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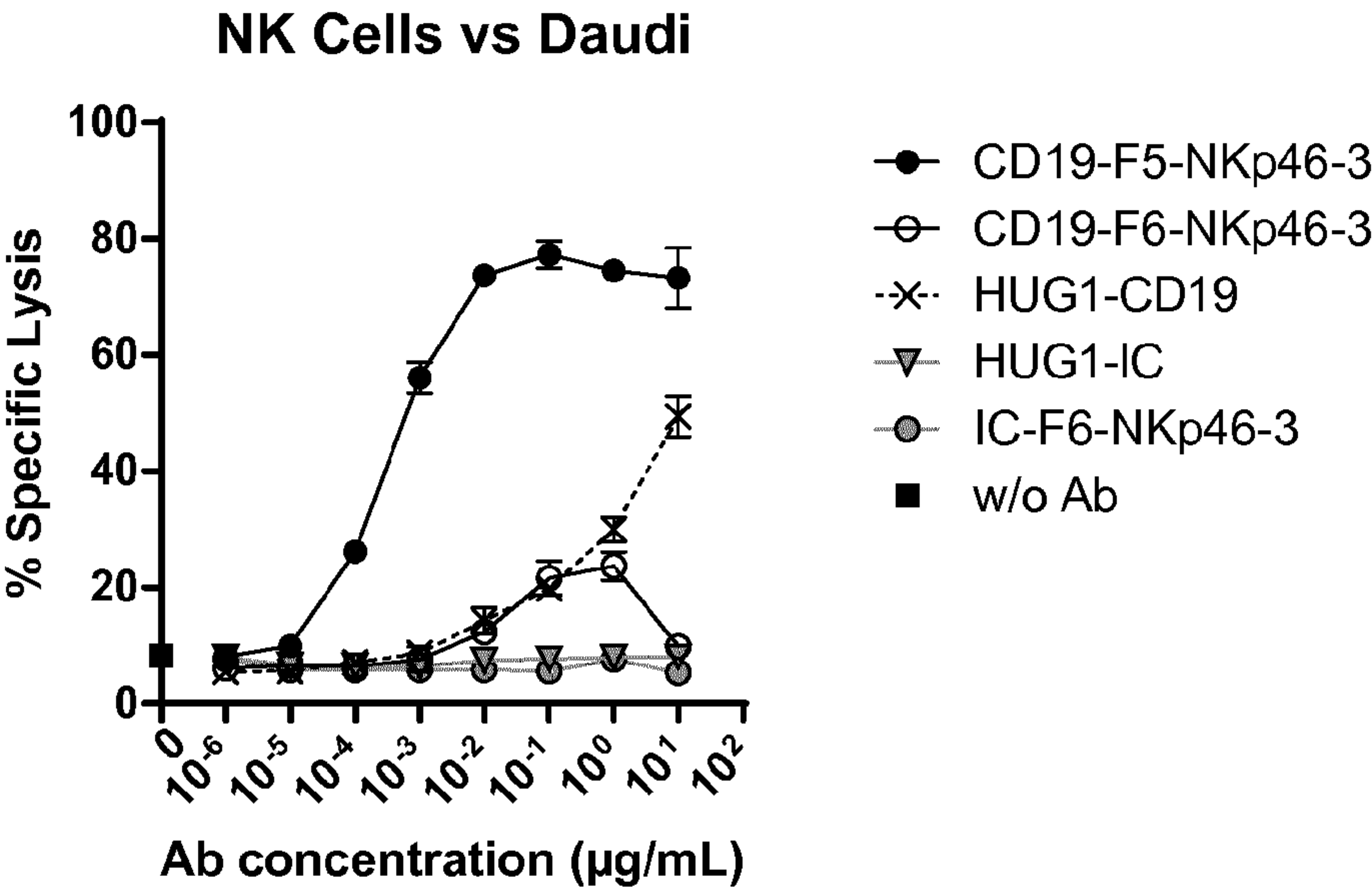
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(54) Title: MULTISPECIFIC NK ENGAGER PROTEINS

Figure 7



(57) **Abrégé/Abstract:**
Multispecific proteins that bind and specifically redirect NK cells to lyse a target cell of interest are provided without non-specific activation of NK cells in absence of target cells. The proteins have utility in the treatment of disease, notably cancer or infectious disease.

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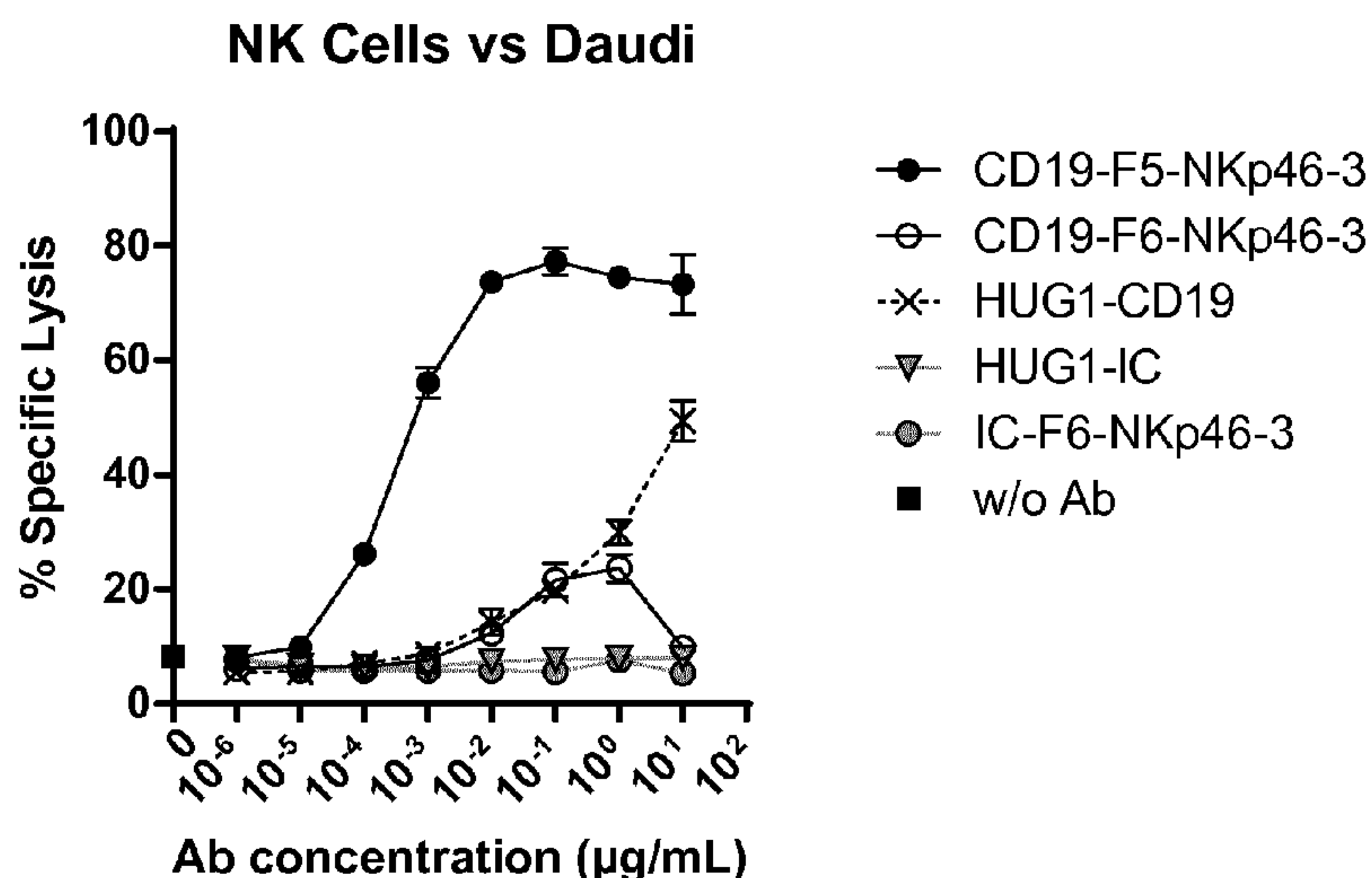
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(54) Title: MULTISPECIFIC NK ENGAGER PROTEINS

Figure 7



(57) Abstract: Multispecific proteins that bind and specifically redirect NK cells to lyse a target cell of interest are provided without non-specific activation of NK cells in absence of target cells. The proteins have utility in the treatment of disease, notably cancer or infectious disease.

