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ISSN: 1942-0862

ISSN: (Print) (Online) Journal homepage: <https://www.tandfonline.com/loi/kmab20>

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To cite this article: Kerry A. Whalen, Kavya Rakhra, Naveen K. Mehta, Alexander Steinle, Jennifer S. Michaelson & Patrick A. Baeuerle (2023) Engaging natural killer cells for cancer therapy via NKG2D, CD16A and other receptors, mAbs, 15:1, 2208697, DOI: [10.1080/19420862.2023.2208697](https://doi.org/10.1080/19420862.2023.2208697)

To link to this article: <https://doi.org/10.1080/19420862.2023.2208697>



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Published online: 10 May 2023.



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REVIEW



Engaging natural killer cells for cancer therapy via NKG2D, CD16A and other receptors

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ABSTRACT

The field of immuno-oncology has revolutionized cancer patient care and improved survival and quality of life for patients. Much of the focus in the field has been on exploiting the power of the adaptive immune response through therapeutic targeting of T cells. While these approaches have markedly advanced the field, some challenges remain, and the clinical benefit of T cell therapies does not extend to all patients or tumor indications. Alternative strategies, such as engaging the innate immune system, have become an intense area of focus in the field. In particular, the engagement of natural killer (NK) cells as potent effectors of the innate immune response has emerged as a promising modality in immunotherapy. Here, we review therapeutic approaches for selective engagement of NK cells for cancer therapy, with a particular focus on targeting the key activating receptors NK Group 2D (NKG2D) and cluster of differentiation 16A (CD16A).

ARTICLE HISTORY

Received 4 January 2023
Revised 21 April 2023
Accepted 26 April 2023

KEYWORDS

ADCC; CD16A; MICA; MICB; monoclonal antibody; NK cell; NKG2D

Introduction

In contrast to T cells, natural killer (NK) cells act as a first line of defense and have the ability to detect and clear tumor or virally infected cells without any requirement for prior antigen-specific activation or differentiation. As such, NK cells possess several attractive properties justifying the development of agents that harness their therapeutic potential. Here, we review recent advances in the use of antibody-based therapies targeting the NKG2D/ligand axis to deliver a key activating signal that can synergize with CD16A signaling, allowing NK cells to mediate tumor cell lysis. The intricate balance between activating versus inhibitory signals, which is a hallmark of NK cells, calls for the engagement of multiple activating receptors, a combination of receptor agonists with inhibitors of NK cell checkpoints, and/or combination with T cell therapies, in order to maximize therapeutic efficacy in patients.

Like T cells, NK cells are increasingly recognized as having high cytotoxic potential when appropriately engaged. This is most evident when NK cells are transfected to express a chimeric antigen receptor (CAR) identical to those used to engineer CAR-T cells. When equipped with a strong activating receptor specific for a cell surface antigen, NK cell therapies have been shown to drive complete responses in patients.¹ Likewise, a T cell receptor complex (TCR) in NK cells enhanced their effector function in a similar manner to T-cells.^{2,3}

NK cell therapies have several advantages over T cell therapies. Most notable is their potential for allogenic, off-the-shelf

use because they avoid graft-versus-host reactivity due to the lack of a TCR. In addition, NK cell therapies are typically associated with less severe toxicities, including immune effector cell-associated neurotoxicity syndrome (ICANS) and cytokine release syndrome (CRS), as compared to certain T cell-based therapies.^{4–6} However, there remain several key challenges for NK cell-based cell therapies, including inefficient transgene delivery, NK cell manufacturing complexities, exhaustion and limited persistence of genetically engineered NK cells.⁷

One of the most significant revolutions in cancer therapy is the development of monoclonal antibodies (mAbs) that target T cells rather than cancer cells to effectively treat, and in some cases, enable durable responses, in cancer patients. Unlike cell therapies, mAbs engaging NK cells, much like those targeting T cells, have the potential to reach every effector NK cell, rely on standardized mAb manufacturing methods, and enable predictable pharmacokinetic properties.

NK cell engagement has long been leveraged in the clinic by IgG1 mAb-based cancer therapies exerting antibody-dependent cellular cytotoxicity (ADCC) activity. ADCC is primarily mediated by an interaction between the CD16A/Fc-gamma receptor IIIA (FcγRIIIa) expressed on NK cells and the Fc gamma domain 1 (Fcγ1) of mAbs that recognize tumor-associated cell surface antigens on cancer cells. Many chimeric, human, and humanized IgG1 antibody therapeutics, such as rituximab (targeting CD20), daratumumab (CD38), and trastuzumab (human epidermal growth factor receptor 2- HER2), engage CD16A.⁸ However, these antibodies leave another key

NK cell-activating receptor, namely NKG2D, untapped. NKG2D is a potent modulator of NK cell activation that has recently emerged as an important target in the immunoncology field.^{9,10} Strategies to therapeutically harness the activity of NKG2D are still in early development.

The biology of NK cells versus T cells in the context of cancer therapy

Among immune cells, NK cells are most closely related to cytotoxic T cells. The latter encompasses T cell subsets, including CD8+ and CD4+ T cells, gamma-delta T cells, and natural killer T (NKT) cells, all of which contain cytotoxic granules filled with cysteine proteases, called granzymes, and a pore-forming protein called perforin. NK cells, NKT cells and gamma-delta T cells belong to the innate immune system and serve as a first line of defense against pathogens, while CD8+ and CD4+ T cells are elements of the adaptive immune system, which are highly specific for pathogenic antigens, but first need to be primed, selected, and expanded in response to peptide antigen stimuli.¹¹ Like T cells, NK cells are cytotoxic by virtue of having secretory granules filled with the same granzymes and a variant of perforin.¹² Once delivered into a cytolytic synapse formed between NK cell and target cell, perforin forms a pore in the target cell membrane that enables transmembrane delivery of granzymes. Inside the target cell, granzyme B activates procaspases 3 and 7, eliciting programmed cell death, or apoptosis, while other granzymes such as granzyme A, H, K and M cleave numerous other protein substrates, causing target cell damage.¹³ Like T cells, NK cells also release inflammatory cytokines and chemokines upon activation.¹⁴

