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Designing multivalent proteins based on natural killer cell receptors and their ligands as immunotherapy for cancer

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Abstract

Introduction—Natural killer (NK) cells are an important component of the innate immune system that play a key role in host immunity against cancer. NK cell recognition and activation is based on cell surface receptors recognizing specific ligands that are expressed on many types of tumor cells. Some of these receptors are capable of activating NK cell function while other receptors inhibit NK cell function. Therapeutic approaches to treat cancer have been developed based on preventing NK cell inhibition or using NK cell receptors and their ligands to activate NK cells or T cells to destroy tumor cells.

Areas covered—This article describes the various strategies for targeting NK cell receptors and NK cell receptor ligands using multivalent proteins to activate immunity against cancer. Expert opinion: NK cell receptors work in synergy to activate NK cell effector responses. Effective anti-cancer strategies will need to not only kill tumor cells but must also lead to the destruction of the tumor microenvironment. Immunotherapy based on NK cells and their receptors has the capacity to accomplish this through triggering lymphocyte cytotoxicity and cytokine production.

Keywords

cancer immunotherapy; bispecific; BiTE; NK cell; NKG2D; CD16

1. Introduction

Despite extensive efforts to improve cancer treatment, many malignancies still remain incurable. Therefore, innovative immunologic approaches for the treatment of cancer have attracted much interest. In this context, natural killer (NK) cell receptor and NK cell receptor ligand-based approaches to trigger immunity against cancer are attractive strategies for targeting cancer and could provide a means to treat many different types of cancer. NK cells are innate lymphoid cells that can readily recognize and destroy tumor cells. A large number of NK cell receptors and NK cell receptor ligands have been identified. Some of these molecules inhibit NK cell function whereas others allow NK cells to recognize malignant

Declaration of interest

CL Sentman and Dartmouth College have filed for patent protection on NK receptor-based bispecific proteins. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

cells. The effector responses of NK cells are mediated by their cytolytic activity and ability to secrete cytokines.

NK cells require external signals to begin the process of cell activation, which is usually regulated by an interplay of activating and inhibitory receptors [1, 2]. NK cell activating receptors, such as Natural Killer Group 2D (NKG2D), NKp30 (CD337), NKp44 (CD336), and NKp46 (CD335), are able to recognize a large panel of molecules that are highly expressed on multiple tumor types [1, 3]. Antibody blockade studies have indicated that different tumor cells express various ligands for these receptors. Human NKG2D homodimers interact with the major histocompatibility complex (MHC) class I chain-related proteins A and B (MICA and MICB) as well as UL16-binding proteins (ULBPs) [3, 4]. Another group of potent activating receptors are the natural cytotoxicity receptors (NCRs). NCRs are prominently expressed on NK cells, and NCRs account for much of the NK cell recognition of tumor cells [3, 5]. NKp30 and NKp46 are constitutively expressed on NK cells, whereas NKp44 is expressed on activated NK cells. Perhaps the most studied and best understood NK cell activating receptor is the low-affinity Fc receptor, CD16, or Fc γ RIII. Ninety percent of human NK cells express CD16, which provides them with the ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) [1].

Therapies that utilize NK cell receptors and/or their ligands have shown preclinical success for the treatment of cancer and many are now under clinical development (Table 1). Monoclonal antibodies (mAbs) have been used as targeted cancer therapies and although significant improvements have been observed in cancer patients, none of the mAbs tested is individually capable of curing cancer [6]. Progress in antibody engineering has allowed for the generation of multivalent antibodies, which are artificial molecules generated using either chemical cross-linking, fusion of two different hybridoma cell lines, or genetic approaches involving recombinant DNA technology to create molecules that simultaneously bind two or more antigens [7].