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MULTISPECIFIC NK ENGAGER PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/271,459 filed December 28, 2015 and PCT patent application No. PCT/EP2015/064063 filed 23 June 2015; both of which are incorporated herein by reference in their entirety; including any drawings and sequence listings.

REFERENCE TO THE SEQUENCE LISTING

The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled "NKp46-6 PCT_ST25.txt", created June 22nd, 2016, which is 355 KB in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

Multispecific proteins that bind and specifically redirect effector cells to lyse a target cell of interest via multiple activating receptors are provided. The proteins have utility in the treatment of disease, notably cancer or infectious disease.

BACKGROUND

Bispecific antibodies binding two different epitopes offer opportunities for increasing specificity, broadening potency, and utilizing novel mechanisms of action that cannot be achieved with a traditional monoclonal antibody. A variety of formats for bispecific antibodies that bind to two targets simultaneously have been reported. Cross-linking two different receptors using a bispecific antibody to inhibit a signaling pathway has shown utility in a number of applications (see, e.g., Jackman, et al., (2010) *J. Biol. Chem.* 285:20850-20859). Bispecific antibodies have also been used to neutralize two different receptors. In other approaches, bispecific antibodies have been used to recruit immune effector cells, where T-cell activation is achieved in proximity to tumor cells by the bispecific antibody which binds receptors simultaneously on the two different cell types (see Baeuerle, P. A., et al, (2009) *Cancer Res* 69(12):4941-4). These antibodies have been referred to as "Bispecific T-cell engager antibodies" (or "BiTE" antibodies). However, in order to fully activate the T-cell, this T-cell and a cluster of BiTEs must interact on the surface of a target cell. Due to the difficulties of finding antibody variable regions which are functional in the BiTE format, to date only a single immune cell receptor (CD3) has been targeted, in the CD19 x CD3 specific antibody blinatumamab. Bispecific antibodies developed to date also include those

which link the CD3 complex on T cells to a tumor-associated antigen. Also, bispecific antibodies having one arm which binds CD16 (FcγRIIIa) and another which bound to an antigen of interest such as CD19 have been developed (see Kellner et al. (2011) *Cancer Lett.* 303(2): 128-139).

5 Natural killer (NK) cells are a subpopulation of lymphocytes that are involved in non-conventional immunity. NK cells provide an efficient immunosurveillance mechanism by which undesired cells such as tumor or virally-infected cells can be eliminated. Characteristics and biological properties of NK cells include the expression of surface antigens including CD16, CD56 and/or CD57, the absence of the α/β or γ/δ TCR complex on
10 the cell surface; the ability to bind to and kill cells that fail to express "self" MHC/HLA antigens by the activation of specific cytolytic enzymes, the ability to kill tumor cells or other diseased cells that express a ligand for NK activating receptors, and the ability to release protein molecules called cytokines that stimulate or inhibit the immune response.

NK cell activity is regulated by a complex mechanism that involves both activating
15 and inhibitory signals. Several distinct NK cell receptors have been identified that play an important role in the NK cell mediated recognition and killing of HLA Class I deficient target cells. One receptor, although not specific to NK cells, is FcγR3a (CD16) which is responsible for NK cell mediated ADCC. NK cells also express a range of other activating and co-activating receptors, including CD137 (4-1BB). Agonist antibodies against anti-4-1BB are in
20 clinical trials in patients with solid tumors, including melanoma, renal carcinoma, and ovarian cancer, and have shown strong activity in different cancer models, including breast cancer, sarcoma, glioma, colon carcinoma, myeloma, and mastocytoma.

Another NK cell receptor is NKp46, a member of the Ig superfamily. NKp46, is specific to NK cells and the cross-linking thereof, induced by specific mAbs, leads to a strong
25 NK cell activation resulting in increased intracellular Ca⁺⁺ levels, the triggering of cytotoxicity, and lymphokine release. International patent publication number WO2005/105858 (Innate Pharma) discloses the use of monospecific full-length IgG anti-NKp46 antibodies that bind Fcγ receptors for treating hematological malignancies that are Fcγ-positive. Fc γ receptors expressed on tumor cells (e.g. B cell malignancies) were proposed to interact with the Fc
30 domain of the anti-NKp46 antibodies which bound NK cells, such that the activated NK cells are brought into close proximity with target cells via the two reactive portions of the antibody (i.e., the antigen-recognizing domain and the Fc domain), thereby enhancing the efficiency of the treatment.

To date, no NK cell-specific bispecific antibodies have been reported. Rather depleting
35 agents that recruit NK cytotoxicity such as anti-tumor antibodies are typically full-length IgG1 antibodies that mediate ADCC via CD16. Despite the existence of a variety of formats for

bispecific antibodies, there remains a need in the art for multispecific proteins with new and well-defined mechanisms of action, particularly those that can provide therapeutic advantages over full-length antibodies.

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SUMMARY OF THE INVENTION

The present invention arises from the discovery of functional multi-specific proteins (e.g. a polypeptide, a single chain protein, a multi-chain protein, including but not limited to antibody-based protein formats) that binds NKp46 on NK cells and to an antigen of interest on a target cell, and is capable of redirecting NK cells to lyse a target cell that expresses the antigen of interest, e.g. a cell that contributes to disease. Provided, inter alia, is a multispecific protein comprising a first antigen binding domain and a second antigen binding domain, wherein one of the first or second antigen binding domains binds to a human NKp46 polypeptide and the other binds an antigen of interest, wherein the multispecific protein binds the NKp46 polypeptide monovalently, and wherein the multispecific protein is capable of directing an NKp46-expressing NK cell to lyse a target cell expressing the antigen of interest. Advantageously, in one embodiment, the presence of NK cells and target cells, the multispecific protein can bind (i) to antigen of interest on target cells and (ii) to NKp46 on NK cells, and, when bound to both antigen of interest on target cells and NKp46, can induce signaling in and/or activation of the NK cells through NKp46 (the protein acts as an NKp46 agonist), thereby promoting activation of NK cells and/or lysis of target cells, notably via the activating signal transmitted by NKp46.

In some embodiment, the multispecific protein comprises at least a portion of a human Fc domain, e.g. an Fc domain that is bound by FcRn.

In certain embodiments, the multispecific antibody is designed to have decreased or substantially lack FcγR binding compared to a conventional full-length human IgG1 antibody. Optionally the multispecific protein has decreased or abolished binding to a human CD16, CD32A, CD32B and/or CD64 polypeptide, compared to a full length wild type human IgG1 antibody. In other embodiments, the multispecific protein is designed to retain substantial FcγR binding, e.g., compared to a conventional full-length human IgG1 antibody. Optionally the multispecific protein binds (e.g. via its Fc domain) to a human CD16, CD32A, CD32B and/or CD64 polypeptide.

The present invention arises in part from the observation that multispecific proteins which bind to an antigen of interest and which are further engineered to bind to NKp46 on NK cells monovalently, and to CD16 or CD16A, exhibit an enhanced capability to promote NK cell-mediated target cell lysis (relative to conventional antibodies). Through comparison of molecules having different functionalities, it was observed that such multispecific proteins

elicited the individual and combined effects of these receptors thereby better promoting the lysis of target cells by NK cells. As described in detail herein, various functional multispecific proteins were constructed that bind (a) NKp46 and CD16 on NK cells and (b) to an antigen of interest on a target cell, which are capable of redirecting NK cells to lyse a target cell that expresses the antigen of interest, e.g. a cell that contributes to a disease such as cancer or infection. Moreover, despite binding to NKp46 on NK cells, advantageously these multispecific proteins do not induce lysis of NK cells themselves.

