular cytotoxicity;

ABSTRACT

NK cells are specialized immune effector cells crucial for triggering immune responses against aberrant cells. Although recent advancements have concentrated on creating or releasing T-cell responses specific to tumor Ags, the clinical advantages of this approach have been limited to certain groups of patients and tumor types. This emphasizes the need for alternative strategies. One pioneering approach involves broadening and enhancing anti-tumor immune responses by targeting innate immunity. Consequently, the advent of bi-, tri-, and multi-specific Abs has facilitated the advancement of targeted cancer immunotherapies by redirecting immune effector cells to eradicate tumor cells. These Abs enable the simultaneous binding of surface Ags on tumor cells and the activation of receptors on innate immune cells, such as NK cells, with the ability to facilitate Ab-dependent cellular cytotoxicity to enhance their immunotherapeutic effectiveness in patients with solid tumors. Here, we review the recent advances in NK cell engagers (NKCEs) focusing on NK cell-activating receptors CD16A and NKp46. In addition, we provide an overview of the ongoing clinical trials investigating the safety, efficacy, and potential of NKCEs.

Keywords: NK cells; Antibody-dependent cell cytotoxicity; Immunotherapy; NK cell receptors

INTRODUCTION

Although monoclonal Abs (mAbs) hold great promise for treating various diseases (1,2), certain therapeutic actions require the proximity of 2 different cells, a condition not achievable with a monospecific mAb or combination therapy. Since the initial proposal of bispecific Abs (bsAbs) targeting independent epitopes in the 1960s (3,4), extensive translational and clinical studies have been conducted. One potential mechanism of action of bsAbs, specifically NK cell engager (NKCE), involves the recruitment of NK cells by concurrently binding to a tumor-associated Ag (TAA) and a specific receptor on the surface of NK cells (5,6). This approach aims to leverage the immune function of NK cells for enhanced efficacy in tumor therapy (7,8).

NK cells express CD16A, also known as FcγRIIIa, which is engaged with low affinity via the fragment crystallizable (Fc) region of the IgG Abs bound to TAAs (9). These IgG molecules facilitate Ab-dependent cellular cytotoxicity (ADCC), a potent mechanism for the destruction of tumor cells by NK cells (10). Rituximab, an anti-CD20 Ab, is the first cytotoxic mAb

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ADCP, Ab-dependent cellular phagocytosis; AML, acute myeloid leukemia ; BCMA, B-cell maturation Ag; BiKE, bispecific killer cell engager; bsAb, bispecific Ab; CAR, chimeric Ag receptor; CR, complete response; DAP12, DNAX-activating protein of 12 kDa; DLT, dose limiting toxicity; DNAM-1, DNAX accessory molecule-1; EGFR, epidermal growth factor receptor; Fc, fragment crystallizable; HCC, hepatocellular carcinoma; HER2, human epidermal growth factor receptor; HL, Hodgkin's lymphoma; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; IV, intravenous ; KIR, killer immunoglobulin-like receptor; mAb, monoclonal Ab; MDS, myelodysplasia; MM, multiple myeloma; NCR, natural cytotoxicity receptor; NKCE, NK cell engager; NKG2D, NK group protein 2 family member D; NSCLC, non-small cell lung cancer; ORR, overall response rate; PK, pharmacokinetic; PKC, protein kinase C; PR, partial response; RR, relapsed/refractory; SD, stable disease; Syk, spleen tyrosine kinase; TAA, tumor-associated Ag; TME, tumor microenvironment; TRAE, treatment related adverse event; TriKE, trispecific killer engager.

Author Contributions

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with ADCC capability that was approved for the treatment of non-Hodgkin's lymphoma (HL) in 1997 (11). Following this milestone, over 30 cytotoxic Abs have been developed, and substantial efforts have been dedicated to enhancing the effectiveness of these therapeutic Abs through Fc engineering (12). Experiments using preclinical models and observed clinical outcomes in patients have demonstrated that ADCC is one of the key mechanisms contributing significantly to the therapeutic impact of numerous approved Abs (13). These drugs include rituximab, cetuximab, and trastuzumab (14). Therefore, binding affinity to CD16A appears to be a crucial factor (9).

To enhance both affinity and cytotoxicity, novel Ab-like molecules have been created to target CD16A with a greater binding strength than the natural Fc portion of the IgG1 Ab (13). One example of these molecules is the bispecific killer cell engager (BiKE), which has one binding arm directed at CD16A and the other at a TAA (6). An advanced version, known as trispecific killer engagers (TriKEs), improves BiKEs by incorporating CD16A and TAA-targeting single-chain variable fragments linked with human IL-15 or other cytokine moieties, providing an additional stimulatory effect on NK cell proliferation and activation (15).

Leading companies, such as Innate Pharma and Affimed that are developing NKCEs are currently developing trifunctional NKCEs that incorporate Ab fragments targeting 2 NK cell-activating receptors (CD16A and NKp46) along with a TAA encompassing CD19, CD20, and the epidermal growth factor receptor (EGFR) (16,17). NKCE-mediated targeting of NK cell-activating receptors enhances NK cell cytotoxicity and cytokine production (5). This strategy offers substantial clinical advantages and strengthens the interaction between the 2 cell types to increase NK cell effector function, which can enhance anti-tumor cytotoxic activity. Here, we review the current status and advances in NKCE strategies primarily focusing on CD16A and NKp46 in the preclinical and clinical development of cancer immunotherapy.

CHARACTERISTICS AND FUNCTION OF NK CELLS

NK cells are innate immune cells involved in the first line of defense, representing 5%–15% of the lymphocytes in human peripheral blood, with the ability to target and eliminate virus-infected cells or tumor cells without prior sensitization (18). NK cells are defined as CD3 negative CD56 positive lymphocytes in the human peripheral blood that play important roles in cytotoxic activity and immune regulation (18,19). They can be divided into two major subtypes—CD56 dim NK cells and CD56 bright NK cells—based on the expression levels of the surface marker CD56. Approximately 90%–95% of blood NK cells are CD56 dim NK cells (20). CD56 dim NK cells release cytolytic granules such as perforin and granzyme B and express high surface levels of CD16A (FcγRIIIa), thereby promptly mediating potent cytotoxic activity (20). The remaining 5%–10% of blood NK cells are CD56 bright NK cells (21). CD56 bright NK cells express low levels of CD16A and release high amounts of IFN-γ in response to stimulation by cytokines (21).

NK cells kill target cells by ADCC, releasing perforin and granzyme contained within cytotoxic granules and mediating apoptosis signals through death receptor pathways via the expression of factor-associated suicide ligand or TNF-related apoptosis-inducing ligand (22). NK cells actively circulate in the body and are located at tumor sites to eliminate cancer cells (23). Consequently, they penetrate the tumor microenvironment (TME), resulting in improved overall survival in individuals with various cancer types (24).

The administration of allogeneic NK cells is considered to be a safer therapeutic option compared to allogeneic T cell treatments, primarily because NK cells do not cause graft-versus-host reactions, which are a significant concern with allogeneic T cell therapies (25). NK cell-based immunotherapies may reduce the likelihood of inducing cytokine release syndrome and neurotoxicity (25,26). Moreover, allogeneic NK cells are not limited by the MHC which binds to inhibitory receptors of NK cells and contributes to the self-tolerance of NK cells (18,26).