What is fundamentally different between cytotoxic T cells and NK cells is the mechanism by which they recognize and are activated by target cells. T cells recognize their target cells

via their TCR, which has six distinct subunits and is among the most intricate receptor complexes in our body.^{15,16} The TCRs of CD8+ and CD4+ T cells utilize their variant alpha and beta subunits to recognize specific peptide/major histocompatibility complexes (MHC) displayed on target cells. A density as low as five copies of a specific peptide/MHC complex on a target cell is sufficient for a specific T cell clone to recognize target cells and induce their lysis.¹⁷ This relies on a powerful signal amplification downstream of the TCR. Some effector cells of the innate immune system, including gamma-delta T cells and NKT cells, instead utilize an invariant TCR to recognize metabolites presented by butyrophilins and lipids presented by CD1d, respectively.^{18,19} In contrast, NK cell activation does not depend on one “master” activating receptor equivalent to the TCR; instead, NK cell activity is regulated by the balance of activating and inhibitory signals transduced by several cell surface receptors.²⁰ A comprehensive description of these receptors is found in several recent reviews.^{21–23} The interactions between tumor cells and T cells or NK cells that are the focus of this review are shown in Figure 1. Briefly, NK cell-activating and inhibitory receptors fall into two classes, human leukocyte antigen (HLA) and non-HLA specific. HLA-specific activating receptors include the activating killer Ig-like receptors (aKIR)s and Natural killer group 2C (NKG2C)/CD94 heterodimer. Activation of these receptors via ligand engagement triggers NK-mediated effector functions.²⁴ Non-HLA-specific activating receptors include CD16A and NKG2D, as well the natural cytotoxicity receptors (NCRs), NKp30, NKp44 and NKp46. NCRs play a primary role in cytotoxicity of tumor cells and in regulating both innate and adaptive immune responses.²⁵ Lastly, there are several activating co-receptors, such as DNAX accessory molecule-1

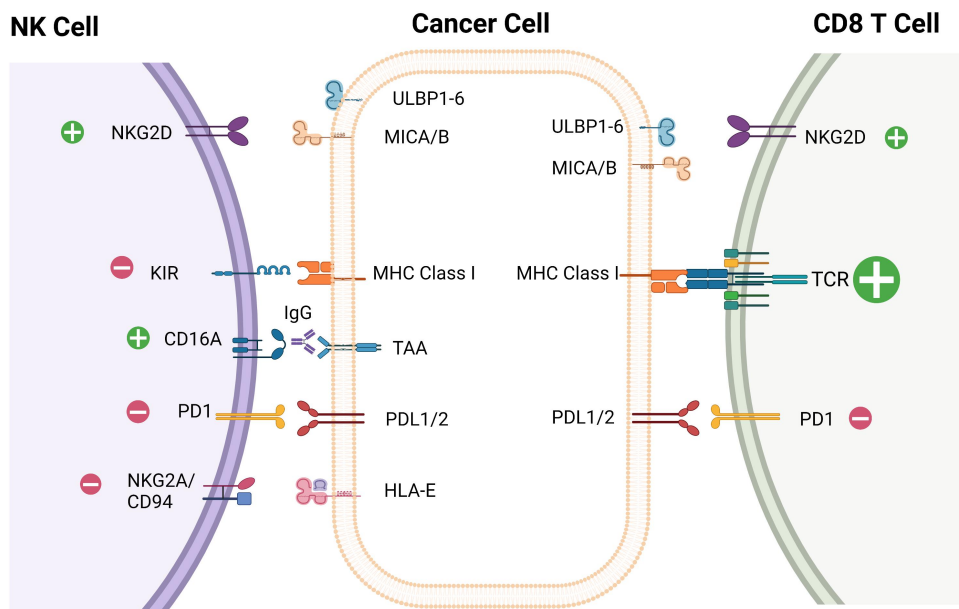


Figure 1. Select activating and inhibitory receptors on NK cells and CD8 T cells and their corresponding ligands. NK cell activation is regulated by the balance of signals from activating and inhibitory receptors present on the cell surface, whereas T cell signalling is driven primarily through a master activating receptor, the TCR, in addition to multiple co-stimulatory and co-inhibitory receptors. Select activating receptors (+) are presented as green positive signal; select inhibitory receptors (-) are depicted by a red negative signal.

(DNAM-1), 2B4 and NKP80, that promote immune cell cross talk and tumor cell killing through amplification of signals coming from NCRs and NKG2D.^{26–28}

HLA-specific inhibitory receptors present on NK cells are important for negatively regulating cellular activity upon engagement with HLA molecules, including the inhibitory killer Ig-like receptors (iKIRs)/CD158 family and the CD94/Natural killer group 2A (NKG2A) (CD94/CD159a) heterodimer.²⁹ Several clinical trials are ongoing with mAbs designed to reinvigorate NK cells by blocking inhibitory ligands on tumor cells or the inhibitory receptor, including anti-NKG2A monalizumab and anti-killer cell immunoglobulin like receptor, three Ig domains and long cytoplasmic tail 2 (KIR3DL2) lacutamab, which are under development by Innate Pharma (NCT05321147, NCT04984837).^{30–32} Antibodies targeting killer cell immunoglobulin like receptor, three Ig domains and long cytoplasmic tail 1–3 (KIR2DL1-L3) have shown limited success in the clinic. Lirilumab, a mAb that targets multiple iKIRs, has failed to meet its clinical endpoints.^{33,34}

In addition to the HLA-specific inhibitory receptors, non-HLA inhibitory receptors also provide important regulatory control for NK cells. This includes the immune checkpoint molecules programmed cell death 1 receptor (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), cluster of differentiation 96 (CD96), sialic acid binding Ig like lectin 7 (siglec-7), leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1), and inhibitor receptor protein (Irp60).^{29,35–39} PD-1, considered the prototypic immune checkpoint molecule, negatively regulates T cell function upon interaction with programmed death-ligand 1 and 2 (PD-L1 and PD-L2), on cancer cells and on immune cells in the tumor microenvironment (TME) and this axis has been targeted with great success in the clinic with antagonist mAb therapies.^{40,41} Tumor-experienced NK cells have also been shown to express PD-1 and PD-L1 in both preclinical and clinical settings.^{42–49} Preclinical data have demonstrated the importance of this pathway in suppressing NK cell activity and may contribute to the activity of anti-PD-1/PD-L1 mAb therapies in the clinic.^{42,48}

In summary, NK cell activity is kept in a delicate balance and tightly controlled by a network of multiple regulatory proteins. Given the inhibitory milieu for NK cell signals in the TME, it is attractive to engage NK cell-activating receptors, such as CD16A and NKG2D, and an inhibitory receptor, such as PD-1, to unleash tumor cell lysis by NK cells.