The use of multivalent antibodies in anti-cancer therapy is an approach that brings new advantages to first-generation antibodies. For example, multivalent antibodies can directly activate different effector cells of the immune system, leading to the stimulation of the innate and the adaptive arms of the immune response [8]. The unique ability of multivalent antibodies to simultaneously bind two or three distinct epitopes is the basis of their therapeutic function. Multivalent antibodies can function as adaptor molecules between effector cells (T cells, NK cells, neutrophils, or macrophages) and tumor cells, cross-linking activating receptors and triggering effector stimulation in the presence of the tumor, resulting in tumor cell destruction [9].

Evidence from clinical trials in which antibodies were used to treat cancer patients has shown the importance of the particular antigen targets and antibodies involved. Important parameters to consider in the design of antibodies for cancer treatment include therapeutic strategy, antibody affinity and avidity, antibody design, and the need to understand pharmacokinetic and pharmacodynamic properties [10]. At present, a new generation of antibody-derived molecules is being evaluated in clinical trials [11, 12]. These include bispecific, multivalent antibodies such as bispecific T cell engagers (BiTEs) as well as

bispecific and trispecific killer cell engagers (BiKEs and TriKEs). This review focuses on the development and potential use of multivalent antibodies that target NK cell receptors and NK cell receptor ligands to activate immunity against tumors.

2. Monoclonal antibodies

NK cells can recognize antibody-coated targets through CD16 and mediate ADCC. Rituximab, a mAb directed against CD20, has demonstrated efficacy in non-Hodgkin's lymphoma [13, 14]. Specific CD16 polymorphisms have been shown to influence NK cell-mediated ADCC, with certain polymorphisms (e.g. FcγRIIIa-V158F polymorphism) resulting in a stronger IgG binding [15]. These findings are clinically relevant, as supported by the observation made by Cartron *et al.* that the V158F polymorphism is associated with higher responses to rituximab therapy in patients with follicular lymphoma [16]. Other mAbs have been developed that also induce NK cell ADCC, and these include trastuzumab (anti-Her2 for breast cancer), alemtuzumab (anti-CD52 for chronic lymphocytic leukemia (CLL)), and cetuximab (anti-epidermal growth factor receptor (EGFR) for colorectal cancer) [17, 18].

Under normal circumstances, healthy cells and many tumor cells express ligands for inhibitory receptors and thus prevent activation of NK cells. One strategy to enhance NK cell activity is to block these inhibitory receptors and allow 'missing-self' recognition to occur. Studies using mAbs or small molecular weight inhibitors to block murine inhibitory Ly49 receptors or human killer-cell immunoglobulin-like receptors (KIR) showed the potential of generating anti-tumor immunity by releasing a brake on NK cell activity [19–23]. In certain conditions, such as a growing tumor, the expression of ligands for inhibitory receptors can be reduced, and the lack of inhibitory signals may allow activation of NK cells [24]. Inhibitory KIRs on human NK cells bind to self-HLA class I and inhibit NK cell function. Blocking antibodies directed against inhibitory KIRs have therapeutic potential by preventing the interaction of KIRs with their cognate HLA class I and thus allow NK cells to become activated against MHC class I+ tumor cells. A monoclonal antibody, 1-7F9, has been generated that recognizes inhibitory KIRs KIR2DL1, KIR2DL2, and KIR2DL3 [25]. Preclinical characterization of 1-7F9 demonstrated that blocking KIRs with this antibody increased lysis of primary acute myeloid leukemia (AML) blasts. In transgenic mice engineered to express KIR2DL3, HLA-Cw3+ splenocytes (a HLA target for KIR2DL3) were rejected after addition of 1-7F9. In a NOD-SCID mouse tumor model, human NK cells alone were unable to protect against a lethal dose of AML cells, but pre-administration of 1-7F9 mAbs resulted in long-term survival by providing specific blockade of KIRs and thereby boosting NK cell-mediated killing of HLA-matched AML blasts *in vivo* [25]. This experimental data set has provided a clinical basis for initiating a phase I clinical trial to investigate the efficacy of anti-KIR mAb therapy in AML (NCT01256073).