Also provided are novel formats for multispecific Fc proteins that are capable of activating CD16 and NKp46 and which can be used to promote NK-mediated killing of desired target cells. The potency in target cell killing demonstrated by the subject multispecific proteins which bind CD16 and which in addition bind to NKp46 and to an antigen of interest is believed to arise at least in part from an induction and upregulation of the co-activating receptor CD137 which is expressed on the surface NK cells. Particularly, CD137 upregulation occurs on resting NK cells, in the absence of target cells (as well as in the presence of target cells), and without the induction of CD16-mediated lysis of NK cells. NK cells with increased CD137 expression are known to be highly active against target cells (e.g. tumor cells) expressing CD137L (CD137 ligand). Consequently, the instant multispecific proteins which bind CD16 in addition to monovalently binding NKp46 can provide a means to upregulate CD137. Further advantageously, the multispecific protein when binding additionally to an antigen of interest expressed by a target cell, can elicit a multi-pronged recognition of target cells that involves multiple activating receptors expressed on effector cells. Also, despite an ability to cause upregulation of CD137 on NK cells that is comparable to human IgG1 antibodies (in the absence of target cells), surprisingly the multispecific proteins are far more potent in inducing NK cell-mediated lysis of tumor cells than human IgG1 antibodies. This would suggest that the subject multispecific proteins trigger the combined effects of NKp46-, CD16- and/or CD137-mediated activation thereby providing for a synergistic or additive enhancement in the induction of NK cell cytotoxicity by CD16⁺ NKp46⁺ NK cells. Additionally, independently of any contribution of CD137, these multispecific proteins advantageously are able to potently mobilize both CD16⁺ and CD16⁻ NK cells (all NK cells are NKp46⁺).

Furthermore, despite that the subject multispecific proteins are bound by CD16, unexpectedly they do not induce or increase down-modulation or internalization of the antigen of interest, even when targeting antigens of interest known to be susceptible to down-modulation or internalization when bound by conventional antibodies (such as full length human IgG1's). Based thereon, the subject multispecific proteins should be well suited for targeting antigens of interest expressed by target cells, e.g., tumor or infected

cells, including antigens which are known to be capable of undergoing down-modulation or internalization when bound by conventional antibodies (e.g. antibodies with human IgG1 Fc domains that retain CD16 binding). This is a huge therapeutic benefit since it is known in the art that antigen internalization can substantially impede the ability of conventional human IgG1 antibodies to mediate ADCC against a target cell.

Therefore, in one embodiment, multispecific proteins are provided which bind to an antigen of interest expressed on the surface of a cell monovalently, wherein the protein does not increase or induce down-modulation or intracellular internalization of the antigen of interest.

In another embodiment the invention provides a multispecific protein that comprises an antigen binding domain that binds to a human a NKp46 polypeptide monovalently (e.g., via a single antigen binding domain), and which is capable of binding to human CD16, and which when incubated in soluble form with effector cells expressing a NKp46 polypeptide and CD16 (e.g. human NKp46⁺CD16⁺ NK cells), optionally in the absence and/or presence of target cells, further optionally in the absence of other cells, can cause an increase or induction of CD137 polypeptide expression on the surface of the effector cells (e.g. without inducing detectable lysis of the NK cells). Optionally, the multispecific protein further binds to an antigen of interest.

In another embodiment the invention provides a multispecific protein that comprises (i) a first antigen binding domain ("ABD") that binds to a human NKp46 polypeptide, (ii) an Fc domain that binds to human CD16A, and (iii) a second antigen binding domain that binds to an antigen of interest expressed by a target cell. In one embodiment, the multispecific protein is capable of directing an NKp46-expressing NK cell to lyse a target cell expressing the antigen of interest, wherein said lysis of the target cell is mediated by NKp46-signaling, wherein optionally said lysis of the target cell is mediated by a combination of Nkp46-mediated signaling and CD16-mediated antibody-dependent cell-mediated cytotoxicity ("ADCC").

In another embodiment the invention provides a multispecific antigen binding protein which comprises (i) a monovalent antigen binding polypeptide ("ABD") which binds to a human NKp46 polypeptide (i) an ABD which binds to an antigen of interest, wherein said antigen of interest optionally comprises an antigen expressed by a tumor cell or an infectious agent and (iii) a CD16A binding polypeptide, optionally an Fc polypeptide which Fc polypeptide is optionally modified to enhance CD16A binding relative to the corresponding wild-type Fc polypeptide, wherein the multispecific protein is capable of directing an NKp46-expressing NK cell to lyse a target cell expressing the antigen of interest by NKp46-signaling.

In another embodiment the invention provides an isolated multispecific Fc-protein comprising a first antigen binding domain, a second antigen binding domain, and an Fc domain or portion thereof, which optionally may be modified, that is capable of binding human CD16 or CD16A, wherein one of the first or second antigen binding domains binds to a human NKp46 polypeptide and the other binds an antigen of interest, and wherein the multispecific protein is capable of directing an NKp46-expressing NK cell to lyse a target cell expressing the antigen of interest. In one embodiment, the protein causes lysis of the target cell by enhancing or inducing NKp46-signaling, wherein optionally lysis of the target cell is mediated by a combination of Nkp46 signaling and CD16-mediated antibody-dependent cell-mediated cytotoxicity ("ADCC"). Optionally, the multispecific protein further comprises a third or fourth or more antigen binding domains that each bind to an antigen of interest, e.g., one other than a NKp46 polypeptide, i.e., an antigen of interest expressed by a target cell such as a tumor cell or infectious agent, wherein the antigen of interest is the same or different from the antigen of interest bound by the second antigen binding domain. In one embodiment, the third antigen binding domain binds to the same antigen of interest as the second antigen binding domain, optionally further wherein the third antigen binding domain binds to the same epitope or a different epitope on the antigen of interest as the second antigen binding domain. In one embodiment, the antigen of interest is a cancer antigen. In one embodiment, the antigen of interest is a protein expressed (optionally over-expressed) on the surface of malignant immune cells, e.g. cells involved in a hematological malignancy, leukemia cells, lymphoma cells, a CD19 protein, a CD20 protein, etc. In one embodiment, the protein is used to treat a hematological malignancy, e.g., a leukemia or lymphoma cells. In another embodiment, the antigen of interest is a protein expressed (optionally over-expressed) on the surface of infected cells or by an infectious agent such as virally, bacterially or parasite infected cells.

Optionally, the subject multispecific polypeptides when incubated in soluble form with effector cells expressing NKp46 and CD16 (e.g. human NKp46⁺CD16⁺ NK cells), optionally in the presence or absence of other cells (e.g. target cells), can elicit an increase or induction of CD137 polypeptide expression on the surface of the NK cells (e.g. without inducing detectable lysis of the NK cells). Optionally, in the presence of target cells expressing the antigen of interest and NKp46⁺CD16⁺ NK cells, the multispecific protein can induce the activation of NK cells and/or lysis of target cells, in particular via the activating signal(s) transmitted by any combination of NKp46, CD16 and/or CD137.

In some embodiments, the multispecific protein binds to NKp46 in monovalent fashion. In one aspect of any embodiment, the multispecific protein comprises an Nkp46-binding ABD which binds to an NKp46 polypeptide monovalently.

In some embodiments in the presence of target cells and NK cells, the multispecific protein is capable of inducing an increase in cell surface CD137, e.g., on NK cells that express NK46 and CD16 (NKp46⁺CD16⁺ NK cells).

In some embodiments in the presence of target cells and NK cells, the multispecific protein is capable of inducing signaling by NK cells through NKp46.

In some embodiments in the presence of target cells and NK cells, the multispecific protein lacks the ability to induce NKp46-mediated signaling or cellular activation of an NK cell (independently of CD16), when incubated with NK cells in the absence of target cells (cells expressing the antigen of interest); for example the multispecific protein lacks the ability to induce NKp46-mediated signaling or NK cell activation when incubated with NK cells in the absence of target cells, when the protein is modified to comprise an Fc domain that does not bind CD16 (e.g. an Fc region containing a N297S substitution); or the multispecific protein lacks the ability to induce NKp46-mediated signaling or NK cell activation when incubated with NKp46⁺CD16⁻ NK cells, in the absence of target cells.

In any of the multispecific proteins described herein, such multispecific protein potentially possesses the following characteristics:

(a) capable of inducing human NK cells that express CD16 and NKp46 (NKp46⁺CD16⁺ NK cells) to lyse target cells expressing the antigen of interest, when incubated in the presence of the NK cells and target cells; and

(b) capable of inducing an increase in cell surface CD137 when incubated (e.g. in soluble form) with NK cells that express CD16 and NKp46, optionally in the presence of target cells, or in the absence of target cells.

In another embodiment the invention provides multispecific protein formats adapted for use in an NKp46-based NK cell engager, including antibody-based formats comprising antigen binding domain(s) and/or constant region domain(s) from immunoglobulins capable of forming a dimeric Fc domain that binds to human CD16 (and optionally further binding to FcRn and/or other human Fcγ receptors). By combining the NK-selective expression of NKp46 with multispecific (e.g. bispecific) antibody formats in which the multispecific proteins retain and/or have increased binding to human Fcγ receptor via an Fc domain, the invention provides multispecific antibody formats with favorable pharmacology due to FcRn binding which can direct NK cell cytotoxicity to a target of interest, and which further possess increased ability to lyse target cells via the combined action of activating receptors CD16, NKp46, and in addition optionally CD137.