NK cells express an array of activating and inhibitory receptors that regulate their activities (**Fig. 1**). DNAX accessory molecule-1 (DNAM-1), NKp30, NKp44, NKp46, and NK group protein 2 family member D (NKG2D) are representative NK cell-activating receptors that recognize stress-induced ligands on tumor cells and activate NK cells to lyse tumor target cells by releasing cytolytic granules such as perforin and granzyme B (18).

DNAM-1 binds to its ligands, CD112 and CD155, on tumor cells to activate NK cells (27). Natural cytotoxicity receptors (NCRs), NKp30 (NCR3), NKp44 (NCR2), and NKp46 (NCR1) are important mediators that trigger cytotoxic effects in NK cells (28). NKp30 is a 30 kDa NK cell receptor protein that is expressed on both mature resting and activated NK cells, similar to NKp46 (28). Unlike NKp30 and NKp46, the expression of NKp44 is triggered by activation signals induced by cytokines, such as IL-2 and IL-15 (29). Therefore, NKp44 can be an activation marker for NK cells, and decreased levels of NKp44 are correlated with the reduced anti-tumor activity of NK cells (29). B7-H6 and BAG6 bind to NKp30 and trigger NK cell activation (30). NKp30 and NKp46 trigger activation signals of NK cells through immunoreceptor tyrosine-based activation motif (ITAM)-containing proteins, FcRγ, and CD3ζ. NKp44 activates NK cells through signals derived from DNAX-activating protein of 12 kDa (DAP12) (31). NKp44 is a 44 kDa transmembrane glycoprotein associated with the adaptor protein DAP12, which contains ITAM for signal activation (31). Ligand-bound NKp44 promotes the target killing activity of NK cells through releasing IFN-γ and TNF-α (32). NKp44 also contains an inhibitory cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) that mediates the inhibitory activity of NK cells, indicating a dual function of NKp44 (33). NKp44 and NKp46 can bind to hemagglutinins of viral envelope proteins such as influenza, resulting in the activation of NK cell cytotoxicity (34). NKp46 (NCR1, CD335) is an activating NK cell-surface glycoprotein associated with CD3ζ and FcRγ and induces cytotoxic activity and cytokine release of NK cells (34).

NKG2D mediates the cytotoxicity of NK cells through activation of the PI3K signaling pathway by interacting with its ligands of MHC class I chain-related proteins A and B and UL16-binding proteins (34). CD16 is another effective activating receptor of NK cells involved in ADCC and is promoted by binding to the Fc region of IgG Abs to target Ab-bound cancer cells (34). Additionally, NK cells produce various cytokines and chemokines including IFN-γ, CCL3, CCL4, CCL5, and IL-10 that are important for immune regulation involving dendritic cells and T cells (35).

NK cells avoid killing normal cells and detect “missing self” cancer cells using their inhibitory receptors (35). Killer immunoglobulin-like receptors (KIRs), which are NK cell inhibitory receptors, bind to MHC-I, which is expressed in normal cells but not in cancer cells (35). Other NK cell inhibitory receptors, such as NKG2A, CD96, lymphocyte-activation gene 3, T cell immunoreceptor with Ig and ITIM domains, and T cell immunoglobulin and mucin domain-containing protein 3 also negatively regulate NK cell activation by mediating their inhibitory signals (35).

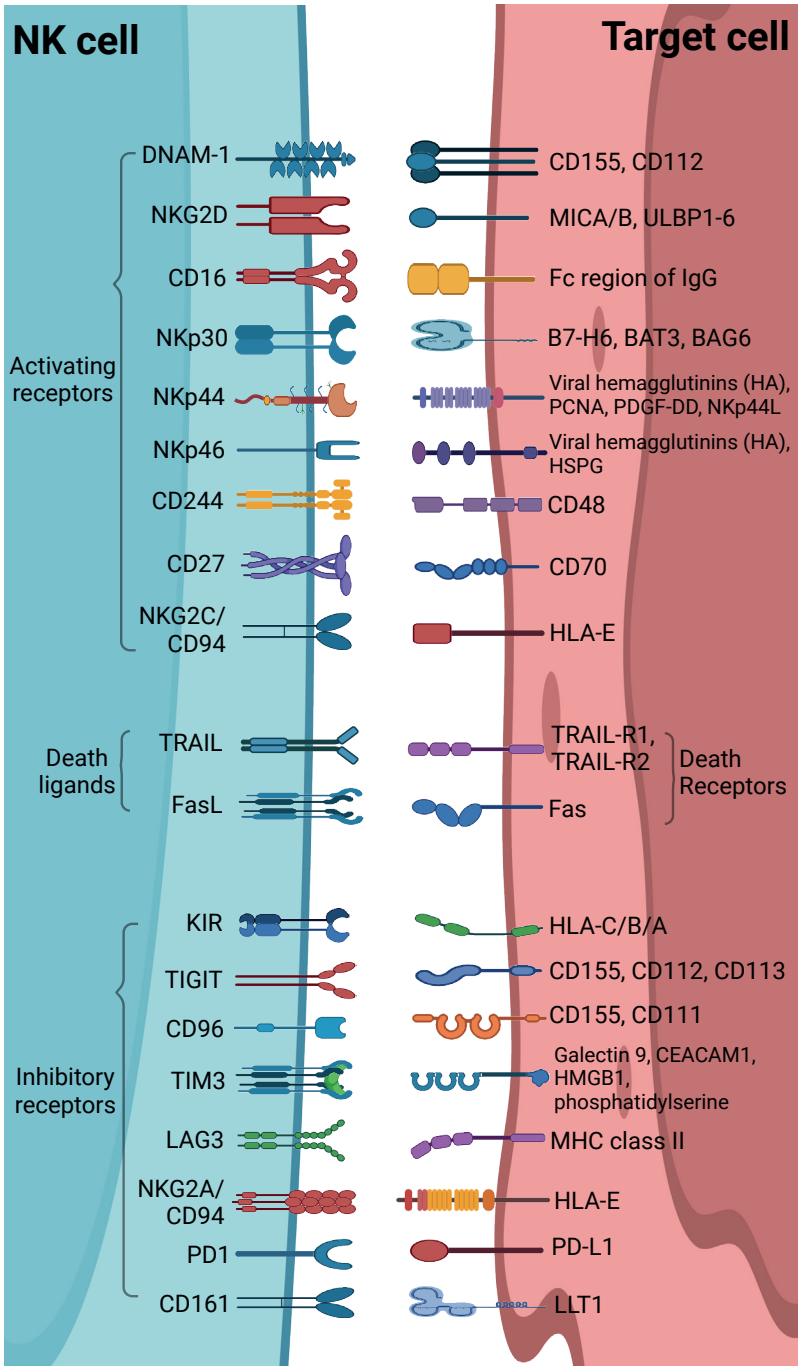

Figure 1. Receptors and ligands of NK cells.
This figure illustrates the array of inhibitory and activating receptors expressed on the surface of NK cells, alongside their corresponding ligands presented on target cells. The diagram provides a comprehensive overview of the interactions between NK cell receptors, such as NKp46, NKp44, NKG2D, and their respective ligands (Created with BioRender.com).