Leveraging the CD16A/Fc-gamma receptor IIIA to engage NK cells

Fc gamma receptors (FcγRs) are a family of proteins expressed on the surface of immune cells which bind the Fc portion of antibodies to mediate immune effector functions.⁵⁰ All FcγRs are activating, with the exception of FcγRIIB (CD32B), which is the only known inhibitory receptor and is the predominant FcγR present on B cells where it serves as a critical mediator of B-cell homeostasis.⁵¹ Activating receptors include the high-

affinity FcγRI (CD64), a receptor primarily responsible for mediating antibody-dependent cellular phagocytosis (ADCP), the highly abundant FcγRIIA (CD32A), a receptor that facilitates phagocytosis and endocytosis, and FcγRIIb (CD16B), a receptor present on granulocytes that mediates opsonization of microbes.^{52,53} Importantly, all of these receptors are notably absent in NK cells.

The most well-characterized and predominant activating receptor on NK cells is FcγRIIIA, or CD16A, which binds to the Fcγ domain of various IgG antibody isotypes. Importantly, CD16A is expressed only on mature NK cells, which is the CD56dim subset present primarily in the periphery, whereas the majority of NK cells present in tissues are the less mature, CD56bright population and express low levels of CD16A.^{20,54,55} NK cells are naturally coated with serum IgG bound to their CD16A receptors via a low-affinity interaction.⁵⁶ CD16A, transmembrane receptor with two extracellular Ig-like domains and a short cytoplasmic tail, lacks an intrinsic signaling domain and thus requires immunoreceptor tyrosine-based activation motif (ITAM)-bearing signaling proteins, CD3 zeta (CD3ζ) and/or Fc epsilon RI (FcεR1), to transduce signals upon binding to IgG. Upon CD16A engagement, recruitment and phosphorylation of other kinases result in calcium influx and degranulation. Calcium influx is one of the major signals for triggering degranulation and ADCC and enables nuclear factor of activated T-cells (NFAT) translocation into the nucleus to induce the transcriptional program to drive an inflammatory response.⁵⁷

MAbs for cancer therapy are designed to simultaneously bind a cell surface antigen on cancer cells and CD16A on NK cells. These mAbs function like an NK cell-engaging bispecific antibody by bridging NK cells with cancer target cells for redirected lysis via ADCC. It is the human IgG1 isotype (and murine IgG2a/c ortholog) that most effectively mediates ADCC, as well as complement-dependent cytotoxicity (CDC) and ADCP.⁵⁸ Support for a role of CD16A in the anti-tumor activity of hIgG1 mAb therapies comes from a polymorphism of CD16A that reduces its affinity for the Fc gamma domain of mAbs.⁵⁹ The lower affinity isoform of CD16A is associated with decreased response rates for rituximab in non-Hodgkin lymphoma.⁶⁰ Additional evidence for the role of CD16A in NK cell-mediated ADCC comes from modifications of the Fcγ1 domain of mAbs that can enhance their affinity for FcγRs resulting in more potent target cell lysis.⁶¹ These modifications include specific mutations in the Fcγ1 domain or prevention of fucosylation in the N-linked carbohydrate moiety of hIgG1. Amino acid (AA) substitutions in the Fcγ1 domain of IgG1, for example S239D/I332E and S298A/Q333A/K334A, have been shown to increase the affinity of binding to CD16A and enhance ADCC, and have been covered in detail in recent reviews.^{61–66} Prevention of fucosylation in the N-linked carbohydrate moiety of hIgG1 is another way to augment Fc-effector functions, as the presence of fucose has been shown to sterically hinder optimal interaction between IgG1 and CD16A.⁶⁷ Afucosylation can be achieved by genetic modification of the host biosynthesis pathways, post-translational enzymatic modification or through the use of small molecule inhibitors.^{68–71} The most robust methods of complete afucosylation include deletions of either the fucosyltransferase (FUT8) enzyme or the

enzyme responsible for generating a key intermediate of fucose, GDP mannose 4,6-dehydratase (GMD), in mammalian host systems.^{70,71}

Approved Fc-enhanced antibodies include the Fcγ1-modified antibody margetuximab (targeting HER-2) and the afucosylated antibody mogamulizumab (targeting C-C chemokine receptor type 4-CCR4).^{72,73} In addition to introducing antibodies to enhance Fc functionality, other anti-tumor agents, such as lenalidomide, a degrader of the multi-functional protease cereblon have been reported to enhance NK cell activity by lowering the threshold required for NK cell activation by CD16A and other receptors.^{74,75} The combination of lenalidomide and the anti-CD38 IgG1 mAb, daratumumab, is now standard-of-care treatment for multiple myeloma (MM) patients.⁷⁶

Activating CD16A through an anti-CD16A-specific antibody rather than modification of the Fcγ domain binding is an alternate approach to activating NK cells. This approach has the advantage of preventing co-engagement of CD16B, the competitive and highly abundant glycosylphosphatidylinositol (GPI)-anchored FcγR expressed on granulocytes, which has been shown to act as a decoy receptor on neutrophils, reducing ADCC activity.^{50,77} Furthermore, engaging CD16A with an engineered binder reduces competitive binding of excess native serum IgG1 antibodies to CD16A. Affimed's AFM13, a CD30/CD16A-bispecific tandem diabody (Tandab) that exemplifies this approach, has shown encouraging clinical activity in Hodgkin lymphoma (HL) patients when administered in combination with the anti-PD-1 mAb pembrolizumab.^{78,79} Intriguingly, a cell therapy generated by coating cord blood-derived NK cells with the Tandab has shown a very high rate of complete responses in HL patients.⁸⁰ RO7297089, a bispecific antibody from Genentech that targets both B-cell maturation antigen (BCMA) and a unique binding site on CD16A resulting in increased tumor cell killing compared to Fc-enhanced tumor-targeting antibodies, was tested in a phase 1 study but does not appear to be under further development.^{81,82} GT-Biopharma has generated both bi- and tri-specific NK cell engagers (NKCEs) using camelid nanobody technology. These molecules contain a camelid nanobody that binds CD16A, a single-chain variable fragment (scFv) that recognizes a tumor antigen and a human wild-type interleukin 15 molecule to enhance NK cell activity. GTB-3560 targeting CD33 is currently in IND-enabling development (NCT03214666).^{83,84}

Since NK cell activation is governed by multiple receptors, efforts are underway to simultaneously engage other activating receptors beyond CD16A, such as NKp30 and NKp46, as reviewed by Phung et al and Demaria et al.^{85,86} Compass Therapeutics generated CTX-8573, a bispecific engager which contains an anti-NKp30 fragment antigen-binding (Fab) fragment linked to the C-terminus of an anti-BCMA IgG1 antibody containing an afucosylated Fc gamma domain for enhanced CD16A engagement, but development has been terminated.⁸⁷ Innate Pharma aims to target activating receptors NKp46, CD16A, and tumor-associated antigens via their Antibody-based NK cell Engager Therapeutics (ANKET) platform, which consists of both trivalent and tetravalent NKCEs. Trivalent engagers include IPH6401, a BCMA-targeting ANKET, and IPH6101, which targets CD123 and is currently

in Phase 1 clinical trials in hematological malignancies. The tetravalent ANKET IPH6501, which targets CD20 and contains an interleukin-2 variant (IL-2 v), is currently in preclinical development (NCT05086315).^{88,89}