In another aspect of any embodiment described herein, the multispecific protein can be characterized by a lack of agonist activity for NKp46 when incubated with Fcγ receptor-

negative NK cells (e.g. as purified NKp46⁺CD16⁻ NK cells) and in the absence of target cells (e.g. cells expressing the antigen of interest).

In another embodiment the invention provides a method for identifying, testing and/or producing a multispecific protein that binds NKp46 and CD16 on an NK cell and an antigen of interest expressed by a target cell, the method comprising:

(a) assessing whether the multispecific protein is capable of inducing an increase in cell surface CD137 on NK cells when incubated with CD16-expressing NK cells (e.g. NKp46⁺CD16⁺ NK cells); and

(b) assessing whether the multispecific protein has the ability to induce NK cells that express CD16 and NKp46 to lyse target cells, when incubated in the presence of the NK cells and target cells.

Optionally, in any of the foregoing the NK cells are purified NK cells.

In another embodiment the invention provides a method for identifying, testing and/or producing a multispecific protein, the method comprising providing a plurality of multispecific proteins that bind NKp46 and CD16 on an NK cell and an antigen of interest expressed by a target cell:

(a) assessing whether each multispecific protein has the ability to induce NK cells that express CD16 and NKp46 to lyse target cells, when incubated in the presence of the NK cells and target cells;

(b) optionally, further assessing each multispecific protein for the ability to induce an increase in cell surface CD137 when incubated with CD16-expressing NK cells (e.g. NKp46⁺CD16⁺ NK cells); and

(c) selecting a multispecific protein (e.g. for use as a medicament, for further evaluation, for further production, etc.) if the multispecific protein:

a. has the ability to induce NK cells that express CD16 and NKp46 to lyse target cells, when incubated in the presence of the NK cells and target cells, and

b. optionally, has the ability to induce an increase in cell surface CD137 when incubated with CD16-expressing NK cells.

In another embodiment the invention provides a method for identifying, testing and/or producing a multispecific protein, the method comprising providing a plurality of multispecific proteins that bind NKp46 and CD16 on an NK cell and an antigen of interest expressed by a target cell:

(a) assessing each multispecific protein to determine whether it is capable of inducing NKp46-mediated signaling or NK cell activation of an NK cell

independently of CD16, when incubated with NK cells in the absence of target cells;

(b) assessing each multispecific protein to determine whether the multispecific protein has the ability to induce NK cells to lyse target cells, when incubated in the presence of the NK cells and target cells;

(c) optionally, further assessing each multispecific protein to determine whether it is capable of inducing an increase in cell surface CD137 when incubated with CD16-expressing NK cells (e.g. NKp46⁺CD16⁺ NK cells); and

(d) selecting a multispecific protein (e.g. for use as a medicament, for further evaluation, for further production, etc.) if the multispecific protein:

a. lacks the ability to induce NKp46-mediated signaling or NK cell activation in an NK cell independently of CD16, when incubated with NK cells in absence of target cells;

b. has the ability to induce NK cells to lyse target cells, when incubated with NK cells and target cells, and

c. optionally, has the ability to induce an increase in cell surface CD137 when incubated with CD16-expressing NK cells.

In another embodiment the invention provides a multispecific protein that binds human CD16 (e.g. polypeptide, non-antibody polypeptide, or antibody), comprising: (a) a first antigen binding domain; and (b) a second antigen binding domain, wherein one of the first antigen binding domains binds NKp46 and the other binds to an antigen of interest on a target cell (other than NKp46), wherein the multispecific protein is capable of directing NKp46-expressing NK cells to lyse said target cell. In one embodiment, the protein comprises at least a portion of a human Fc domain, optionally wherein the Fc domain is dimeric, and is bound by human CD16 (and further optionally wherein the Fc domain is also bound by FcRn), and still further optionally wherein the multispecific antibody comprises an Fc domain comprising a modification (compared to a wild-type Fc domain) that results in increased binding to CD16; in one embodiment, the Fc domain is interposed between the two ABDs (one ABD is placed N- terminal and the other is C- terminal to the Fc domain).

In one aspect, the multispecific protein comprises two or more polypeptide chains, i.e. it comprises a multi-chain protein. For example, the multispecific protein or multi-chain protein can be a dimer, trimer or tetramer or may comprise more than 4 polypeptide chains.

An antigen binding domain positioned on a polypeptide chain can itself bind to its target (i.e., NKp46 or an antigen of interest) (e.g. an scFv or single antigen binding domain) or can optionally bind its target when it is in association or together with one or more complementary protein domains (antigen binding domain or domains) positioned on a

different polypeptide chain, wherein these polypeptide chains associate to form a multimer (e.g. dimer, trimer, etc.).

In one aspect, the multispecific protein binds an NKp46 polypeptide (e.g. expressed on the surface of a NK cell) in monovalent fashion. In one aspect, the multispecific protein binds the antigen of interest in monovalent fashion.

In another embodiment the invention provides multispecific proteins having a structure in which the freedom of motion (intrachain domain motion) or flexibility of one or more antigen binding domains (ABDs) is increased, e.g. compared to the ABDs of a conventional human IgG antibody. In one embodiment, provided is a multispecific protein comprising a structure that permits the antigen binding site of the first antigen binding domain and the antigen binding site of the second antigen binding domain to be separated by a distance that results in enhanced function, e.g., the ability of the multispecific protein to induce NKp46 signaling and lysis of target cells, e.g., optionally a distance of less than 80 ångström (Å). Multispecific proteins wherein the ABDs possess greater flexibility and/or are separated by an optimized distance may enhance the formation of a lytic NKp46-target synapse, thereby potentiating NKp46-mediated signaling.

In one embodiment, the invention provides multispecific proteins having increased freedom of motion of the antigen binding domains (e.g. compared to the ABDs of a conventional human IgG antibody, e.g., a human IgG1 antibody). One example of such a protein is a monomeric or multimeric Fc domain-containing protein (e.g. a heterodimer or heterotrimer) in which an antigen binding domain (e.g., the ABD that binds NKp46 or the ABD that bind the antigen of interest) is linked or fused to an Fc domain via a flexible linker. The linker can provide flexibility or freedom of motion of one or more ABDs by conferring the ability to bend thereby potentially decreasing the angle between the ABD and the Fc domain (or between the two ABDs) at the linker. Optionally, both antigen binding domains (and optionally more if additional ABDs are present in the multispecific protein) are linked or fused to the Fc domain via a linker, typically a flexible peptide linker. Optionally, other sequences or domains such as constant domains which optionally may be modified to alter (enhance or inhibit) one or more effector functions are placed between the Fc domain and an ABD, e.g. such that the ABD is fused to the Fc domain via a flexible linker and a constant region. The antigen binding domain can for example be comprised of variable region(s), a dAb, a VhH or a non-Ig scaffold. The antigen binding domain may be present in its entirety on a single polypeptide chain or may be formed from the association with a domain present on a separate polypeptide chain. Optionally, the protein with increased freedom of motion permits the protein to adopt a conformation in which the distance between the NKp46 binding site

and the antigen of interest binding site is less than that observed in proteins in which both binding domains were Fabs, or less than in full length antibodies.

An ABD can be connected to the Fc domain (or CH2 or CH3 domain thereof) via a flexible linker (optionally via intervening sequences such as constant region domains or portions thereof, e.g. CH1 or C κ). The linker can be a polypeptide linker, for example peptide linkers comprising a length of at least 5 residues, at least 10 residues, at least 15 residues, at least 20 residues, at least 25 residues, at least 30 residues or more. In other embodiments, the linker comprises a length of between 2-4 residues, between 2-4 residues, between 2-6 residues, between 2-8 residues, between 2-10 residues, between 2-12 residues, between 2-14 residues, between 2-16 residues, between 2-18 residues, between 2-20 residues, between 2-30 residues, between 10-24 residues, between 10-26 residues, between 10-30 residues, or between 10-50 residues. Optionally a linker comprises an amino acid sequence derived from an antibody constant region, e.g., an N-terminal CH1 or hinge sequence. Optionally a linker comprises the amino acid sequence RTVA. Optionally a linker is a flexible linker predominantly or exclusively comprised of glycine and/or serine residues, e.g., comprising the amino acid sequence GEGTSTGS(G₂S)₂GGAD or the amino acid sequence (G₄S)₃.