NKCE

The use of Abs targeting activating receptors enhances NK cell cytotoxicity and cytokine production (7,36). Based on this, a novel class of multifunctional Abs known as NKCEs has

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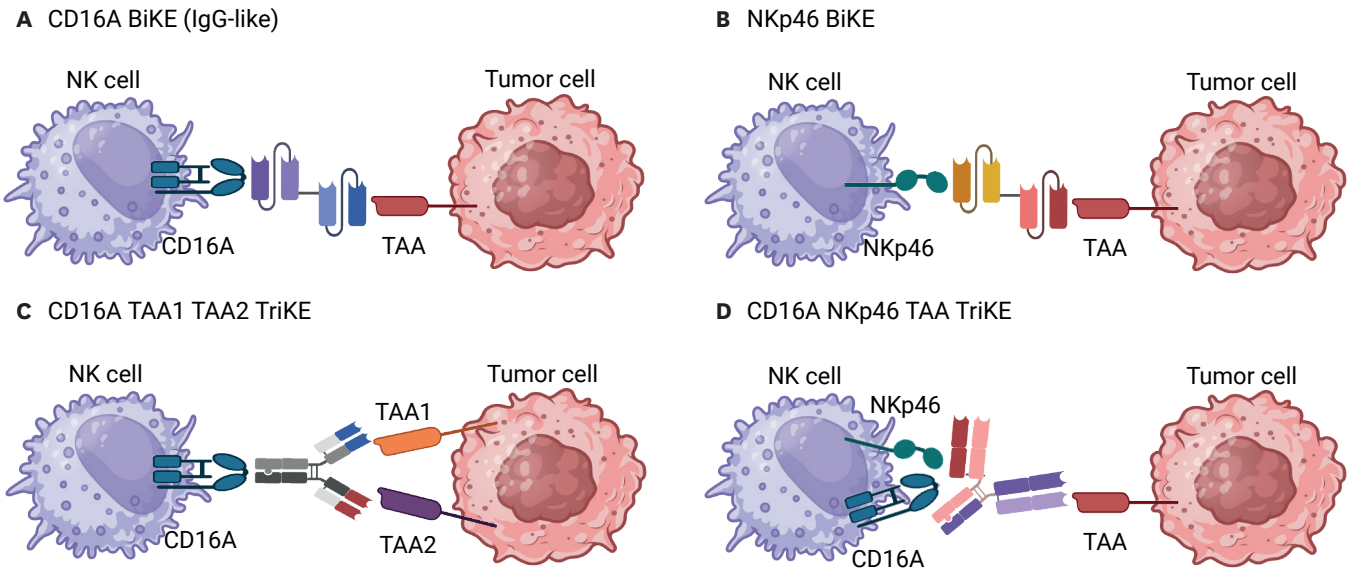

Figure 2. Diverse formats of NKCEs and their interactions with NK cells and tumor cells.
This figure provides a detailed illustration of various formats of NKCEs, including (A) CD16A BiKE (IgG-like), (B) NKp46 BiKE, (C) CD16A TAA1 TAA2 TriKE, and (D) CD16A NKp46 TAA TriKE. Each format is depicted interacting with key activating receptors on NK cells, and their respective ligands on tumor cells. These interactions are crucial for the effective engagement and activation of NK cells, promoting mechanisms such as ADCC, direct cytotoxicity, and cytokine production (Created with BioRender.com).

been developed (5). NKCEs are a novel class of synthetic Abs engineered to concurrently target Ags on tumor cells, functioning as BiKEs or TriKEs, and to activate receptors on NK cells, thereby enhancing the innate effector functions of NK cells (**Fig. 2**) (7,36).

This approach has the potential to offer cost-effective and readily available off-the-shelf solutions in contrast to cellular therapies. Additionally, NKCEs exhibit activity solely in the presence of tumor cells, implying their safety and minimal risk of nonspecific cytokine release (36). Therefore, the combination of NKCE with cell therapy appears to be a compelling alternative to chimeric Ag receptor (CAR) therapy, as it involves equipping patients with transferred effector cells without requiring genetic modifications. NKCEs have demonstrated remarkable efficacy in laboratory settings and preclinical mouse models, and ongoing clinical trials for hematological malignancies have shown encouraging initial outcomes (36).

Various activating receptors of NK cells, such as CD16A (FcγRIIIa), NKG2D, and the NCRs NKp30 and NKp46, are crucial for triggering NK cell-mediated anti-tumor immune responses. These receptors are also utilized in the development of NKCEs (37). Among these receptors, CD16A plays a particularly significant role as it interacts with the Fc region of Abs to initiate ADCC (38). For example, the effectiveness of Food and Drug Administration-approved Abs such as cetuximab, which targets EGFR, and trastuzumab, which targets the erb-b2 receptor tyrosine kinase, relies heavily on the interaction between these Abs and NK cells through CD16A to mediate ADCC (38). Thus, NK cells expressing CD16A are essential for the therapeutic efficacy of these Ab treatments (38).

To enhance ADCC efficacy, extensive progress has been made in developing next-generation Abs with improved ADCC potential, considering the Ab isotype and glycosylation, as well as affinity to the NK cell-activating receptor CD16 (39). Moreover, bi- and tri-specific multifunctional Abs with Fc regions that recognize CD16A on NK cells and tumor Ags have

been designed to sustain ADCC activity (35). These Abs establish a physical connection between NK and malignant cells using BiKEs or TriKEs (35,40). NK cell-engaged BiKEs or TriKEs offer advantages such as lower cost, reduced toxicity, and shorter preparation time than T cell engagers, and numerous BiKEs and TriKEs have been developed as targets in preclinical and clinical trials (5,22). Unlike T cell engagers, which often require personalized manufacturing processes, NKCE can be produced as off-the-shelf products with allogeneic NK cells, eliminating the need for patient-specific manufacturing processes that are required for autologous T cell-based engagers (5,41). The production process for NK cells is generally simpler and more cost-effective due to their natural ability to recognize and kill tumor cells without extensive genetic modification (5,41). This reduces both the time and cost associated with personalized treatments. Additionally, NK cells are less likely to cause graft-versus-host disease compared to T cells, particularly in allogeneic settings (5,18,41). This makes NKCEs in allogeneic settings safer for use in a broader patient population without the need for stringent HLA matching. Moreover, NK cells can target a wide range of tumor types through various activating receptors and can act independently of tumor Ag expression levels (5,18,41). This broad applicability makes NKCEs versatile and effective across different cancer types. Furthermore, NK cells are a part of the innate immune system and have a natural ability to detect and eliminate stressed or transformed cells, including tumor cells (5,18,41). NKCEs enhance this natural surveillance capability, leading to effective tumor cell elimination. T cells, being part of the adaptive immune system, require Ag presentation and prior sensitization to target tumor cells (5,18,41). This adaptive response is highly specific but can be less immediate compared to the innate responses mediated by NK cells.

At the molecular level, NKCEs facilitate the activation of NK cells by inducing the crosslinking of surface receptors (42). NKCEs offer the opportunity to crosslink activating receptors that do not typically interact during natural immune reactions. This phenomenon induces artificial crosstalk between signaling pathways, leading to potent activation of NK cell functions. Trifunctional Ab-based NK cell engager technology molecules that activate both NKp46 and CD16A show a more potent phenotype than a mixture of bispecific molecules that separately activate NKp46 and CD16A (43).