Role of the NKG2D/ligand axis in immune surveillance

Another key activating receptor is NKG2D, which is not only expressed on NK cells, but also on certain T cell populations, including subpopulations of CD8+, NKT and gamma/delta T cells.^{90,91} NKG2D recognizes eight distinct ligands that are upregulated on tumor cells: MHC class I chain-related protein A (MICA), MHC class I chain-related protein sequence B (MICB), and UL-16- binding proteins (ULBPs) named ULBP1 through ULBP6.^{91,92}

Upon engagement with one or more of these ligands, NKG2D triggers a downstream signaling cascade in effector cells leading to the formation of a cytolytic synapse. NKG2D is expressed as a homodimer and lacks its own signaling domain. It therefore relies on binding to the adaptor protein DNAX-activating protein 10 (DAP10), which recruits phosphoinositide 3-kinase (PI3K) and growth factor receptor bound 2 protein (Grb2).⁹³ Activation of these signaling proteins triggers an increase in intracellular calcium concentration, actin cytoskeleton rearrangement, and activation of transcription factors, including NFAT and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). In NK cells, these signals culminate in cytotoxicity by release of granzymes, perforin and cytokines. Direct target cell killing by NKG2D ligation on NK cells has been demonstrated using NKG2D ligands, such as MICA or ULBP2, fused to a mAb that recognizes a tumor-associated antigen.^{94–96} In the case of T cell populations that express NKG2D, the axis may serve a costimulatory role in lowering the threshold for lysis while the primary recognition of target cells is via the TCR.⁹⁷

NKG2D ligation serves an important role in clearing cells with stress-induced damage, including those undergoing nascent carcinogenesis. NKG2D ligands are not expressed on healthy cells, but appear on the cell surface when cells are exposed to a variety of stresses, including metabolic stress, genotoxic stress, irradiation, viral infection, or malignant transformation.^{91,98} As shown in Figure 2, gene expression profiling of 32 different human cancer tissues from The Cancer Genome Atlas (TCGA) database revealed that, compared to other NKG2D ligands, MICA is the most consistently and highly expressed across all solid tumors and hematological malignancies. The second broadest expression pattern is exhibited by MICB, which is closely related in sequence to MICA. This makes both MICA and MICB (MICA/B) attractive pan-cancer targets.

MICA/B contain key structural differences compared to other members of the NKG2D ligand family. While all ligands consist of MHC class I-related polypeptide chains that lack beta-2-microglobulin and peptide association, MICA/B ligands contain alpha 1, alpha 2 and alpha 3 domains, whereas the ULBPs only contain the alpha 1 and alpha 2 domains and have low (25%) sequence identity to MICA/B. Despite differences in protein sequence, co-crystal structures of NKG2D with MICA or with ULBP3 revealed that NKG2D binds to its

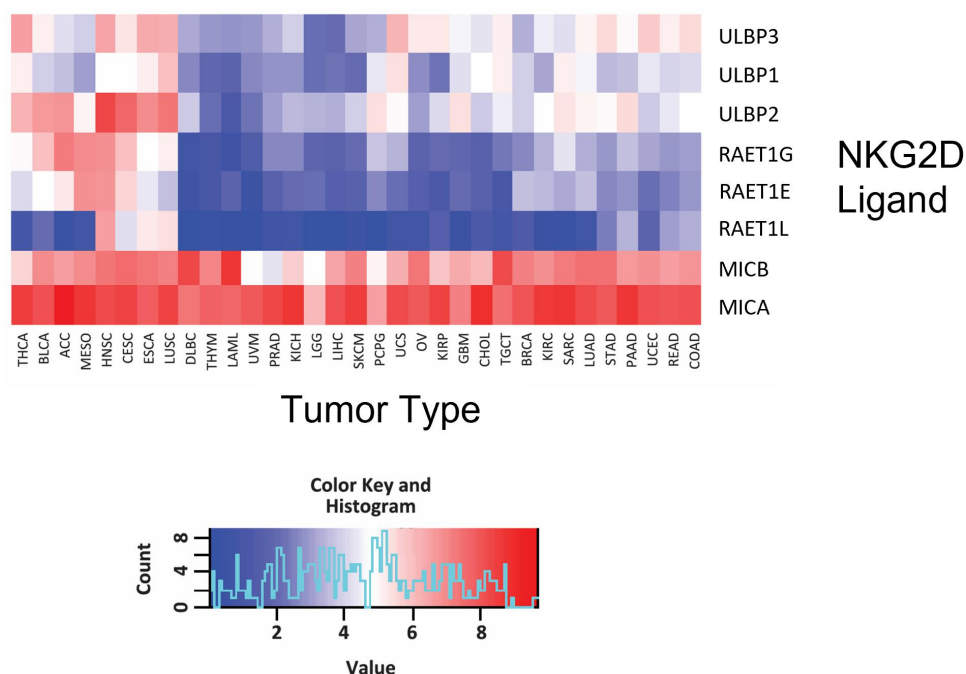


Figure 2. Broad expression of MICA and MICB in human cancers. mRNA gene expression analysis of thirty-two cancer indications from the TCGA database. NKG2D ligands analyzed include MICA, MICB and ULBP1-6. Abbreviations for indications can be found here: <https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations>. Abbreviations are captured in the order they appear starting from left to right. THCA: thyroid carcinoma, BLCA: bladder urothelial carcinoma, ACC: adrenocortical carcinoma, MESO: mesothelioma, HNSC: head and neck squamous cell carcinoma, CESC: cervical squamous cell carcinoma and endocervical carcinoma, ESCA: esophageal carcinoma, LUSC: lung squamous cell carcinoma, DLBC: lymphoid neoplasm diffuse large B-cell lymphoma, THYM: thymoma, LAML: acute myeloid leukaemia, UVM: uveal melanoma, PRAD: prostate adenocarcinoma, KICH: kidney chromophobe, LGG: brain lower grade glioma, LIHC: liver hepatocellular carcinoma, SKCM: skin cutaneous melanoma, PCPG: pheochromocytoma and paraganglioma, UCS: Uterine carcinosarcoma, OV: ovarian serous cystadenocarcinoma, KIRP: kidney renal papillary carcinoma, GBM: glioblastoma multiforme, CHOL: cholangiocarcinoma, TGCT: testicular germ cell tumors, BRCA: breast invasive carcinoma, KIRC: kidney renal clear cell carcinoma, SARC: sarcoma, LUAD: lung adenocarcinoma, STAD: stomach adenocarcinoma, PAAD: pancreatic adenocarcinoma, UCEC: uterine corpus endometrial carcinoma, READ: rectum adenocarcinoma, COAD: colon adenocarcinoma.