In one embodiment, the Fc domain is interposed between the two ABDs (one ABD is placed N-terminal and the other is C-terminal to the Fc domain). The subject multispecific proteins (e.g. dimers, trimers, tetramers) may in some embodiments comprise a domain arrangement as follows, in which domains can be placed on any of the 2, 3 or 4 polypeptide chains, wherein an Fc domain is interposed between the antigen binding domains, and wherein a flexible linker is present between at least one of the ABDs and the Fc domain:

(ABD₁) (Fc domain) (ABD₂).

In another embodiment, the multispecific proteins (e.g. dimers, trimers, tetramers) may comprise a domain arrangement of any of the following in which domains can be placed on any of the 2, 3 or 4 polypeptide chains, wherein the Fc domain is not interposed between ABDs (e.g. the protein has a terminal or distal Fc domain), and wherein a flexible linker is present between at least one of the ABDs and the Fc domain:

(Fc domain) (ABD₁ and ABD₂),

or

(ABD₁ and ABD₂) (Fc domain).

In the above-described domain arrangements, one of ABD₁ and ABD₂ is an antigen binding domain that binds NKp46 and the other is an antigen binding domain that binds an antigen of interest, and wherein the linker is a flexible polypeptide linker. The Fc domain can be a dimeric Fc domain (e.g. that binds human FcRn and/or Fc γ receptors). In one

embodiment, each of ABD₁ and ABD₂ are formed from two variable regions present within tandem variable regions, wherein the variable regions that associate to form a particular ABD can be on the same polypeptide chain or on different polypeptide chains. In another embodiment, one of ABD₁ and ABD₂ comprises a tandem variable region and the other comprises a Fab structure.

The invention also identifies specific epitopes on NKp46 which are well suited for targeting with NKp46 binding moieties, including the multispecific polypeptides disclosed herein. For example, bispecific or multispecific proteins which bind to one or more of these NKp46 epitopes possess advantageous properties, notably high efficacy in causing or directing NK cells to lyse target cells (e.g. via NKp46-mediated signaling). Provided also are CDRs of different anti-NKp46 antibodies suitable for use in the construction of efficient multispecific proteins, e.g., bispecific and trispecific proteins particularly those which potently lyse a target cell of interest, and amino acid and nucleic acid sequences of exemplary multispecific proteins and nucleic acids which encode these proteins.

In one aspect, the protein (and/or the antigen binding domain thereof that binds NKp46) competes for binding to a NKp46 polypeptide with any one or any combination of monoclonal antibodies NKp46-1, -2, -3, -4, -6 or -9 or an Anti-CD19-F5-Anti-NKp46 antibody that comprises such NKp46-1, -2, -3, -4, -6 or -9 bispecific antibodies. In one embodiment, the antigen binding domain that binds NKp46 binds an epitope within (partly or fully within) the D2 (proximal) domain of the NKp46 polypeptide. In one embodiment, the antigen binding domain that binds NKp46 binds an epitope within (partly or fully within) the D1 (distal) domain of the NKp46 polypeptide. In one embodiment, the antigen binding domain that binds NKp46 binds an epitope on an NKp46 polypeptide of SEQ ID NO:1 comprising one, two, three or more residues selected from the residues bound by any one or combination of antibodies NKp46-1, -2, -3, -4, -6 or -9 or an Anti-CD19-F5-Anti-NKp46 antibody that comprises such NKp46-1, -2, -3, -4, -6 or -9 bispecific antibodies. In one embodiment the multispecific protein is capable of binding to human neonatal Fc receptor (FcRn). In one embodiment the multispecific protein has decreased or abolished binding to a human and/or non-human primate (e.g. cynomolgus monkey) Fcγ receptor, e.g., compared to a full length wild type human IgG1 antibody. In one embodiment the multispecific protein is capable of inducing NK-mediated lysis (e.g. as well as or better than a full length human wild-type IgG1 antibody).

In one embodiment of any of the multispecific proteins described herein, the antigen binding domain that binds to an antigen of interest binds to an antigen (e.g. polypeptide) expressed by a target cell which is sought to be lysed by an NK cell. Optionally this antigen is expressed by a cancer cell, or a virally, bacterially or parasite infected cell, an immune cell

that contributes to tumor growth or escape (e.g. a tumor-associated monocyte or macrophage), or a cell that contributes to an autoimmunity, an allergic response or inflammatory disease.

In one embodiment, the multispecific protein binds NKp46 in monovalent fashion. In one embodiment, the multispecific protein binds to the antigen of interest in monovalent fashion. In one embodiment, the multispecific protein binds both NKp46 and the antigen of interest in monovalent fashion. In one embodiment, the multispecific protein binds CD16 via a dimeric Fc domain.

In one embodiment, the first antigen binding domain comprises an antibody heavy chain variable domain and a light chain variable domain. Optionally, both said heavy and light chain variable domains are involved in binding interactions with NKp46.

In one embodiment, the second ABD (and optionally third or more ABDs, when present) comprises an antibody heavy chain variable domain and a light chain variable domain. Optionally, both said heavy and light chain variable domains are involved in binding interactions with the antigen bound by the second antigen binding domain. In one embodiment, the second ABD (and optionally third or more ABD, when present) comprises a non-immunoglobulin scaffold.

Optionally, the Fc domain comprises at least a portion of a CH2 domain and at least a portion of a CH3 domain, and when present in a multispecific polypeptide, is part of a dimeric Fc domain.

In one embodiment, the CH2 domain comprises an amino acid modification, compared to a wild-type CH2 domain. In one embodiment, the CH2 modification increases binding (e.g. increases binding affinity) of the bispecific polypeptide to a human CD16 polypeptide relative to a wild-type human Fc region.

In one embodiment, the CH2 domain and/or CH3 domains are naturally occurring (non-engineered) human CH2 and/or CH3 domains. In one embodiment, the multispecific protein comprises an Fc domain that comprises N-linked glycosylation. In one embodiment, the N-linked glycosylation (at residue N297, Kabat EU numbering) comprises glycan structures typical of those found on IgG-class (e.g. IgG1) immunoglobulins produced in mammalian cells (e.g., CHO cells or other rodent cells, non-human primate or human cells). In one embodiment, the Fc domain comprises modified N-linked glycosylation, e.g. hypofucosylated glycans at N297) which increase binding affinity for a human CD16 polypeptide.

In one embodiment, the Fc-derived polypeptide is a dimer, optionally a homodimer or a heterodimer. In one embodiment, the Fc-derived polypeptide is a heterotrimer. In one embodiment, the Fc-derived polypeptide is a hetero-tetramer.

In one embodiment, heterotrimer proteins are provided comprising two antigen binding domains that are composed of three different polypeptide chains that each comprise at least one V-(CH1/C κ) unit, wherein a first (central) chain comprises two V-(CH1/C κ) units separated by an Fc domain (or portion thereof which optionally binds CD16), and each of the second and third chains comprise one V-(CH1/C κ) unit, wherein one of the V-(CH1/C κ) units of the central chain preferentially undergoes CH1-C κ dimerization with the V-(CH1/C κ) unit of the second chain thereby forming a first antigen binding domain, wherein the other of the V-(CH1/C κ) units of the central chain preferentially undergoes CH1-C κ dimerization with the V-(CH1/C κ) unit of the third chain thereby forming a second antigen binding domain, and wherein the second or third chain further comprise an Fc domain (or portion thereof) placed on the polypeptide chain such that the Fc domain is capable of forming a dimeric Fc domain that binds CD16 together with the Fc domain of the central polypeptide.

In one embodiment, heterotrimer proteins having three antigen binding domains are provided that are composed of three different polypeptide chains that each comprise at least one V-(CH1/C κ) unit, wherein a first (central) chain comprises two V-(CH1/C κ) units and each of the second and third chains comprise one V-(CH1/C κ) unit, wherein one of the V-(CH1/C κ) units of the central chain preferentially undergoes CH1-C κ dimerization with the V-(CH1/C κ) unit of the second chain thereby forming a first antigen binding domain, wherein the other of the V-(CH1/C κ) units of the central chain preferentially undergoes CH1-C κ dimerization with the V-(CH1/C κ) unit of the third chain thereby forming a second antigen binding domain, wherein one of the polypeptide chains further comprises an antigen binding domain (e.g. a tandem variable domain, an scFv) that forms third antigen binding domain, and wherein the first or second chain further comprise an Fc domain (or portion thereof) placed on the polypeptide chain such that the Fc domain is capable of forming a dimeric Fc domain that bind CD16 together with the Fc domain of the central polypeptide.