CD137 or 4-1BB is a co-stimulatory molecule of the tumor necrosis factor (TNF) receptor family. On resting NK cells, its expression is low, however CD16 activation induces CD137 expression [26]. CD137 can be activated by binding to its natural ligand or it can be triggered with an agonistic mAb. Upon binding of CD16 with rituximab-coated tumor cells, CD137 is upregulated on NK cells and addition of an CD137 agonist increased NK cell-

mediated ADCC [27]. A similar effect was observed using a combination of anti-CD137 and trastuzumab (anti-human EGFR 2 (HER2/HER2/neu)) to eliminate breast cancer cells more efficiently *in vitro* and *in vivo* in xenotransplant models of human breast cancer, including one using a human primary breast tumor [28]. Lenalidomide, a drug that is presently used in the treatment of multiple myeloma, has demonstrated enhanced NK cell-mediated ADCC in combination with rituximab [29]. An alternative to combining drug therapy is to combine NK cell-stimulating cytokines. Stimulation of NK cells with IL-2, IL-12, IL-15, IL-18 or type-I interferon (IFN) have all been shown to activate NK cells resulting in increased expression of adhesion molecules, cytokine induced activating receptors (e.g. NKP44), perforin, granzymes, FasL, TRAIL as well as increased proliferation and cytokine production *in vitro* [30–32].

Recently, an inhibitory mechanism that dampens CD16 signaling was discovered. Cytokine activation and target cell recognition through activating receptors, such as CD16, led to a rapid and striking decrease in CD16 expression [33]. A disintegrin and metalloprotease-17 (ADAM17) is expressed by NK cells and leads to shedding of CD16. Activated NK cells lose CD16 (FcγRIII) and CD62L through ADAM17 activity expressed on NK cells and may thereby directly impact the efficacy of Fc-mediated cytotoxicity. Selective inhibition of ADAM17 increased NK cell function by preserving CD16 on the NK cell surface and thereby enhanced ADCC [33]. Combined, these findings support the concept of targeting ADAM17 in order to prevent CD16 shedding and thus increase the efficacy of therapeutic antibodies.

3. NKG2D and NK2GD ligands

NKG2D is a potent activating receptor on NK cells whose ligands are widely expressed on tumor cells but only in a limited manner on normal tissue. The restricted tissue expression of such ligands makes them prime candidates for tumor-specific recognition. Upon interaction with its ligands, NKG2D can trigger NK cell-mediated cytotoxicity. NKG2D recognizes eight ligands in humans, and these ligands consist of the MHC class I chain-related protein (MIC) family (MICA and MICB) and the UL16-binding protein (ULBP1 - 6) family [4, 34, 35]. In mice, NKG2D ligands include the retinoic acid early inducible (Rae) gene family, the H60 family, and mouse ULBP-like-1 (MULT-1) [36–38]. The ligands are very different in sequence, and NKG2D recognition is species-specific for its ligands. Inhibition of NKG2D function may lead to an increased susceptibility to tumor development in some mouse tumor models demonstrating a role for NKG2D in immune surveillance of tumors [39, 40].

Several therapies that target NKG2D or its ligands have shown therapeutic potential. The use of NKG2D based chimeric antigen receptor (CAR) T cells to target specific ligands on tumors leads to long-term survival in tumor models [41–44]. NKG2D can be involved in anti-tumor responses induced via IL-2 and IL-12 therapy, and also through CTLA-4 inhibitory receptor blockade [45–47]. A NKG2D-Fc fusion protein was shown to efficiently trigger NK cell ADCC against NKG2D ligand-expressing tumor cells [48, 49]. Novel strategies that exploit the NKG2D activating receptor are represented by bispecific mAbs directed against an NKG2D-tumor-associated antigen or by fusion proteins that link

NKG2D ligands to an anti-tumor antigen Fv region to bring NKG2D⁺ effector cells to tumor cells [50–52].