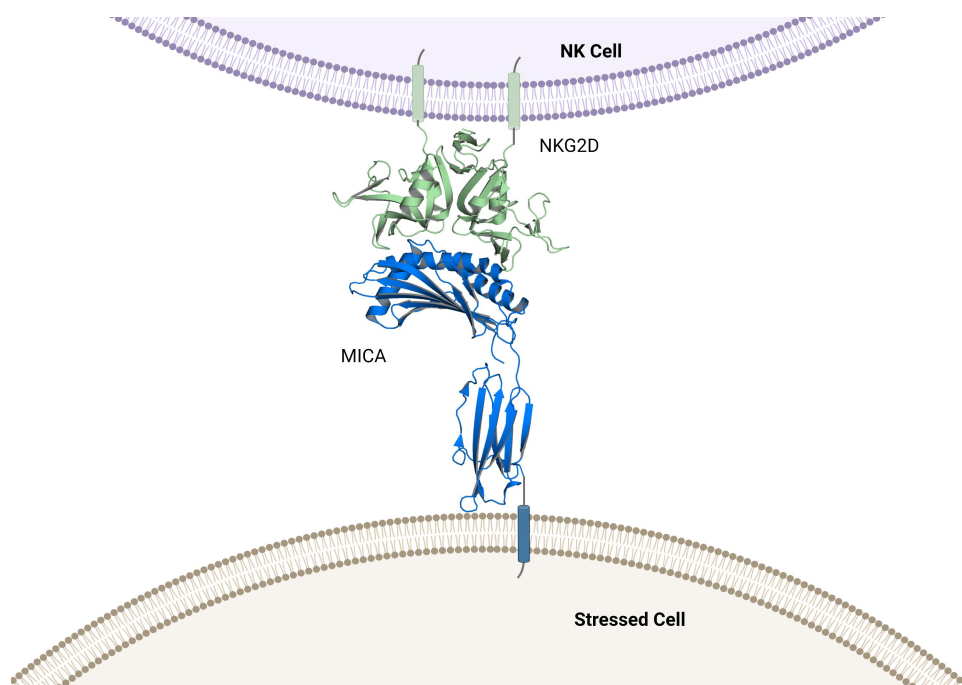


Figure 3. Structure of the NKG2D-MICA complex. . MICA is shown in blue. The alpha 1 and alpha 2 domains of MICA form the interface with the NKG2D receptor, depicted in green. The arrow indicates the region containing proteolytic cleavage sites of MICA which are proximal to the cell membrane. (Figure adapted from <https://www.rcsb.org/>, PDB 1HYR (complex of MICA and NKG2D)).

ligands through topologically similar but distinct sets of interface residues of the alpha 1/alpha 2 domains using a rigid adaptation mode.⁹⁹ Of note, the alpha 3 domain of MICA, which connects the other two domains to the plasma membrane of NK cells, is not involved in this interaction (Figure 3).¹⁰⁰

Cancer cells avoid NKG2D-mediated NK cell lysis by exploiting the unique alpha 3 region of MICA/B, which is the target for proteolytic shedding of the extracellular domain (ECD) from the cancer cell surface. Shedding is enabled by multiple proteases that most cancer cells express on their cell surface or release into the TME. It has been reported that matrix metalloproteinase-9 (MMP-9)/ matrix metalloproteinase-14, (MMP-14), a disintegrin and metalloproteinase-10 (ADAM10), and a disintegrin and metalloproteinase-17 (ADAM17) are all able to recognize and cleave sequences located in the stalk region between the alpha 3 domain and the transmembrane domain of MICA.^{101–103} Several cleavage sites have been mapped by mutational analysis that are proximal to the transmembrane domain of MICA (Figure 3). A prerequisite for cleavage of MICA/B appears to be the reduction of a single disulfide bridge in the alpha 3 domain by protein disulfide isomerase endoplasmic reticulum protein 5 (ERP5), which may enable MICA/B to adopt a protease-sensitive conformation.¹⁰⁴

Shedding of MICA/B from the tumor cell membrane leads to an accumulation of soluble MICA/B in the blood of cancer patients.^{105,106} The potentially pivotal role of the NKG2D/ligand axis in immune surveillance is evident from the negative prognostic value of soluble MICA in a variety of cancers, including solid tumors, such as hepatocellular carcinoma (HCC), as well as hematological malignancies, like MM. This has been corroborated in a meta-analysis including many other cancer indications.¹⁰⁷ Of note, expression of MICA on cancer cells, as observed in tumor biopsies, is a positive prognostic factor for improved overall survival.¹⁰⁸

Collectively, these data suggest that therapies preventing MICA/B shedding, and thereby preserving MICA/B on the cell surface to promote NKG2D signaling, may have therapeutic potential in treating diverse cancers. However, MHC-related proteins MICA/B exhibit a high degree of polymorphism, which poses a potential challenge with this approach. Nearly 150 alleles have been identified for MICA and 50 alleles for MICB across the population.¹⁰⁹ When polymorphic amino acid residues are present in target epitopes of mAbs, they may negatively impact antibody binding affinity across allelic variants and reduce target recognition in the diverse patient population. Great care must therefore be taken to identify mAbs that broadly recognize MICA/B alleles. Given the high sequence homology between MICA/B, it is possible to discover cross-reactive mAbs that recognize both NKG2D ligands. A MICA/B cross-reactive mAb with coverage of the most common MICA/B alleles should be able to overcome the challenges posed by polymorphisms, since each tumor may express up to four relevant target alleles for MICA and MICB.

Nevertheless, given the large number of activating and inhibitory receptors on NK cells, even if NKG2D signaling is preserved or enhanced, its activity is contextually dependent on the NK cell activation status. This justifies the development

of therapeutic strategies with multi-modal mechanisms of action that combine NKG2D agonism with additional activating signals for NK cells.

Therapies leveraging the NKG2D-ligand axis for cancer therapy

The compelling role of the NKG2D-MICA/B axis in cancer biology has triggered numerous attempts to leverage these pathways for therapeutic targeting. Approaches include engineered T or NK cells, NK cell engagers, antibody-drug conjugates (ADCs), vaccines and mAbs (Figure 4). Therapeutics in active clinical development targeting the axis are highlighted in Table 1.