In one embodiment, the invention provides a heteromultimeric, e.g. heterodimeric, bispecific protein comprising: (a) a first polypeptide chain comprising a first variable region (V), fused to a CH1 or C κ domain, wherein the V-(CH1/C κ) unit is in turn fused to a first terminus (N- or C- terminus) of a human Fc domain (a full Fc domain or a portion thereof); (b) a second polypeptide chain comprising a first variable region (V) fused to a CH1 or C κ domain that is complementary with the CH1 or C κ of the first chain to form a CH1-C κ dimer, wherein the V-(CH1/C κ) unit is fused to at least a human Fc domain (a full Fc domain or a portion thereof), wherein the two first variable regions form an antigen binding domain that binds a first antigen of interest in monovalent fashion, and (c) an antigen binding domain that binds a second antigen (optionally together with a complementary antigen binding

domain), and optionally a second CH1 or C κ domain, fused to a second terminus (N- or C-terminus) of the Fc domain of the first polypeptide such that the Fc domain is interposed between the V-(CH1/C κ) unit and the antigen binding domain that binds a second antigen, and wherein the Fc domains (or portions thereof) of the first and second chains of the heteromultimeric protein form a dimeric Fc domain capable of being bound by human CD16 polypeptide, e.g. a dimeric Fc domain comprising N-linked glycosylation at residue N297 (Kabat EU numbering). Optionally the first and second polypeptide chains are bound by non-covalent bonds and optionally further interchain disulfide bonds, e.g. formed between respective CH1 and C κ domains and/or between respective hinge domains. Optionally a V-(CH1/C κ) unit is fused to a human Fc domain directly, or via intervening sequences, e.g. linkers, other protein domain(s), etc. Optionally, one of the antigens is NKp46 and another of the antigens is one expressed on the surface of a cell that is to be lysed by the immune cell, e.g. a cancer or viral or bacterial antigen; optionally further, wherein such antigen present on the surface of a cell that is to be lysed by the immune cell, e.g., NK cell, is a protein that is known to undergo intracellular internalization, notably when bound by an antibody (e.g. a full length antibody of an isotype such as human IgG1 or IgG3 that is bound by CD16).

In one embodiment, the multispecific polypeptide or protein is monoclonal. In one embodiment, the multispecific polypeptide or protein is purified. In one embodiment, the multispecific polypeptide or protein is isolated. In another embodiment the multispecific polypeptide or protein is expressed by a cell, e.g., a human immune cell such as a human NK cell.

In one embodiment of the above heteromultimeric polypeptide or protein, the polypeptide or protein is a heterodimer, wherein the antigen binding domain for a second antigen is an scFv, optionally an scFv that binds NKp46.

In one embodiment of the afore-described heteromultimeric polypeptides or proteins, the heteromultimeric polypeptide or protein is a heterotrimer, comprising an antigen binding domain for a second antigen which comprises or consists of an heavy or light chain variable region, and the heteromultimeric polypeptide or protein further comprises a third polypeptide chain comprising or consisting of a variable region (V) fused to a CH1 or C κ domain that is complementary with the CH1 or C κ of the first chain to form a CH1-C κ dimer wherein the variable region that is the antigen binding domain for a second antigen of the first polypeptide and the variable region of the third chain form an antigen binding domain. The double dimerization yields a trimer. The CH1 or C κ constant region of the third polypeptide is selected to be complementary to the second CH1 or C κ constant region of the first polypeptide chain (but not complementary to the first CH1/C κ of the first polypeptide chain).

In one aspect a heterodimeric polypeptide according to the invention comprises:

(a) a first polypeptide chain comprising, from N- to C- terminus, a first variable domain (V), a CH1 of C κ constant region, a Fc domain or portion thereof which optionally binds CD16, a second variable domain and third variable domain; and

5 (b) a second polypeptide chain comprising, from N- to C- terminus, a first variable domain (V), a CH1 or C κ constant region, an Fc domain or portion thereof, wherein the CH1 or C κ constant region is selected to be complementary to the CH1 or C κ constant region of the first polypeptide chain such that the first and second polypeptides form a CH1-C κ heterodimer in which the first variable domain of the first polypeptide chain and the first
10 variable domain of the second polypeptide form an antigen binding domain that binds the first antigen of interest; and wherein a second variable domain and third variable domain forms an antigen binding domain that binds the second antigen of interest, and wherein the Fc domains (or portions thereof) of the first and second chains of the heteromultimeric protein form a dimeric Fc domain capable of being bound by human CD16 polypeptide, e.g.
15 a dimeric Fc domain comprising N-linked glycosylation at residue N297 (Kabat EU numbering).

In another aspect the invention provides a heterodimeric polypeptide which comprises:

(a) a first polypeptide chain comprising, from N- to C- terminus, a second variable
20 domain and third variable domain, a Fc domain or portion thereof, a first variable domain (V), and a CH1 of C κ constant region; and

(b) a second polypeptide chain comprising, from N- to C- terminus, a first variable
25 domain (V), a CH1 or C κ constant region, and a Fc domain or portion thereof, wherein the CH1 or C κ constant region is selected to be complementary to the CH1 or C κ constant region of the first polypeptide chain such that the first and second polypeptides form a CH1-C κ heterodimer in which the first variable domain of the first polypeptide chain and the first
30 variable domain of the second polypeptide form an antigen binding domain that binds to the first antigen of interest; and wherein a second variable domain and third variable domain forms an antigen binding domain that binds to a second antigen of interest, wherein the Fc domains (or portions thereof) of the first and second chains of the heteromultimeric protein form a dimeric Fc domain capable of being bound by human CD16 polypeptide, e.g. a
dimeric Fc domain comprising IgG-type N-linked glycosylation at residue N297 (Kabat EU numbering).

In another aspect the invention provides a heterodimeric polypeptide which
35 comprises:

(a) a first polypeptide chain comprising, from N- to C- terminus, a first variable domain (V) fused to a first CH1 or C κ constant region, an Fc domain or portion thereof, and a second variable domain (V) fused to a second CH1 or C κ constant region;

(b) a second polypeptide chain comprising, from N- to C- terminus, a variable domain fused to a CH1 or C κ constant region selected to be complementary to the first (but not the second) CH1 or C κ constant region of the first polypeptide chain such that the first and second polypeptides form a CH1-C κ heterodimer, and an Fc domain or portion thereof; and

(c) a third polypeptide chain comprising, from N- to C- terminus, a variable domain fused to a CH1 or C κ constant region, wherein the CH1 or C κ constant region is selected to be complementary to the second (but not the first) variable domain and second CH1 or C κ constant region of the first polypeptide chain, wherein the Fc domains (or portions thereof) of the first and second chains of the heteromultimeric protein form a dimeric Fc domain capable of being bound by a human CD16 polypeptide, e.g. a dimeric Fc domain comprising IgG-type N-linked glycosylation at residue N297 (Kabat EU numbering). The first and third polypeptides will therefore form a CH1-C κ heterodimer formed between the CH1 or C κ constant region of the third polypeptide and the second CH1 or C κ constant region of the first polypeptide, but not between the CH1 or C κ constant region of the third polypeptide and the first CH1 or C κ constant region of the first polypeptide. The first, second and third polypeptides form a CH1-C κ heterotrimer, and wherein the first variable domain of the first polypeptide chain and the variable domain of the second polypeptide chain form an antigen binding domain specific for a first antigen of interest, and the second variable domain of the first polypeptide chain and the variable domain on the third polypeptide chain form an antigen binding domain specific for a second antigen of interest.

In another embodiment, the above-described heteromultimeric polypeptides or proteins may comprise one or more additional polypeptide chains.

Any the heteromultimeric polypeptides or proteins described herein may comprise a dimeric Fc domain capable of being bound by human CD16 polypeptide, e.g. a dimeric Fc domain comprising N-linked glycosylation at residue N297 (Kabat EU numbering).

Optionally, the CH1 and/or C κ domain are fused via a hinge region to the Fc domain. Optionally the hinge, CH2 and/or CH3 comprises one or more amino acid modifications which increase binding affinity for human CD16. Optionally the hinge, CH2 and/or CH3 comprises an amino acid modification which increases binding affinity for human FcRn. Optionally the amino acid modifications that increase binding affinity for CD16 may also increase affinity for one or more other human Fc γ receptors. Optionally the hinge, CH2

and/or CH3 comprise an amino acid modification which reduces or substantially abolishes binding to an inhibitory human Fcγ receptor (e.g. CD32B) and/or to an Fcγ receptor other than CD16 (e.g. CD32A and/or CD64). In any embodiment described herein, the CH1 and Cκ domains optionally can be of human origin.