4. Bispecific T cell Engager (BiTE)

A novel format of bispecific antibodies are bispecific T cell engager (BiTE) molecules, which target CD3 on T cells and an antigen on tumor cells to activate T cells to attack and destroy tumor cells (Figure 1A). BiTEs direct a host's T cells' cytotoxic activity against antigen expressing tumor cells [53]. Similar to other bispecific antibodies, but unlike ordinary mAbs, BiTEs form a direct link between T cells and tumor cells. This causes T cells to exert cytotoxic activity on tumor cells by releasing granules containing perforin and granzymes, independent of the presence of MHC class I or co-stimulatory molecules. The BiTE platform can be used to bind a broad range of cell surface target antigens by incorporating single chain variable fragments (scFvs) with distinct specificities [54].

Blinatumomab (MT103) was the first BiTE developed and has advanced the farthest in clinical testing. It targets CD3 on T cells and CD19, which is expressed on most B-lineage malignancies but is absent in hematopoietic stem cells and plasma cells [55]. In December of 2014, the U. S. Food and Drug Administration (FDA) granted approval for blinatumomab as second-line treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia (R/R ALL) under the trade name of BLINCYTO™. Currently, phase III clinical trials are undergoing to investigate blinatumomab in ALL (NCT02013167, NCT02393859).

A BiTE platform has been used to create a B7H6-specific BiTE. B7H6 is a specific ligand for the NK cell-activating receptor NKp30 [56]. B7H6 is expressed on various primary human tumors, however it is not expressed on healthy tissues [57]. It has been demonstrated that B7H6-specific BiTE therapy prolongs survival in a B7H6⁺ lymphoma tumor model through both IFN- γ and perforin effector mechanisms and protects surviving mice against lymphoma tumor rechallenge [56]. This B7H6-specific BiTE decreased tumor burden in models of murine melanoma and ovarian cancer indicating the potential of the B7H6 BiTE to treat both B7H6⁺ hematological and solid tumors.

A combination of anti-CD3 scFv and anti-CD138 scFv with the hIgG1 Fc (hIgFc) sequence was developed to form a novel BiTE with an additional hIgFc (BiTE-hIgFc, STL001) [58]. BiTE-hIgFc (STL001) activated T cells effectively and induced cytotoxicity against multiple myeloma cells *in vitro* and *in vivo* [58]. It is also the first BiTE to target CD138, CD3, and FcR, which are expressed on multiple myeloma cells, T cells and NK cells, respectively. An advantage of the BiTE-hIgFc (STL001) construct is that the antibody fragment could be replaced with that of antibodies that are directed against other tumor cells as a means to produce BiTE-hIgFc recombinants that activate T cells and stimulate anti-tumor activity *in vivo*.

5. Bispecific proteins

In vivo, a bispecific protein consisting of the extracellular region of NKG2D linked to an anti-CD3 scFv was shown to promote regression of lymphoma and melanoma tumors [59].

NKG2D-anti-CD3 binds NKG2D ligands on tumor cells and CD3 on T cells leading to T cell killing of tumor cells, cytokine production, and initiates a host anti-tumor immune response. This bispecific protein allows T cells to use NKG2D recognition as a primary triggering receptor. This results in a sustained protection from rechallenge with the same tumor cells. A recombinant ULBP2-BB4 bispecific protein was designed to simultaneously bind the NK cell NKG2D (via the ULBP2 moiety) and CD138 (via the BB4 moiety), which is overexpressed on a variety of malignancies, including multiple myeloma. Addition of ULBP2-BB4 protein increases IFN- γ secretion through activation of primary NK cells. *In vitro*, ULBP2-BB4 also enhances NK cell-mediated lysis of CD138+ human multiple myeloma cell lines, U-266 and RPMI-8226, and primary malignant plasma cells. In a subcutaneous tumor model in nude mice, the combination of ULBP2-BB4 protein and human peripheral blood lymphocytes abrogated tumor growth, which supports the potential clinical use of ULBP2-BB4 in multiple myeloma [51].