Celyad Oncology has developed two autologous (CYAD-01, CYAD-02) and one allogenic (CYAD-101) T cell therapies that express a CAR based on the ECD of NKG2D. Although CYAD-01 and CYAD-101 are no longer in clinical development, CYAD-02 is being evaluated in acute myeloid leukemia (AML) and myeloid dysplastic syndrome (MDS) patients in a Phase 1 study where two patients were reported to have achieved a complete marrow response (NCT04167696).^{110–114}

Nkarta Therapeutics is developing NKX101, an allogenic NK cell therapy based on NK cells collected from normal human donors by apheresis and expanded ex vivo. Like Celyad, the CAR utilizes the ECD of NKG2D to target NKG2D ligands on tumor cells; the CAR-NK cells also express an interleukin-15 (IL-15) receptor/IL-15 fusion protein, which allows for the enhancement of NK cell proliferation, cytotoxicity, cytokine release and maintenance.¹¹⁵ NKX101 is being evaluated in a Phase 1 study of relapsed or refractory AML and MDS patients (NCT04623944).¹¹⁶ Of five AML patients treated, three were recently reported to have achieved a complete response with hematological recovery.¹¹⁷ Other clinical trials of CAR T or CAR gamma-delta T cells that incorporate the ECD of NKG2D are ongoing and are reviewed by Curio et al.¹¹⁸

Fate Therapeutics has developed an allogenic MICA/B-directed NK cell therapy, FT536, based on the company's human-induced pluripotent stem cell (iPSC) platform. Rather than using the ECD of NKG2D to target MICA/B, FT536 expresses a CAR harboring a scFv domain with specificity for the alpha 3 domain of MICA/B, which prevents shedding of MICA/B from the tumor cell surface. This approach was shown to have superior anti-tumor activity as compared to an NKG2D-based CAR in preclinical models.¹¹⁹ The engineered NK cells are further enhanced by expression of a high-affinity, non-cleavable version of CD16A (for combination with mAb therapies), as well as an IL-15 receptor/IL-15 fusion protein and knockout of the CD38 gene for enhanced NK cell activity and augmented metabolic fitness and persistence.¹²⁰ Fate recently announced that it is discontinuing development of the program.¹²¹

While these cell therapies speak to the industry's interest in targeting NKG2D ligands for cancer therapy and have shown initial signs of efficacy, all involve complex manufacturing of genetically engineered cells and will likely be limited by the number of cells that can be safely administered to patients. Furthermore, therapies using the ECD of NKG2D for MICA/B

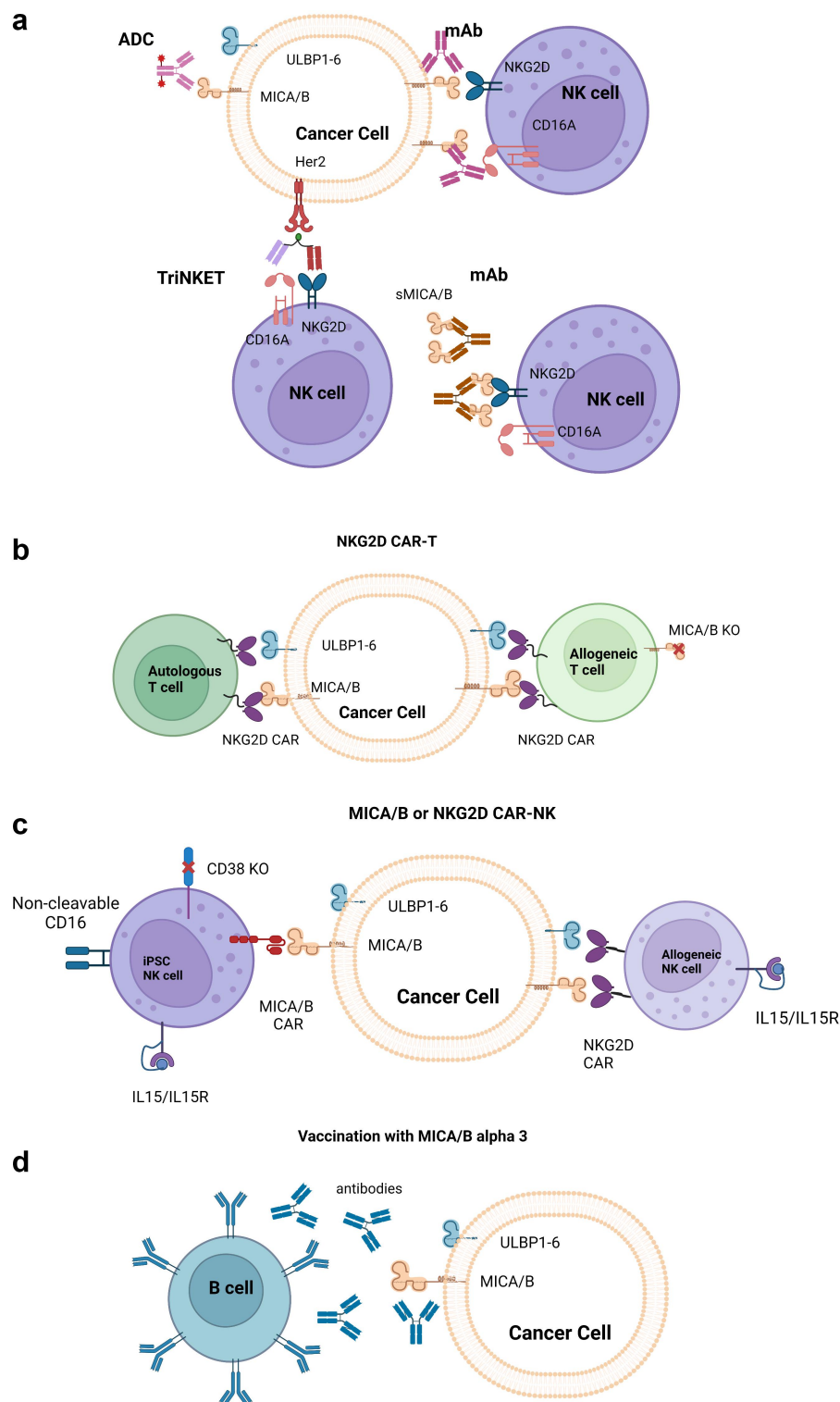
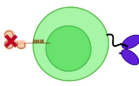
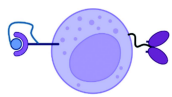