At the cellular level, NKCEs may enhance NK cell proliferation, viability, and sustainability, as evidenced in humanized mouse models through several mechanisms (5,41). NKCEs activate NK cells by engaging specific surface receptors, such as CD16A and NKp46, which leads to enhanced cytotoxic activity and subsequent proliferation of NK cells (5,41). This targeted activation ensures that NK cells are continuously stimulated, promoting their survival and expansion (5,41). Furthermore, NKCEs often incorporate cytokine support, such as IL-15, which is crucial for NK cell proliferation and maintenance (5,41). This cytokine environment supports the long-term viability and functionality of NK cells within the humanized mouse models (5,41). The design of NKCEs also facilitates the formation of effective immune synapses between NK cells and their target cells, enhancing the efficiency of NK cell-mediated cytotoxicity and sustaining their activity over extended periods (5,41). Additionally, NKCEs have shown potential to augment the infiltration of NK cells into solid tumors. This augmentation may be facilitated by modulating the TME, altering the expression of adhesion molecules, and increasing the production of chemokines and cytokines that attract NK cells to the tumor site (44). For example, studies have indicated that certain chemokines, such as CXCL10 and CCL5, can play a role in directing NK cells into tumors (44).

However, continuous stimulation in the context of NKCE therapies can result in the exhaustion of NK cells, characterized by reduced cytotoxic activity and proliferation (36). This issue can be addressed by utilizing molecules with short half-lives or by implementing adjusted injection protocols to restrict the ongoing activation of NK cells (36). Additionally, NK cells may encounter an immunosuppressive environment in the TME, potentially limiting their effectiveness (45). The TME can be rich in immunosuppressive factors such as TGF-β, prostaglandin E2, and adenosine, which inhibit NK cell function (45).

Combining NKCEs with treatments that block immune checkpoints on NK cells or inhibit immunosuppressive factors released at the tumor site emerges as an appealing strategy to enhance NKCE efficacy (46). For instance, checkpoint inhibitors targeting receptors such as NKG2A or PD-1 on NK cells can alleviate suppression and restore NK cell activity. Additionally, targeting immunosuppressive molecules like TGF-β, prostaglandin E2, and adenosine with specific inhibitors can further potentiate the anti-tumor effects of NKCEs (46).

THE BIOLOGY OF CD16A

CD16A is a surface receptor on NK cells and a member of the immunoglobulin superfamily with two extracellular Ig-like domains (9). It is expressed at low levels in active cytokine-releasing $CD56^{bright}$ NK cells but is highly expressed in highly cytotoxic $CD56^{dim}$ NK cells (26). CD16A recognizes the constant Fc region of IgG Abs bound to tumor Ags to trigger strong downstream signals, resulting in anti-tumor responses mediated through a potent cytotoxic mechanism of ADCC, with the degranulation of perforin and granzymes to target tumor cells (**Fig. 3**) (9).

CD16A signalling is triggered by the phosphorylation of intracellular ITAM, which is induced by Ag-bound IgG interacting with CD16A (**Fig. 3**) (9). Since CD16A does not have ITAM in its intracellular domain, ITAM domain bearing CD3ζ and FcεRIγ are required (9). In addition, CD16A is phosphorylated by protein kinase C (PKC) (9). Phosphorylated CD16A promotes cytokine production, whereas non-phosphorylated CD16A results in potent degranulation (47). Once CD16A is phosphorylated, an Src kinase Lck phosphorylates ITAM domains located in CD3ζ and FcεRIγ (38). Phosphorylated ITAMs then recruit and phosphorylate spleen tyrosine kinase (Syk), zeta-chain-associated protein kinase-70 (ZAP-70), and Syk family kinases (38). The downstream PI3K converts phosphatidylinositol (4,5)-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate through phospholipase C-γ (38). Then, downstream diacylglycerol activates the PKC family, and another downstream target, inositol triphosphate, promotes calcium release from the endoplasmic reticulum to the cytosol, which is a major signal for ADCC triggering (38). Additionally, ERK2 MAPK pathways are activated by CD16A engagement in ADCC (9).

THE BIOLOGY OF NKp46

NKp46 (NCR1, CD335) is a 46kDa glycoprotein which is a member of the Ig superfamily (43). It is expressed on CD56 dim CD16+ and CD56 bright CD16− human NK cells regardless of their activation status (48). Cross-linking with anti-NKp46 mAb promotes enhanced cytotoxicity and secretion of IFN-γ and TNF-α (41). Reduced NK cell cytotoxicity has been observed by blocking NKp46 signaling using specific mAbs in various cancer cells (41).

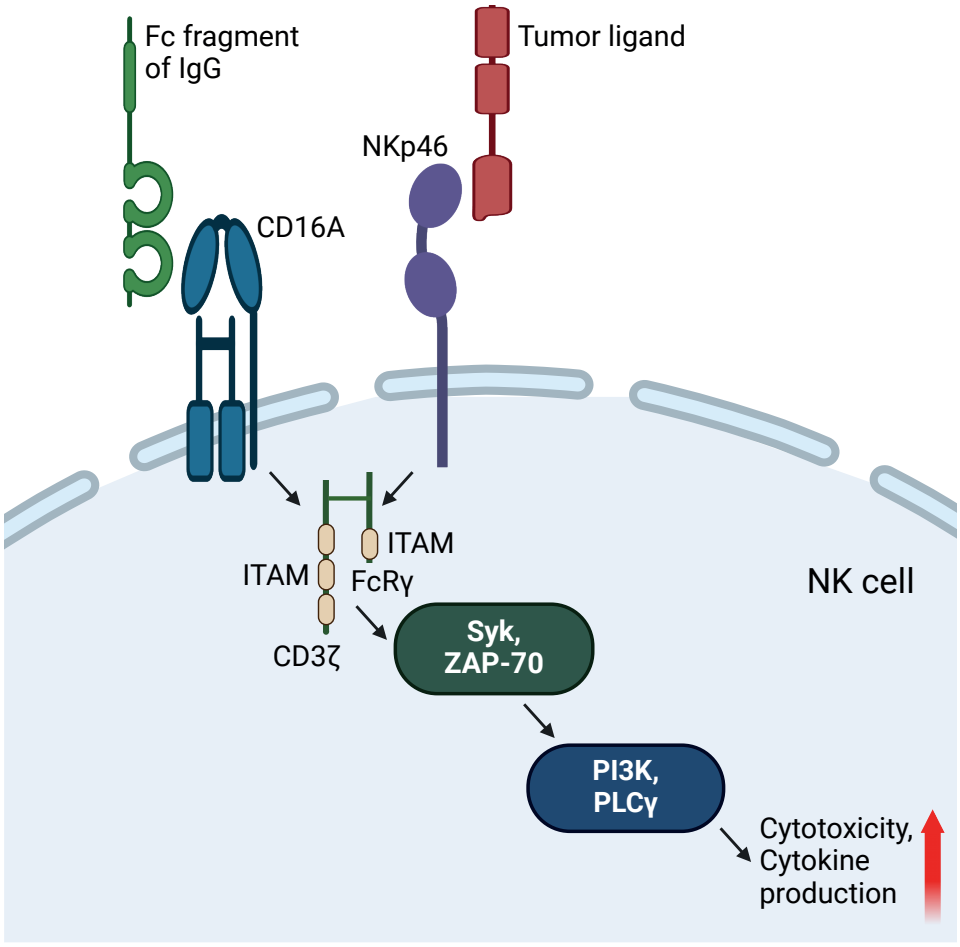

Figure 3. Mechanism of CD16A and NKp46 in NK cell functions.
This figure elucidates the mechanisms by which CD16A and NKp46 receptors enhance NK cell functions. The binding of the Fc fragment of IgG to CD16A, and the interaction of tumor-associated ligands with NKp46, trigger signal transduction pathways. These pathways involve ITAMs, CD3ζ, and FcRγ, leading to the activation of downstream signaling molecules such as Syk, ZAP-70, PI3K, and PLCγ. This cascade of signaling events results in increased cytotoxic activity and cytokine production, thereby augmenting the immune response against tumor cells (Created with BioRender.com).