MICA is a ligand of NKG2D and plays an important role in NK cell-mediated immune recognition. Patients treated with blocking anti-CTLA-4 antibodies or with GM-CSF expressing autologous tumor cells generated high amounts of anti-MICA antibodies that were associated with improved therapeutic efficacy and anti-tumor cytotoxicity [47]. Anti-MICA antibodies were associated with lower circulating soluble MICA and increased cross-presentation of tumor antigens by dendritic cells. This supports the idea that treatment with anti-MICA antibodies may promote tumor elimination while stimulating cytotoxic T-lymphocyte (CTL) activity through dendritic cell cross-presentation.

To reconstitute the immune surveillance function of NK cells, a fusion protein rG7S-MICA was constructed consisting of extracellular domains of human MICA and an anti-CD24 single chain antibody fragment (rG7S). *In vitro*, rG7S-MICA triggered NK cell killing (through NKG2D) of CD24+ human hepatocellular carcinoma (HCC) cells [60]. *In vivo*, rG7S-MICA effectively recruited NK cells to tumor tissue in HCC-bearing nude mice, induced the release of cytokines, and showed anti-tumor activity, supporting the use of rG7S-MICA in the clinic.

6. Bispecific and Trispecific Killer Cell Engagers

Triggering NK cell effector functions via CD16 is a viable strategy to treat cancer as demonstrated by bispecific and trispecific killer cell engagers (BiKEs and TriKEs) [61]. BiKEs specifically target CD16 expressed on effector NK cells and a single tumor associated antigen to trigger NK cell cytotoxicity in an analogous manner to T cell targeting with BiTEs (Figure 1B). TriKEs target CD16 and two tumor associated antigens (Figure 1C). BiKEs and TriKEs are currently being developed for clinical use [62–66] and have advantages over first generation antibodies because they trigger effector cells, inducing target-specific cytotoxicity, cytokine and chemokine production, which results in greater NK cell anti-tumor activity [62]. Gleason *et al.* demonstrated that a CD16xCD19 BiKE and CD16xCD19xCD22 TriKE directly triggered NK cell activation through CD16 and thereby increased NK cell cytolytic activity and cytokine production against several CD19-expressing tumor cell lines. The same group also established a CD16xCD33 BiKE, tested its activity using refractory AML tumor samples *in vitro* and showed that cytotoxicity by NK

cells could overcome the inhibitory effect of KIR signaling [61, 62, 64]. A novel drug recognizing CD16 and epithelial cell adhesion molecule (EpCAM), EpCAMxCD16 (AFM13), is currently undergoing clinical trials in patients with non-Hodgkin's lymphoma (NCT02321592). EpCAM16 increased the destruction of many types of carcinomas *in vitro*, including breast, colon, prostate, head and neck [63]. EpCAM16 could activate resting NK cells to effectively kill tumor cells and produce cytokines. Analysis of CD107a expression indicated degranulation and killing by the EpCAM16 activated NK cells but not by IL-12/IL-18 activated NK cells. Bispecific antibodies are able to induce NK cell killing of tumor cells that are normally not susceptible to NK cells [63].

7. Challenges for the use of multivalent proteins targeting NK cell receptors

There are challenges for the use of specific targeting strategies in cancer, and targeting through NK cell receptors is no different. Unless the target itself is essential for tumor cell survival, there is a risk that antigen negative variants of tumors will selectively grow out over time. This risk is not unique to the approaches highlighted in this review, and this has been documented for the use of chemotherapies, antibodies, and adoptive T cell therapies [67–69]. This risk can be mitigated to an extent by using methods that target more than one target antigen on tumor cells, such as through TriKEs or by using NKG2D. In order for the strategies described herein to be effective they require the activation of T cells or NK cells. If a patient has poorly functioning effector cells or if the effector cells are inhibited within the tumor microenvironment, for example through T regulatory cells, PD-L1, or indoleamine-2,3-dioxygenase IDO action, then the multivalent proteins would be less effective at eliminating those tumors [70–72]. In those cases, there are approaches to prevent such immunosuppression with specific inhibitors or in combination with other immunotherapies [73].