Figure 4. Schematic representation of therapeutic modalities targeting the MICA/B pathway. (a) Protein-based therapeutics targeting the MICA/B pathway including an ADC that delivers a cytotoxic payload to MICA/B-expressing tumor cells, mAbs that induce immune-mediated tumor cell killing by preventing shedding of cell surface MICA/B or by binding to sMICA/B to form a NKG2D-activating complex, and a TriNKET that simultaneously binds NKG2D and CD16A on NK cells and targets Her2-expressing tumor cells. (b) Allogeneic or autologous NKG2D based r CAR)T-cell therapies activated by NKG2D ligands including MICA/B and (c) an allogeneic NKG2D based CAR NK cell therapy and iPSC-derived allogeneic MICA/B scFV-based CAR NK cell therapy activated by NKG2D ligands including MICA/B. (d) Vaccine-induced production of antibodies targeting the MICA/B alpha 3 domain.

targeting will be hampered by the frequent shedding of the two NKG2D ligands. In addition, there is a potential for lack of specificity given the ability of the NKG2D ECD to interact with other NKG2D ligands for which the safety consequences are

unknown. Lastly, MICA/B can be expressed on cellular products as a consequence of stressful cell isolation and expansion, which may trigger fratricide and thereby limit the manufacturing yield of engineered cells.

Table 1. Therapeutics targeting NKG2D in active clinical development. Abbreviations used: CAR: Chimeric antigen receptor, NKG2D: Natural Killer Group 2D.

Company (Asset)	Modality	Molecule properties	Structure	Indication	Development Phase/ Clinical trial number
Cullinan Oncology (CLN-619)	Monoclonal antibody	Tumor target: MICA/B NK cell target: NKG2D, CD16A		Advanced solid tumors	Phase 1 NCT05117476
Dragonfly Therapeutics (DF1001)	NK cell engager	Tumor target : HER2 NK cell target: NKG2D NK cell target: CD16A		Advanced solid tumors	Phase 1/2 NCT04143711
Celyad (CYAD-02)	Allogeneic CAR T-cell therapy	Target: All NKG2D ligands CAR: NKG2D extracellular domain Additional modification: MICA/B knockdown to reduce fratricide		Acute myeloid leukemia Myelodysplastic syndrome	Phase 1 NCT04167696
Nkarta (NKX101)	Human donor allogeneic CAR NK cell therapy	Target: All NKG2D ligands CAR: NKG2D extracellular domain Additional modification: IL-15 R/IL15 fusion		Acute myeloid leukemia Myelodysplastic syndrome	Phase 1 NCT04623944

Biologics, such as mAbs and antibody constructs like NKCEs, represent an alternative approach to leveraging the NKG2D pathway. NKCEs consisting of the ECD of NKG2D fused to Fab fragments directed against CD16A (NKG2D-CD16) have demonstrated potent target cell lysis in preclinical studies.¹²² NKCEs that use the MICA ECD fused to scFvs against tumor- or TME-associated antigens, such as vascular endothelial growth factor receptor 2 (VEGFR2), CD24, CD20 and BCMA, have also been generated.^{96,123–125} Such constructs were tested preclinically and demonstrated specific NK cell activation and target cell lysis. However, the potential cleavage of the ECD of MICA by proteases in the TME is a liability. This is the case for those NKCEs that incorporate full-length, unmodified MICA ECD, such as the VEGFR2 and CD24 constructs. In addition, the affinity of MICA for NKG2D is weak and may limit the potency of these molecules, supporting the use of higher-affinity/avidity constructs such as those from Dragonfly Therapeutics and Xencor, which simultaneously bind to tumor-associated antigens and NK cells via CD16A and NKG2D. DF1001, a Tri-specific NKCE (TriNKET) from Dragonfly Therapeutics, is composed of three antibody fragments simultaneously targeting tumor cells via HER2 and NK cells via both CD16A and NKG2D. DF1001 is currently being evaluated in Phase 1/2 clinical studies of patients with advanced solid tumors (NCT04143711). Xencor's XmAb[®] bispecific NKE molecule, which targets B7 homolog 3 protein (B7H3) and NKG2D, and is currently in preclinical development, is another example of a NKCE that leverages NKG2D and CD16A to promote NK cell activation and lysis of tumor cells.¹²⁶ These approaches are similar to the ANKET platform described above, with the addition of an NKG2D-targeting arm.

Several mAbs have been generated that directly target MICA/B. These antibodies all aim to augment the NKG2D signaling axis and have diverse mechanisms of action. One

strategy involves targeting soluble MICA/B (sMICA/B) as a means to modulate the interaction of sMICA/B with NKG2D, which is thought to be detrimental to NK cell function via impairment of NKG2D functionality.^{106,127–130} For example, the antibody B10G5 binds to shed sMICA/B to stimulate NK cells through two proposed mechanisms of action, namely clearance of sMICA/B and formation of antibody/MICA/B complexes that may agonize NKG2D on NK and T cells. B10G5 has been demonstrated to restore NK cell function and remodel the TME, resulting in tumor growth inhibition in preclinical models.^{131–133}

Innate Pharma recently reported results for the MICA/B targeted ADC MICAB1. The mAb used to generate the ADC was selected due to its ability to stimulate a high level of MICA/B internalization and engineered to be Fc-silent. The antibody is coupled to first-generation pyrrolobenzodiazepine dimers (PBD) as a cytotoxic payload. Treatment with MICAB1 in preclinical models was effective at single, low doses in both human xenograft and patient-derived xenograft models.¹³⁴ The current development status of this agent is unknown.

The generation of therapeutics that inhibit shedding of MICA/B is an emerging strategy to leverage NKG2D ligands.^{135,136} One approach to achieve this is the induction of antibodies in response to vaccination with the alpha 3 domain of MICA/B. These antibodies can prevent shedding by binding MICA/B at the proteolytic cleavage site. Robust anti-tumor responses dependent upon both NK and T cells were generated in response to vaccination in preclinical studies.¹³⁷ Furthermore, engaging multiple effector cells mediated protective immunity against tumors with common escape mechanisms, such as loss of MHC-I expression.¹³⁷ A potential limitation to this approach is the lack of control over how much antibody is produced and the functionality of the antibody response.