NKp46 is involved in the recognition and lysis of target cells by NK cells (**Fig. 3**) (28). It recognizes and binds to specific ligands on target cells, such as tumor cells and virally infected cells. This activation leads to the release of cytotoxic granules containing perforin and granzymes, which induce apoptosis in the target cells (49). Known ligands include viral hemagglutinins and stress-induced cellular proteins, and the binding specificity is mediated through the extracellular Ig-like domains (49).

Additionally, NKp46 engagement stimulates the production of cytokines such as IFN-γ, which further enhances the immune response. NKp46 consists of two extracellular Ig-like domains of the C2 type. NKp46 mediates cytotoxic activity of NK cells by inducing signaling through ITAM, CD3ζ and FcRγ (**Fig. 3**) (31). The signal transduction pathway of NKp46 involves its association with CD3ζ or FcεRIγ adaptor proteins, which contain ITAMs (50). Upon ligand binding, these ITAMs become phosphorylated, initiating a cascade of downstream signaling events that lead to NK cell activation and target cell lysis (50).

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Table 1. Clinical trials of NKCE based on CD16A and NKp46

Engager name	National clinical trial number	Title	Status	Conditions	Interventions	NK receptor	Tumor receptor	Phase	Clinical outcomes	Sponsor/ Collaborators	Start date
AFM13	NCT01221571	A Study to Assess AFM13 in Patients With Hodgkin Lymphoma	Completed	Hodgkin lymphoma	Drug: AFM 13	CD16A	CD30	Phase 1	Favorable safety profile, partial remission (11.5%), SD (50%), and overall disease control rate (61.5%) at doses of 0.01–7 mg/kg ORR (23%) and overall disease control rate (77%) at doses of 1.5 mg/kg or higher (53,54)	Affimed GmbH	October 2010
AFM13	NCT02665650	Study of the Combination of AFM13 and Pembrolizumab in Patients With Relapsed or Refractory Classical Hodgkin Lymphoma	Completed	Hodgkin lymphoma	Biological: AFM13 Biological: Pembrolizumab	CD16A	CD30	Phase 1	Safe and well-tolerated, ORR: 83% for the overall population, half-life of up to 20.6 h (56)	Affimed GmbH/Merck Sharp & Dohme LLC The Leukemia and Lymphoma Society	May 2016
AFM13	NCT03192202	AFM13 in Relapsed/ Refractory Cutaneous Lymphomas	Completed	Lymphoma, T-cell, cutaneous	Drug: AFM13	CD16A	CD30	Phase 1 Phase 2	Safe and well-tolerated, with significant clinical responses ORR (40%) The activation marker CD69 on NK increased in responders compared to non-responders (57)	Ahmed Sawas, MD Columbia, University	July 17, 2017

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DEVELOPMENT STATUS AND CLINICAL TRIALS OF NKCEs BASED ON CD16A AND NKp46

AFM-13

Affimed's AFM13-NK, the first product to advance to clinical trials, involves non-engineered umbilical cord blood-NK cells pre-conjugated with AFM13 tetravalent bsAbs (17). AFM13 binds to CD16A on NK cells and CD30-positive tumor cells, such as those found in HL and peripheral T-cell lymphoma, thereby enhancing NK cell-mediated ADCC (17). The results of pre-clinical study of AFM13 have been published in 2021, identified AFM13 as a promising therapeutic agent in combination with IL-12/15/18-activated peripheral blood or cord blood NK cells to treat CD30 positive lymphomas *in vitro* and *in vivo* (51).

Clinical trials of AFM13 NK cell engagers are presented in **Table 1**. NCT01221571 is a phase 1 dose escalation study that evaluated the safety of AFM13 in patients with CD30 positive refractory and relapsed HL (52). Patients received AFM13 at doses of 0.01 to 7 mg/kg body weight (53). Partial remission was observed in 11.5% of evaluable patients, with stable disease (SD) rate of 50% and overall disease control rate of 61.5% (53). Significant NK cell activation and decrease in soluble CD30 in peripheral blood were observed, although the most favorable clinical outcome was only partial response (PR, 11.5%) (54). Among patients administered AFM13 at doses of 1.5 mg/kg or higher, the overall response rate (ORR) was 23%, and the overall disease control rate was 77% in those who had undergone extensive prior treatment (54). This phase 1 study demonstrated that AFM13 is a promising therapeutic agent for patients with relapsed or refractory HL, with a favorable safety profile, positive immunological activity, measurable disease response, and significant immunogenicity (53). NK cells from patients treated with AFM13 showed enhanced anti-tumor activity against HL (55).

NCT02665650 is a phase 1 dose escalation study that tested the safety of Affimed's AFM13 in combination with pembrolizumab in patients with relapsed or refractory HL (52). Patients were infused with AFM13 at escalating doses intravenously for 25 wk and a fixed dose of pembrolizumab intravenously for 52 wk (52). AFM13 in combination with pembrolizumab showed an ORR of 83% in the overall population, and well tolerated in patients with relapsed/ refractory (RR) HL (56). Pharmacokinetics of AFM13 in combination with pembrolizumab showed a half-life of up

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Table 1. (Continued) Clinical trials of NKCE based on CD16A and NKp46