8. Expert opinion

NK cells have the ability to recognize many different types of tumor cells and several of these receptors are also expressed on T cell subsets. Bryceson *et al.* demonstrated that there is a hierarchy and synergy among activation receptors on NK cells. Responses by resting NK cells, in the absence of cytokines, can be induced by combining the activity of several activating receptors [74]. They also found that CD16 was unique in its ability to mediate ADCC and to activate significant cytotoxicity and cytokine secretion when triggered alone. Each of the receptors tested did contribute to signaling in resting NK cells as demonstrated through synergistic activation by specific combinations of receptors. Many of the ligands recognized by NK cell receptors represent the body's way to flag altered or defective cells and support immune activation. The amount of ligand expression on the cell surface can also be modulated by ligand shedding, secretion of ligands, or excretion in microvesicles. The extent that such regulation of ligands affects immune targeting remains to be determined. In fact, commonly used chemotherapeutic agents can trigger pathways of DNA-damage and promote the expression of activating NK cell receptor ligands [75–78]. Thus, there may be opportunities to pair chemotherapy treatment with targeted immunotherapy against these ligands.

An effective therapy against cancer will not only need to kill tumor cells alone, but must also destroy the tumor microenvironment. As tumors grow, they induce nearby normal cells to support tumor growth and survival leading to a local microenvironment that contains a number of different cells with the capacity to inhibit anti-tumor immune responses. There are a variety of different mechanisms involving cell surface molecules, enzymes, metabolites, and cytokines that are present within the tumor microenvironment and inhibit effective immune responses against tumor cells [79, 80]. A key challenge in treating tumors is that by the time they become clinically relevant, they have established an immunosuppressive microenvironment that supports tumor survival and resists anti-tumor immunity [70]. It is within the tumor microenvironment that the battle to defeat cancer will be won or lost.

Immunotherapy has the capacity to engage new immune cells to attack tumors and overcome many of these microenvironment challenges through release of cytotoxic mediators and cytokines. These immune effector responses will not only kill tumor cells, but they modify the tumor microenvironment and activate pro-inflammatory processes to induce anti-tumor innate and adaptive immunity. As we learn more about the key effector mechanisms needed to change the tumor microenvironment, it will become possible to design proteins to harness these functions of the immune system. One of the challenges of understanding how bispecific proteins function is that much of the research and development work has focused on direct killing of tumor cells. While this is a key and important mechanism of action, it is not the only important mechanism that needs to be involved to lead to tumor elimination and long-term patient survival.

The use of mAb therapy in oncology has shown promising results in the last decade [10]. A large number of mAb therapies have been FDA approved and are currently in use in the clinic. To add additional functionality to mAbs, bispecific antibodies that target effector lymphocytes against tumors were created. The initial studies led to insights in protein stability, production, immunogenicity, side effects and overall efficacy [81]. Their early development was hampered by difficulties in producing these antibodies with both the yield and purity required for clinical purposes. Progress in antibody engineering has led to alternative antibody formats with improved therapeutic properties, including multivalent proteins [82].

The therapeutic approaches highlighted in this review use NK cell receptor/ligand interactions as a way to trigger powerful cytotoxicity and cytokines from NK cells and T cells to attack a variety of tumors and modulate tumor growth. Preclinical success and broad applicability of multivalent proteins based on NK cell recognition strategies bring promise as new therapies in the treatment of cancer.