5 In one aspect of any of the embodiments described herein, the bispecific protein binds more strongly or avidly (has a greater binding affinity) for the antigen of interest (e.g. a cancer or viral or other infectious agent antigen) than it binds NKp46. Such antibodies may possess advantageous pharmacological properties. In one aspect of any of the embodiments herein, the polypeptide has a K_d (monovalent binding affinity) to NKp46 of less than 10⁻⁷ M, preferably less than 10⁻⁸ M, or preferably less than 10⁻⁹ M; optionally the polypeptide has a K_d for binding (monovalent binding affinity) to a cancer, viral, bacterial or other antigen that is less than (i.e. has better binding affinity than) the K_d (monovalent binding affinity) to a NKp46 polypeptide. In one aspect of any of the embodiments described herein, the polypeptide has a K_d (monovalent binding affinity) to NKp46 of between 10⁻⁷ M (100 nanomolar) and 10⁻¹⁰ M (0.1 nanomolar) for binding to a NKp46 polypeptide. In one aspect of any of the embodiments disclosed herein, the polypeptide has a K_d (monovalent binding affinity) to NKp46 of between 10⁻⁸ M (10 nanomolar) and 10⁻¹⁰ M (0.1 nanomolar). In one aspect of any of the embodiments herein the multimeric polypeptide has a K_d (monovalent binding affinity) to NKp46 of between 10⁻⁸ M (10 nanomolar) and 10⁻⁹ M (1 nanomolar).
20 Binding can be assessed as in the Examples herein, e.g. by surface plasmon resonance.

In one aspect of any of the embodiments of the invention, the antigen binding domain that binds NKp46 binds to at least one residue on NKp46 corresponding to any of the amino acid residues bound by any one of monoclonal antibodies NKp46-1, -2, -3, -4, -6 or -9 or the Anti-CD19-Anti-NKp46-1, -2, -3, -4, -6 or -9 bispecific antibodies. In one aspect, the antigen binding domain that binds NKp46 binds at least 1, 2, 3, 4 or more amino acids of NKp46 within the epitope bound by any one or combination of monoclonal antibodies NKp46-1, -2, -3, -4, -6 or -9 or the Anti-CD19-Anti-NKp46-1, -2, -3, -4, -6 or -9 bispecific antibodies. In one aspect of any of the embodiments of the invention, the antigen binding domain that binds NKp46 binds to the same epitope on a NKp46 polypeptide as any of monoclonal antibodies NKp46-1, -2, -3, -4, -6 or -9 or to any of the Anti-CD19-Anti-NKp46-1, -2, -3, -4, -6 or -9 bispecific antibodies. In one embodiment, the antigen binding domain that binds NKp46 binds to an epitope on the NKp46 polypeptide of SEQ ID NO:1 wherein the epitope comprises one, two, three or more residues selected from the group of residues bound by any of antibodies NKp46-1, -2, -3, -4, -6 or -9.
30

In some embodiments, the protein that binds NKp46 exhibits significantly lower binding for a mutant NKp46 polypeptide in which a residue bound by any of antibodies NKp46-1, -2, -3, -4, -6 or -9 is substituted with a different amino acid, compared to a wild-type NKp46 polypeptide of SEQ ID NO: 1.

In one aspect of any of the embodiments of the invention, the protein that binds NKp46 competes for binding to a NKp46 polypeptide with any one or any combination of monoclonal antibodies NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9, or any of the Anti-CD19-anti-NKp46-1, -2, -3, -4, -6 or -9 bispecific antibodies. In one embodiment, the protein that binds NKp46 competes for binding to an NKp46 polypeptide with an antibody selected from the group consisting of:

- (a) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 3 and 4 (NKp46-1);
- (b) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 5 and 6 (NKp46-2);
- (c) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 7 and 8 (NKp46-3);
- (d) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 9 and 10 (NKp46-4);
- (e) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 11 and 12 (NKp46-6); and
- (f) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 13 and 14 (NKp46-9).

In one embodiment, the invention provides a protein (or nucleic acid encoding such) that specifically binds NKp46 (e.g. a monospecific monoclonal antibody or fragment, a multispecific protein or fragment, a bispecific antibody, etc.) that competes for binding to an NKp46 polypeptide with an antibody selected from the group consisting of:

- (a) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 3 and 4 (NKp46-1);
- (b) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 5 and 6 (NKp46-2);
- (c) (a) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 7 and 8 (NKp46-3);
- (d) (a) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 9 and 10 (NKp46-4);
- (e) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 11 and 12 (NKp46-6); and

- (f) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 13 and 14 (NKp46-9).

In one aspect of any of the embodiments of the invention, the antigen binding domain that binds NKp46 comprises the hypervariable regions of any one of monoclonal antibodies NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 or a combination of any of the foregoing.

In one aspect of any of the embodiments of the invention, the antigen binding domain that binds NKp46 has a heavy and/or light chain variable region having one, two or three CDRs of the respective heavy and/or light chain of an antibody selected from the group consisting of antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 and NKp46-9.

In one embodiment, an isolated multispecific protein that binds NKp46 according to the invention comprises or an antigen binding domain thereof comprises heavy chain CDR1, 2 and 3 and light chain CDR 1, 2 and 3 of any of the antibodies selected from the group consisting of:

- (a) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 3 and 4 (NKp46-1);
- (b) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 5 and 6 (NKp46-2);
- (c) (a) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 7 and 8 (NKp46-3);
- (d) (a) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 9 and 10 (NKp46-4);
- (e) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 11 and 12 (NKp46-6); and
- (f) an antibody having respectively a V_H and V_L region of SEQ ID NOS: 13 and 14 (NKp46-9).

In one embodiment, the invention provides an antibody (e.g. a full length monospecific antibody or a bispecific antibody) or antigen binding domain (which optionally may be humanized, chimerized or affinity matured) that binds NKp46 and comprises:

- (a) (i) a polypeptide chain comprising a CDR 1, 2 and 3 of the heavy chain variable region of NKp46-1 of Table A, and (ii) a polypeptide chain comprising a CDR 1, 2 and 3 of the light chain variable region of NKp46-1 of Table A;
- (b) (i) a polypeptide chain comprising a CDR 1, 2 and 3 of the heavy chain variable region of NKp46-2 of Table A and (ii) a polypeptide chain comprising a CDR 1, 2 and 3 of the light chain variable region of NKp46-2 of Table A;

(c) (i) a polypeptide chain comprising a CDR 1, 2 and 3 of the heavy chain variable region of NKp46-3 of Table A and (ii) a polypeptide chain comprising a CDR 1, 2 and 3 of the light chain variable region of NKp46-3 of Table A;

(d) (i) a polypeptide chain comprising a CDR 1, 2 and 3 of the heavy chain variable region of NKp46-4 of Table A and (ii) a polypeptide chain comprising a CDR 1, 2 and 3 of the light chain variable region of NKp46-4 of Table A;

(e) (i) a polypeptide chain comprising a CDR 1, 2 and 3 of the heavy chain variable region of NKp46-6 of Table A and (ii) a polypeptide chain comprising CDR 1, 2 and 3 of the light chain variable region of NKp46-6 of Table A; or

(f) (i) a polypeptide chain comprising a CDR 1, 2 and 3 of the heavy chain variable region of NKp46-9 of Table A and (ii) a polypeptide chain comprising a CDR 1, 2 and 3 of the light chain variable region of NKp46-9 of Table A.

In one aspect, the invention provides a protein (a monomeric or multimeric protein) that specifically binds NKp46 (e.g. a monospecific monoclonal antibody, a multispecific protein, a bispecific antibody) that binds the same or overlapping epitope on NKp46 as an antibody selected from the group consisting of antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 and NKp46-9. The isolated polypeptide may be, for example, a monospecific monoclonal antibody, a multispecific polypeptide or a bispecific antibody.