Engager name	National clinical trial number	Title	Status	Conditions	Interventions	NK receptor	Tumor receptor	Phase	Clinical outcomes	Sponsor/ Collaorators	Start date
AFM13	NCT04074746	Modified Immune Cells (AFM13-NK) and A Monoclonal Antibody (AFM13) in Treating Patients With Recurrent or Refractory CD30 Positive Hodgkin or Non-Hodgkin Lymphomas	Active (not recruiting)	Recurrent anaplastic large cell lymphoma, recurrent B-cell non-Hodgkin lymphoma, recurrent classic Hodgkin lymphoma, recurrent mycosis fungoides, recurrent peripheral T-cell lymphoma, not otherwise specified, refractory anaplastic large cell lymphoma, refractory B-cell non-Hodgkin lymphoma, refractory classic Hodgkin lymphoma, refractory mycosis fungoides, refractory peripheral T-cell lymphoma, not otherwise specified	Biological: Anti-CD30/CD16A mAb AFM13 Drug: Cyclophosphamide Drug: Fludarabine Drug: Fludarabine Phosphate Biological: Genetically Engineered Lymphocyte Therapy	CD16A	CD30	Phase 1 Phase 2	Favorable safety profile, ORR (92.8%), CR (62%) (58)	M.D. Anderson Cancer Center	July 18, 2020
AFM24	NCT04259450	Study to Assess AFM24 in Advanced Solid Cancers	Recruiting	Advanced solid tumor	Drug: AFM24	CD16A	EGFR	Phase 1 Phase 2	Well-tolerated with favorable safety profile, Tumor control rate: (50%) (60,61)	Affimed GmbH	April 7, 2020
AFM24	NCT05099549	Safety, Tolerability, and Anti-Tumor Activity of AFM24 in Combination With SNK01 in Subjects With Advanced/ Metastatic EGFR-Expressing Cancers	Recruiting	Squamous cell carcinoma of the head and neck, non-small cell lung carcinoma, colorectal neoplasms, advanced solid tumor, refractory tumor, metastatic tumor	Drug: AFM24 Biological: SNK01	CD16A	EGFR	Phase 1 Phase 2	Well-tolerated with favorable safety profile, No DLTs or unexpected toxicities were observed. Grade 1/2 infusion related reactions in 5/6 patients In the low dose cohort (160 mg), 2 patients had SD for 106 days (15 wk) and 100 days (14 wk), respectively At 480 mg, 1 patient with SD had 2% tumor burden reduction and 2 subsequent carcinoembryonic Ag reductions (62)	NKGen Biotech, Inc., Affimed GmbH	November 3, 2021
AFM24	NCT05109442	Study to Assess AFM24 in Combination With Atezolizumab in Selected Advanced/ Metastatic EGFR-expressing Cancers	Recruiting	Advanced solid tumor	Drug: AFM24 Drug: Atezolizumab 840 MG in 14 ML Injection	CD16A	EGFR	Phase 1 Phase 2	Well tolerated with no new or unexpected toxicities compared to each single agent The most common AFM24-related adverse events were infusion-related reactions (grade 1–2, n = 10; grade 3, n = 2) 4 out of 15 patients responded to treatment (1 confirmed CR and 3 PRs) (66)	Affimed GmbH	November 19, 2021
DF1001	NCT04143711	Study of DF1001 in Patients With Advanced Solid Tumors	Recruiting	Solid tumor, adult	Drug: DF1001 Drug: Nivolumab Drug: Nab paclitaxel	CD16A	HER2	Phase 1 Phase 2	Well-tolerated with favorable safety profile, TRAEs were mostly low grade (grade 1–2) No DLT was detected. Responders showed increased inflammatory cytokines, chemokines and NK, CD8 cells in TME (68)	Dragonfly Therapeutics	November 11, 2019

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Table 1. (Continued) Clinical trials of NKCE based on CD16A and NKp46

Engager name	National clinical trial number	Title	Status	Conditions	Interventions	NK receptor	Tumor receptor	Phase	Clinical outcomes	Sponsor/ Collaorators	Start date
DF9001	NCT05597839	Study of DF9001 in Patients With Advanced Solid Tumors	Recruiting	Solid tumor, adult	Drug: DF9001 Drug: Nivolumab	CD16A	EGFR	Phase 1 Phase 2	Well-tolerated with favorable safety profile and encouraging clinical responses Tumor burden reductions across several tumor types, including HER2-low and heavily pre-treated patients (67)	Dragonfly Therapeutics	November 15, 2022
GTB-3550 (CD16/IL-15/CD33) tri-specific killer cell engager (TriKE®)	NCT03214666	GTB-3550 Tri-Specific Killer Engager (TriKE®) for High Risk Hematological Malignancies	Terminated (Development of GTB-3550 halted due to development of the second generation camelid nanobody TriKE drug product, GTB-3650)	High-risk myelodysplastic syndromes, acute myelogenous leukemia, systemic mastocytosis, mast cell leukemia	Drug: GTB-3550 TriKE® Phase I Drug: GTB-3550 TriKE® Phase II	CD16A and IL15R	CD33	Phase 1 Phase 2	Well tolerated, with favorable safety profile No DLTs in doses of 5–150 mcg/kg/day Responders have higher NK cell degranulation than non-responders Increased expression of the maturation marker CD57 and activation receptor NKG2D, while showing decreased expression of inhibitory KIR (70)	GT Biopharma, Inc.	January 1, 2020
RO7297089 (AFM26)/ RG6296	NCT04434469	A Study Evaluating The Safety And Pharmacokinetics Of Escalating Doses Of RO7297089 In Patients With Relapsed Or Refractory Multiple Myeloma	Completed	Refractory multiple myeloma, relapsed multiple myeloma	Drug: RO7297089	CD16A	BCMA	Phase 1	Well tolerated at doses up to 1,850 mg, PR (7%), minimal response (7%), SD (52%). (72)	Genentech, Inc.	July 8, 2020
SAR443579	NCT05086315	First-in-human Study of SAR443579 Infusion in Male and Female Participants of at Least 12 Years of Age With Relapsed or Refractory Acute Myeloid Leukemia (R/R AML), B-cell Acute Lymphoblastic Leukemia (B-ALL) or High Risk-myelodysplasia (HR-MDS)	Recruiting	Acute lymphocytic keukaemia, acute myeloid leukaemia refractory, myelodysplastic syndrome	Drug: SAR443579	NKp46/ CD16A	CD123	Phase 1 Phase 2	No DLTs were observed Well tolerated up to doses of 3000 μg/kg once a week The most common treatment emergent adverse events included infusion-related reactions (n = 10 [43.5%]) and nausea (n = 7 [30.4%]) Two cases of cytokine release syndrome (grade 1, n = 1; grade 2, n = 1) No case of immune effector cell-associated neurotoxicity syndrome (75)	Sanofi	December 8, 2021

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to 20.6 h (56). AFM13 induced promising NK cell activation and a decrease of soluble CD30 in peripheral blood of HL patients (56). This trial was found to be safe and well-tolerated, with manageable adverse events and significant clinical responses (56).

NCT03192202 is a phase 1/2, open-label trial evaluating the safety and efficacy of Affimed's AFM13 in patients with relapsed or refractory CD30-positive T-cell cutaneous lymphomas (52). This trial also examines the immunologic changes in the tumor and peripheral blood in response to the variations of dose and method of injection of AFM13 (57). Patients with relapsed or refractory CD30 positive T cell cutaneous lymphomas were infused with AFM13 intravenously (1.5 mg/kg intravenous [IV] weekly, 7 mg/kg IV weekly, 7 mg/kg continuous IV infusion over 5 days weekly and 200 mg IV weekly) (57). The results showed a high ORR of 40% among heavily pretreated patients with CD30 positive T cell lymphomas (57). Circulating NK cells were decreased in peripheral blood during therapy, with recovery noted post-therapy (57). The expression of NK cell activation marker CD69 was increased in patients with response compared to non-responders (57). Infiltrated NK cells were increased and circulating CD4+ CD25+ Tregs were decreased in patients with response (57). This trial was found to be safe and well-tolerated, with significant clinical responses observed in many patients (52).

NCT04074746 is a phase 1/2 M.D. Anderson Cancer Center-sponsored trial to evaluate the safety and efficiency of modified umbilical cord blood-derived NK cells in combination with AFM13 (AFM13-NK) and AFM13 monotherapy in patients with CD30 positive HL or recurrent/refractory non-HL (52). Specifically, this study aims to determine the safety and recommended phase 2 dose of umbilical cord blood-derived NK cells preloaded with the bsAb AFM13 (AFM13-NK), followed by IV administration of AFM13 in patients with RR CD30-positive lymphomas (52). Cord blood-derived cytokine (IL-12/IL-15/IL-18)-induced memory-like NK cells pre-associated with AFM13 have promising tolerability and activity for patients with RR CD30 positive lymphoma (58). This study demonstrated a high efficacy with 92.8% ORR and 62% complete response (CR), along with a favorable safety profile (58).