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Abbreviations

ADAM17	a disintegrin and metalloprotease-17
ADCC	antibody-dependent cell-mediated cytotoxicity
AML	acute myeloid leukemia
BiKE	bispecific killer engager
BiTE	bispecific T-cell engager
HCC	hepatocellular carcinoma
HLA	human leukocyte antigen
IFN-γ	interferon-gamma
KIR	killer-cell immunoglobulin-like receptor
MICA	MHC class I chain-related protein A
NK	natural killer
PMSA	prostate-specific membrane antigen
TriKE	trispecific killer engager
ULBP	UL16-binding protein

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Article highlight box

- NK cells are important components of the innate immune system that play a key role in host immunity against cancer.
- Therapeutic approaches for cancer immunotherapy have been developed based on preventing NK cell inhibition or using NK cell receptors and their ligands to activate NK cells or T cells to destroy tumor cells.
- Strategies for targeting NK cell receptors and NK cell receptor ligands using multivalent proteins to activate immunity against cancer are being developed. The use of multivalent antibodies in anti-cancer therapy brings new advantages to antibodies, such as bispecific T cell engagers (BiTEs), bispecific and trispecific killer cell engagers (BiKEs and TriKEs).

Figure 1. BiTE-, BiKE- and TriKE-mediated tumor cell killing

(A) BiTEs bind CD3 on T cells and a tumor-associated antigen (TAA) on a cancer cell. T cell activation is triggered only when both single-chain variable fragments bind simultaneously. This will lead to the release of cytotoxic granules, cytokine production, T cell proliferation, and tumor cell death. BiKEs (B) and TriKEs (C) deliver a potent activating signal by binding through CD16 on NK cells. Both BiKEs and TriKEs simultaneously bind to CD16 and one (BiKE) or two (TriKE) TAAs on cancer cells to induce activation of NK cells leading to killing of the tumor cells and proinflammatory cytokine production.

Table 1

Multivalent antibodies currently ongoing in clinical trials.

NAME	FORMAT	TARGET	DEVELOPMENT STAGE
Blinatumomab (MT103)	BiTE	CD19xCD3	FDA approved in 2014
Solitumab (MT110, AMG110)	BiTE	EpCAMxCD3	Phase I
AMG330	BiTE	CD33xCD3	Phase I
AMG211	BiTE	CEAxCD3	Phase I
CD16xCD33	BiKE	CD16xCD33	preclinical development
CD16xCD133	BiKE	CD16xCD133	preclinical development
EpCAM16	BiKE	EpCAMxCD16	preclinical development
AFM13	BiKE	CD30xCD16A	Phase I
CD16xCD19xCD22	TRIKE	CD16xCD19xCD22	Phase II
Catumaxomab (Removab®)	Trifunctional Ab (Triomab ®)	EpcamxCD3xFc	Phase II/III
Ertumaxomab (Rexomun®)	Trifunctional Ab (Triomab ®)	HERxCD3xFc	Phase II
Bi20/FBTA05 (LymphomunTM)	Trifunctional Ab (Triomab ®)	CD20xCD3xFc	Phase I/II
3F8/GD2Bi	Trifunctional Ab (Triomab ®)	GD2xCD3xFc	Phase I/II
EGFRBi	Trifunctional Ab (Triomab ®)	EGFRxCD3xFc	Phase I
Bispecific protein therapy			
ULBP2-BB4	bispecific protein	NKG2D/NKG2D ligands	
UNKG2D-CD3 scFv	bispecific protein	UNKG2D-CD3	
anti-MICA therapy	mAb	MICA	preclinical development
Monalizumab	mAb	NKG2A	Phase II, Phase I,II
αCD16	mAb	CD16	
Lirilumab	mAb	KIR2DL1,2,3	Randomized Phase II, Phase I, Phase II
MGD006	DART	CD123xCD3	Phase I

Abbreviations: Ab, antibody; BiTE, bispecific T-cell engager; BiKE, bispecific killer cell engager; CEA, carcinoembryonic antigen; DART, dual-affinity re-targeting molecules; EGFRBi, epidermal growth factor receptor bispecific antibody; EpCAM, Epithelial cell adhesion molecule; MICA, MHC class I-related chain A; mAb, monoclonal antibody; scFv, single-chain variable fragment; TriKE, trispecific killer cell engager; ULBP, UL16-binding protein