A promising approach to targeting inhibition of MICA/B shedding is being undertaken by Cullinan Oncology with the generation of CLN-619, a humanized IgG1 antibody that is currently the only MICA/B-targeted mAb in the clinic. CLN-619 prevents shedding of MICA/B from cancer cells by binding to the alpha 3 domain and contains an active Fcγ1 domain to drive ADCC and ADCP. As described above, the MICA/B genes are highly polymorphic, yet CLN-619 has been demonstrated to have broad reactivity to all allelic variants tested. Reduced levels of shed MICA and a concomitant increase in MICA on the surface of tumor cells has been observed with CLN-619 treatment. Importantly, the alpha 1 and alpha 2 domains of MICA, which bind to NKG2D, are unencumbered by the bound antibody. In fact, CLN-619 enhances the binding of MICA to NKG2D, which appears to be dependent on the Fcγ1 domain of the antibody that can concomitantly bind to CD16A on NK cells. Notably, CLN-619 shows compelling single-agent activity at low doses in tumor xenograft models, where its activity critically relies upon a functional Fcγ1 domain.^{138,139}

Stabilization of MICA/B by CLN-619 leads to accumulation of these NKG2D ligands on the tumor cell surface, thereby overcoming immune evasion by MICA/B shedding. This likely maximizes ADCC and ADCP functions of the antibody due to increased target antigen density. Importantly, CLN-619 has the potential to activate NK cells via simultaneous and perhaps synergistic engagement of two key activating receptors: NKG2D and CD16A. Cooperation between the two receptors is supported by published data showing engagement NKG2D can lower the activation threshold of several receptors including CD16A.¹⁴⁰ Cooperation between the two receptors may be important when considering NK cell-activating receptors in the TME are often downmodulated^{141–143}. CLN-619 is currently in a Phase 1 clinical trial in patients administered alone or in combination with pembrolizumab in patients with advanced solid tumors (NCT05117476). Given the broad tumor expression of the pathway (Figure 2), it is anticipated that CLN-619 may have the potential for pan-cancer activity.

Conclusions and outlook

While therapeutic success has been achieved with engagement of T cells using multiple modalities, this review highlights the challenges and opportunities to accomplish this by engaging NK cells. Although certain NK cell therapies are beginning to show some promise in the clinic, an off-the-shelf mAb therapy has the advantage of ease of manufacturing and the potential to engage most if not all NK cells in a patient for lysis of cancer cells. However, treatment with mAbs comes with potential liabilities, including the possibility of recruiting exhausted, dysfunctional NK cells. In addition, the intricate interplay between negative and positive regulatory receptors on NK cells makes it difficult to simply engage a single receptor to achieve therapeutic benefit. Nevertheless, one approach that has met with success is leveraging activation of the CD16A receptor on NK cells via tumor antigen-targeted mAbs mediating ADCC. Another attractive target on NK cells is the activating receptor NKG2D. However, the shedding of its most abundantly expressed ligands MICA/B must be addressed in

order to maximize the therapeutic potential of engaging this pathway. To fully unleash their killing potential, NK cells in the immunosuppressive TME may require more than one activating stimulus, for example simultaneous engagement of both CD16A and NKG2D. The ultimate therapeutic potential for NK cell-activating therapeutics in the clinic may be enabled when delivered in combination with therapies that enhance or complement NK cell activation, such as lenalidomide, cytokines or checkpoint inhibitors. Combination with checkpoint inhibitors provides the opportunity for dual activation of both the innate and adaptive immune response and may attenuate exhaustion of both T cells and NK cells expressing PD-1. In summary, engagement of NK cells, particularly via stimulatory mAbs, holds great promise for the future development of novel immuno-oncology therapeutics.

Acknowledgments

Figures 1, 3, and 4 were created using Biorender.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

Abbreviations

AA	Amino acid
ADAM10	a disintegrin and metalloproteinase-10
ADAM17	a disintegrin and metalloproteinase-17
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
ADC(s)	antibody-drug conjugates
aKIR(s)	activating killer Ig-like receptors
AML	acute myeloid leukemia
ANKET	Antibody-based NK cell Engager Therapeutics
B7H3	B7 homolog 3 protein
BCMA	B-cell maturation antigen
CAR	Chimeric antigen receptor
CCR4	C-C chemokine receptor type 4
CD	Cluster of differentiation
CD16A	Cluster of differentiation 16A
CD3ζ	CD3 zeta
CD96	cluster of differentiation 96
CDC	complement-dependent cytotoxicity
CRS	Cytokine release syndrome
DAP10	DNAX-activating protein 10
DNAM-1	DNAX accessory molecule-1
ECD	extracellular domain
ERP5	endoplasmic reticulum protein 5
Fab	fragment antigen-binding
FcεRIγ	Fc epsilon RI
Fcγ1	Fc gamma domain 1
FcγRIIIa	Fc-gamma receptor IIIA
FcγRs	Fc gamma receptors
FUT8	fucosyltransferase
GMD	GDP mannose 4,6-dehydratase
GPI	glycosylphosphatidylinositol
Grb	growth factor receptor bound 2 protein
HCC	hepatocellular carcinoma

HER2	human epidermal growth factor receptor 2
HL	Hodgkin lymphoma
HLA	human leukocyte antigen
ICANs	Immune effector cell-associated neurotoxicity syndrome
iKIR(s)	inhibitory killer Ig-like receptors
IL-15	interleukin-15
IL-2 v	interleukin-2 variant
Ipsc	induced pluripotent stem cell
IRp60	inhibitor receptor protein
ITAM	immunoreceptor tyrosine-based activation motif
KIR2DL1-L3	killer cell immunoglobulin like receptor, three Ig domains and long cytoplasmic tail 1–3
KIR3DL2	killer cell immunoglobulin like receptor three Ig domains and long cytoplasmic tail 2
LAIR-1	leukocyte-associated immunoglobulin-like receptor 1
mAb	monoclonal antibody
MDS	myeloid dysplastic syndrome
MHC	Major histocompatibility complexes
MICA	MHC class I chain-related protein A
MICB	MHC class I chain-related protein sequence B
MICA/B	MICA and MICB
MM	Multiple myeloma
MMP-9	matrix metalloproteinase-9
MMP-14	matrix metalloproteinase –14
NCR	natural cytotoxicity receptors
NFAT	nuclear factor of activated T-cells
NFKB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NKCEs	NK cell engagers
NKG2A	Natural Killer Group 2A
NKG2C	Natural Killer group 2C
NKG2D	Natural Killer Group 2D
NKT	Natural Killer T cell
PBD	pyrrolbenzodiazepine dimers
PD-1	programmed cell death 1 receptor
PD-L1 and PD-L2	programmed death-ligand 1 and 2
PI3K	phosphoinositide 3-kinase
scFv	single-chain variable fragment
siglec-7	sialic acid binding Ig like lectin 7
sMICA/B	soluble MICA/B
Tandab	tandem diabody
TCGA	The Cancer Genome Atlas
TCR	T cell receptor complex
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TME	tumor microenvironment
TriNKET	Tri-specific NKCE
ULBPs	UL-16- binding proteins
VEGFR2	vascular endothelial growth factor receptor 2.

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