AFM-24

Affimed's AFM24 is a bispecific NKCE targeting CD16A on NK cells and EGFR on solid tumor (59). The pre-clinical study of AFM24 showed promising results of effectively inducing ADCC via NK cells against a variety of EGFR-expressing tumor cells regardless of EGFR mutational status *in vitro* (59). In vivo studies with cynomolgus monkeys revealed no notable toxicities (59). Clinical trials of AFM24 NK cell engagers are presented in **Table 1**.

NCT04259450 is a phase 1/2, open-label, multi-center, dose escalation and expansion study testing AFM24 monotherapy in patients with EGFR-positive advanced solid tumors, including renal cell carcinoma, non-small cell lung cancer, and colorectal cancer (52). AFM24 is infused intravenously into patients (17,52). The recommended phase 2 dose of AFM24 was determined at phase 1 dose escalation study by intravenously injecting AFM24 to test safety, efficacy, pharmacokinetic (PK) and pharmacodynamic (60). AFM24 showed safety and was well tolerated with tumor control rate of 50% (60,61).

NCT05099549 is a phase 1/2, open-label, multi-center trial investigating the safety and efficacy of AFM24 in combination with SNK01 in patients with advanced or metastatic EGFR-positive cancers, including squamous cell carcinoma of the head and neck, non-small cell lung cancer (NSCLC), and colorectal neoplasms (52). This triggers specific lysis of EGFR-positive cancer cells by ADCC (17,62). SNK01 is an autologous NK cell therapy candidate from

NKGen Biotech (Santa Ana, CA, USA) and is actively undergoing clinical trials for cancer and neurodegenerative diseases (63). This study employs a dose escalation phase (phase 1) to test escalating doses of AFM24 in combination with a fixed dose of SNK01 and a dose expansion phase (phase 2) (52). This combination study of AFM24 and SNK01 has completed 2 dose cohorts (160 mg followed by 480 mg) (62). Grade 1/2 infusion related reactions in 5/6 patients (62). In the low dose cohort (160 mg), 2 patients had SD for 106 days (15 weeks) and 100 days (14 weeks), respectively (62). At 480 mg, 1 patient with SD had 2% tumor burden reduction and 2 subsequent carcinoembryonic Ag reductions (62). PK results of AFM24 and SNK01 combination are consistent with AFM24 alone without toxic effects (62). This demonstrates that AFM24 in combination with SNK01 is well tolerated with favorable safety profile (62).

NCT05109442 is a phase 1/2, open-label, multi-center, dose escalation and expansion trial testing the safety and efficacy of AFM24 in combination with atezolizumab in patients with EGFR-positive advanced solid tumors, including NSCLC, gastric cancer, and pancreatic/hepatocellular/biliary tract cancer (52). AFM24 uses innate immunity of patients by targeting EGFR on tumor cells and CD16A on NK cells to induce ADCC/Ab-dependent cellular phagocytosis (ADCP) towards tumor cells (64). Atezolizumab is a humanized engineered mAb targeting PD-L1 (65). The combination was well tolerated with no new or unexpected toxicities observed (66). The most common AFM24-related adverse events were infusion-related reactions (grade 1–2, n = 10; grade 3, n = 2) (66). No dose limiting toxicities (DLTs) were reported and clinical activity was observed. 4 out of 15 patients responded to treatment (1 confirmed CR and 3 PRs) (66). This trial demonstrates that AFM24 in combination with atezolizumab is well tolerated with favorable safety in patients with selected advanced/metastatic EGFR-expressing cancers (66).

DF1001 and DF9001

DF1001 and DF9001 are NKCE targeting CD16A on NK cells and human epidermal growth factor receptor 2 (HER2) (DF1001) and EGFR (DF9001) developed by Dragonfly therapeutics (67). Pre-clinical study of DF1001 showed that DF1001 revealed promising anticancer activity with immune cell infiltration including NK cells and CD8+ T cells even in the patients received heavy prior therapy regardless of the level of HER2 expression (67). Preclinical study results of DF9001 demonstrated that it is engaged with immune effector cells including NK cells and promotes anti-tumor activity by triggering expression of PD-L1 in tumor cells to sensitize cold tumors to checkpoint inhibitors (67). Clinical trials of DF1001 and DF9001 NK cell engagers are presented in **Table 1**.

NCT04143711 is a phase 1/2, open label trial to test the safety, tolerability and efficacy of Dragonfly Therapeutics-sponsored DF1001 in patients with advanced solid tumors including metastatic breast cancer, urothelial bladder cancer, gastric cancer, esophageal cancer, gastroesophageal junction cancer, and NSCLC (52). This trial comprises two phases. The first phase is the dose escalation phase in patients with various types of HER2-positive solid tumors (52). In dose escalation study of phase 1, DF1001 is shown to be a safe and well-tolerated NKCE targeting HER2 (68). DF1001 alone showed significant anti-tumor activity targeting HER2 positive refractory solid tumors (68). The effects of 2 combination therapies, DF1001 in combination with nivolumab (PD-1-blocking Ab) or Nab paclitaxel (albumin-bound paclitaxel), will be tested (52). The second phase tests the dose expansion using the best dose determined from the first phase (52). DF1001 was well-tolerated with favorable safety profile in refractory patients with a spectrum of HER2 expression (68). Treatment related adverse events (TRAEs) were mostly low grade (grade 1–2) and no DLT was detected (68). Responders showed increased inflammatory cytokines, chemokines and NK, CD8 cells in TME (68).

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NCT05597839 is a phase 1/2, open-label trial to test the safety, tolerability, pharmacokinetics, biological, and clinical activity of DF9001 as a monotherapy and in combination with nivolumab (a PD-1 checkpoint inhibitor) in adult patients with solid tumors, including head and neck squamous cell carcinoma, colorectal cancer, and NSCLC (52). The study is composed of 2 phases. The first phase is a dose escalation study, and the second phase is a dose expansion, using the best dose determined in the first phase (52). DF9001 in patients with advanced solid tumors has shown a well-tolerated and favorable safety profile with encouraging clinical responses (67). Tumor burden reductions were observed in several tumor types including in HER2-low and heavily pre-treated patients (67).

GTB3550

GTB3550 is a tri-specific killer cell engager targeting CD16A on NK cells and CD33 on hematological malignancies, and simultaneously expressing IL-15 (69). Preclinical study results of GTB3550 demonstrated that it enhances NK cell cytotoxicity, degranulation and release of cytokines against CD33 positive target tumor cells (69). GTB3550 also induced in vivo persistence and expansion of NK cells (69). Clinical trial of GTB3550 NK cell engager is presented in **Table 1**.

NCT03214666 is a phase 1/2, multi-center trial testing the safety and efficacy of a GTB-3550 in patients with CD33-positive high-risk myelodysplastic syndromes, refractory and relapsed acute myeloid leukemia, or advanced systemic mastocytosis (52). GT Biopharma's GTB-3550 enhances the anti-tumor activity of NK cells and targets CD33 positive myeloid derived suppressor cells which mediate immunosuppressive TMEs (52). GTB-3550 TriKE, administered as a monotherapy, safely promoted functional expansion of endogenous NK cells with anti-tumor activity in patients with advanced acute myeloid leukemia (AML) and myelodysplasia (MDS) (70). The result of phase 1 trial shows that GTB-3550 TriKE is safe, promotes a significant expansion of endogenous NK cells, and demonstrates clinical activity (70). This trial demonstrates that GTB-3550 is well tolerated with favorable safety profile and no severe dose-limiting toxicities in patients with CD33-expressing MDS (52). No DLT was observed in doses of 5–150 mcg/kg/day (70). Responders have higher NK cell degranulation (CD107a) than non-responders (70). Increased expression of the maturation marker CD57 and activation receptor NKG2D were observed, while inhibitory KIR was shown to be decreased (70). This study was terminated because the development of GTB-3550 was halted by the development of a second-generation camelid nanobody TriKE drug product, GTB-3650 (52).

RO7297089

RO7297089 is bispecific tetravalent Ab targeting CD16A on NK cells and B-cell maturation Ag (BCMA) on multiple myeloma (MM) (71). Pre-clinical study of RO7297089 showed robust target engagement *in vivo* model of cynomolgus monkeys (71). The clinical trial of RO7297089 is presented in **Table 1**.

NCT04434469 is a phase 1, open-label, multicenter, Genentech-sponsored study to investigate the safety, pharmacokinetics and efficacy of RO7297089 in patients with RRMM (52). In a dose-escalation study, patients with RRMM were intravenously infused with RO7297089 weekly at a starting dose of 60 mg (72). Patients were treated with doses between 60 and 1,850 mg, and RO7297089 was well-tolerated and showed efficacy in RRMM (72). No DLTs were observed among the DLT-evaluable patients, and a recommended phase 2 dose has not been determined (73). This trial demonstrates that RO7297089 shows manageable safety profile (72). The most common adverse events were mild to moderate in severity, with no unexpected safety concerns

in patients with RRMM (72). PR was observed in 7%, minimal response in 7%, and SD in 52% (72). The results of this study were published in 2021, 2022, and 2023 (71-73).

SAR443579

NKp46-NKCE consists of 2 Fabs targeting NKp46 and tumor Ag and an Fc region mediating the interaction with FcγR (36). NKp46-NKCE induces potent NK cell activation and targeted cell lysis without any signs of toxicity (7,43). Innate Pharma's NKCE, IPH6101/SAR443579, which is a trifunctional NKCE based on NKp46/CD16 on NK cells and targeting CD123 on AML, is undergoing clinical development in collaboration with Sanofi after an encouraging anti-tumor activity was observed in preclinical studies (17). Preclinical study of IPH6101/SAR443579 showed that this induces NK cell activation and cytokine release against AML cells without toxic effects (7). Targeting NKp46 has the potential to increase the specificity of NKCE compared with other types of immune cells. Arulanandam et al. engineered and tested the effect of CYT-303. This is a bispecific multifunctional Ab targeting NKp46 on NK cells and glypican-3 of hepatocellular carcinoma (HCC). They showed CYT-303 can induce NK cell cytotoxicity, cytokine production, ADCP, and complement-dependent cytotoxicity against multiple HCC tumor cell lines and tumor spheroids. Moreover, CYT-303 showed no toxic effects in cynomolgus monkeys up to the highest dose demonstrating its potential to be a safe and effective therapeutic agent targeting HCC (74). The clinical trial of SAR443579 is presented in **Table 1**.

NCT05086315 is a phase 1/2, open-label, multi-center trial to test the safety and efficacy of SAR443579 in patients with RR AML, B-cell acute lymphoblastic leukemia or high risk-MDS (52). This trial is a dose escalation and expansion study with intravenously infused SAR443579 (52). SAR443579 was injected intravenously twice or once a week for the first 2 wk, and then once a week for the rest of the cycles (75). This trial demonstrates that SAR443579 shows manageable safety profile with most adverse events being mild to moderate (52). No unexpected severe toxicities were reported in patients with RR hematological malignancies (52). Pharmacokinetics and pharmacodynamics of SAR443579 are evaluated in this trial (52). SAR443579 was well tolerated up to doses of 3,000 μg/kg once a week with recognizable clinical benefits in patients with RR AML (75). No DLTs were observed among the DLT-evaluable patients (75). The most common adverse events were infusion-related reactions (n = 10 [43.5%]) and nausea (n = 7 [30.4%]) (75). Two cases of cytokine release syndrome (grade 1, n = 1; grade 2, n = 1) were observed (75). No case of immune effector cell-associated neurotoxicity syndrome was reported (75).

ICE®

Another NKCE developed by Affimed (ICE®) demonstrated that the addition of ICE® (a tetravalent bispecific CD16A/CD19 against CD19-positive tumor cells) to NK and CAR-NK cells showed improved cytotoxicity of both cell types against target tumor cells (17). They compared NK cells treated with ICE® with CAR-NK cells (17). More specifically, NK cells treated with ICE® exhibit at least the same effects as CAR-NK cells alone or treated by ICE® against target tumor cells (17). This means that NK cells in combination with ICE® are able to have at least comparable effects to that of CAR-NK cells (17). This suggests the potential of allogeneic NK cells with ICE® without spending high cost to additionally engineer NK cells (17). Specifically, the effect of NK cells combined with ICE® was compared to that of anti-CD19 CAR-NK cells alone or in combination with the ICE® (76). Cytotoxic activity was demonstrated by CD19-positive target tumor cell killing assay, measuring released cytokines and degranulation (76). NK cells or CAR-NK cells combined with ICE® showed enhanced ADCC against target tumor

cells compared to NK cells or CAR-NK cells alone (76). These preclinical data presented as a poster at the 2023 annual meeting of Society for Immunotherapy of Cancer, titled "Redirecting NK cell cytotoxicity by innate cell engagers: a differentiated and innovative approach compared to CAR-NK cells" presented by Ciulean et al (76).

SELECTED RECENT PRECLINICAL BiKE STUDIES

Recent preclinical study showed that a BiKE molecule, which includes a single-domain CD16 Ab, an IL-15 linker, and a single-chain variable Ab targeting the glioma-associated Ag IL13Rα2, effectively targets IL13Rα2-positive gliomas (77). Co-culturing peripheral blood-derived NK cells with glioblastoma multiforme patient-derived xenograft lines showed a significant increase in glioma cell killing after BiKE treatment compared to the controls (77).

Additionally, a BiKE referred to as BiKE:E5C1 exhibits high affinity/specificity for the CD16A activating receptor on NK cells and HER2 on cancer cells (78). It leads to elimination of HER2-positive ovarian and breast cancer cells (78).

CONCLUSION

NK cell cancer immunotherapy has remarkable advantages in aspects of safety, lower costs, and availability of "off-the-shelf" solutions compared to T cell-based therapy such as CAR-T cells. Among the various therapeutic strategies involving NK cells, NKCEs have CAR-like specificity, and the combination of NKCE with immune cell therapy has the potential to be a promising alternative to CAR-based immunotherapy without the need for vector-mediated genetic modifications. Additionally, the NKCE clinical trials currently underway have shown promising preliminary results.

NKCEs have the advantages of low cost, readiness for immediate clinical use, multi-Ag targeting, and better safety. Additionally, they are active only in the presence of cancer cells and have a low risk of toxicity derived from cytokine storms. However, chronic stimulation induced by NKCEs promotes NK cell exhaustion and limits their *in vivo* persistence. Combination therapies with immune checkpoint inhibitors that target NK cells appear to be an attractive strategy for enhancing the efficacy of NKCE.

Further in-depth studies should focus on developing novel NK cell engagers with enhanced target specificity, better safety, appropriate strategies for combination therapy, and fewer side effects for cancer treatment.

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