In one aspect the invention provides an isolated multispecific heterotrimeric protein comprising a first polypeptide chain comprising an amino acid sequence which is at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98 or 99% identical to the sequence of a first polypeptide chain of a F5, F13 or T5 protein disclosed herein; a second polypeptide chain comprising an amino acid sequence which is at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98 or 99% identical to the sequence of a second polypeptide chain of the respective F5, F13 or T5 protein disclosed herein; and a third polypeptide chain comprising an amino acid sequence which is at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98 or 99% identical to the sequence of a third polypeptide chain of a F5, F13 or T5 protein disclosed herein. In one aspect, the protein comprises a dimeric Fc domain capable of being bound by a human CD16 polypeptide, e.g. a dimeric Fc domain comprising N-linked glycosylation at residue N297 (Kabat EU numbering). Optionally any or all of the variable regions or CDRs of the first, second and/or third chains are substituted with different variable regions; optionally any or all of the V-CH1/C κ units of the first, second and/or third chains are substituted with different V-CH1/C κ units. Optionally variable regions, CDRs or V-CH1/C κ units are excluded from the sequences that are considered for computing identity; optionally wherein the anti-NKp46 variable regions, CDRs or V-CH1/C κ units are included for computing identity and

the variable regions, CDRs or V-CH1/C_K units for the antigen binding domain that binds the other antigen are excluded from the sequences that are considered for computing identity.

In one embodiment of any of the polypeptides described herein, the multispecific polypeptide is capable of directing NKp46-expressing NK cells to lyse a target cell of interest (e.g. a target cell expressing an antigen other than NKp46).

In one aspect of any of the embodiments described herein, the invention provides a recombinant nucleic acid encoding a first polypeptide chain, and/or a second polypeptide chain, and/or a third polypeptide chain and/or a fourth polypeptide. In one aspect of any of the embodiments described herein, the invention provides a recombinant host cell comprising a nucleic acid encoding a first polypeptide chain, and/or a second polypeptide chain and/or a third polypeptide chain, optionally wherein the host cell produces a multimeric or other protein according to the invention with a yield (final productivity or concentration before or after purification) of at least 1, 2, 3 or 4 mg/L. Also provided is a kit or set of nucleic acids comprising a recombinant nucleic acid encoding a first polypeptide chain of the according to the invention, a recombinant nucleic acid encoding a second polypeptide chain according to the invention, and, optionally, a recombinant nucleic acid encoding a third polypeptide chain according to the invention. Also provided are methods of making dimeric, trimeric and tetrameric proteins according to the invention.

Any of the methods can further be characterized as comprising any step described in the application, including notably in the "Detailed Description of the Invention"). The invention further relates to methods of identifying, testing and/or making proteins described herein. The invention further relates to a multispecific protein obtainable by any of present methods. The disclosure further relates to pharmaceutical or diagnostic formulations containing at least one of the multispecific proteins disclosed herein. The disclosure further relates to methods of using the subject multispecific proteins in methods of treatment or diagnosis.

These and additional advantageous aspects and features of the invention may be further described elsewhere herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows that Anti-CD19-F1-Anti-CD3 does not cause T/B cell aggregation in the presence of B221 (CD19) or JURKAT (CD3) cell lines when separate, but it does cause aggregation of cells when both B221 and JURKAT cells are co-incubated.

Figures 2A to 2F show different domain arrangements of bispecific anti-NKp46 proteins produced.

Figures 3A and **3B** respectively demonstrate that bispecific F1 and F2 format proteins having NKp46 binding region based on NKp46-1, NKp46-2, NKp46-3 or NKp46-4 are able to direct resting NK cells to their CD19-positive Daudi tumor target cells, while isotype control antibody did not lead to the elimination of the Daudi cells. Rituximab (RTX) served as the positive control of ADCC, where the maximal response obtained with RTX (at 10 µg/ml in this assay) was 21.6% specific lysis.

Figure 4A shows that bispecific antibodies having NKp46 and CD19 binding regions in an F2 format protein do not activate resting NK cells in the absence of target cells; by contrast full length anti-NKp46 antibodies as well as positive control alemtuzumab did activate NK cells. **Figure 4B** shows that bispecific anti-NKp46 x anti-CD19 antibodies (including each of the NKp46-1, NKp46-2, NKp46-3 or NKp46-4 binding domains) activated resting NK cells in presence of Daudi target cells, while full-length anti-CD19 showed at best only very low activation of NK cells and neither full-length anti-NKp46 antibodies nor alemtuzumab elicited a substantial increase in activation beyond what was observed in the presence of NK cells alone. **Figure 4C** shows that in the presence of CD19-negative HUT78 cells, none of the bispecific anti-NKp46 x anti-CD19 antibodies (including each of the NKp46-1, NKp46-2, NKp46-3 or NKp46-4 variable regions) activated NK cells. However, the full-length anti-NKp46 antibodies and alemtuzumab resulted in detectable activation of NK cells, i.e., at a similar level observed in presence of NK cells alone. Isotype control antibody did not induce activation.

Figures 5A and **5B** shows that at low effector:target ratios of 1:1 each of the tested bispecific anti-NKp46 x anti-CD19 antibodies activated NK cells in the presence of Daudi cells, and that bispecific anti-NKp46 x anti-CD19 antibodies were far more potent (better elicited lysis of target cells) than a control anti-CD19 antibody as well as a full-length human IgG1 ADCC inducing antibody.

Figures 6A and **6B** show that each NKp46 x CD19 bispecific protein (Format F3, F5 and F6) induced specific lysis of Daudi (Figure 6A) or B221 (Figure 6B) cells by human KHYG-1 CD16-negative hNKp46-positive NK cell line, while rituximab and human IgG1 isotype control (IC) antibodies did not. **Figure 6C** shows that a NKp46 x KIR3DL2 bispecific protein (Format F6) induced specific lysis of HUT78 tumor cells via NKp46 binding (without CD16 binding) comparably to a conventional IgG1 antibody with the same anti-KIR3DL2 variable regions.

Figure 7 shows a NKp46 x CD19 bispecific protein in F5 format whose Fc domain binds CD16 is far more potent in mediating Daudi target cell lysis than a full-length IgG1 anti-CD19 antibody or a F6 format bispecific protein. The figure also shows that a bispecific anti-CD19 in F6 format whose Fc domain does not bind CD16 was as potent in mediating NK cell

lysis of Daudi target cells as the full-length IgG1 anti-CD19 antibody, which is unexpected considering that the control IgG1 anti-CD19 antibody binds CD19 bivalently. At comparable levels of target cell lysis, CD19-F5-NKp46-3 was at least 1000 times more potent than the full-length anti-CD19 IgG1.

Figure 8 shows the results of cytotoxicity assays using fresh NK cells (Daudi cell in the right hand panel and HUT78 cells in the left hand panel); the CD19-F6-NKp46-3 whose Fc domain does not bind CD16 due to a N297 substitution has as mode of action NKp46 triggering when NK cells encounter the target cell, while the CD19-F5-NKp46-3 bispecific protein demonstrated a far higher potency in mediating cytotoxicity toward Daudi cells. Neither the F5 nor F6 proteins mediated any NK cell cytotoxicity towards HUT78 cells.

Figure 9 shows the results of flow cytometry staining of NK cell surface markers showed a strong upregulation of CD137 on the surface of NK cells by F5 proteins (Left-most panel: NK cells vs. Daudi; middle panel: NK cells vs. HUT78; right-most panel: NK cells alone). The full-length anti-CD19 IgG1 antibody that binds CD16 also showed CD137 upregulation, but to a far lesser extent than the CD19-F5-NKp46-3 protein. The CD19-F6-NKp46-3 which functions via NKp46 but not CD16 did not show any CD137 upregulation.

Figure 10 shows the results of cytotoxicity assays which compared the ability of the GA101-F5+-NKp46-1 bispecific protein to a comparison antibody (GA101) containing the same variable regions to lyse Daudi cells. The results therein show that the GA101-F5+-NKp46-1 bispecific protein possesses far higher potency (approximately 10-fold increase in EC₅₀) in mediating cytotoxicity toward Daudi cells than GA101.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used in the specification, "a" or "an" may mean one or more. As used in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one.

Where "comprising" is used, this can optionally be replaced by "consisting essentially of", or optionally by "consisting of".

As used herein, the term "antigen binding domain" or "ABD" refers to a domain comprising a three-dimensional structure capable of immunospecifically binding to an epitope. Thus, in one embodiment, said domain can comprise a hypervariable region, optionally a V_H and/or V_L domain of an antibody chain, optionally at least a V_H domain. In another embodiment, the binding domain may comprise at least one complementarity determining region (CDR) of an antibody chain. In another embodiment, the binding domain may comprise a polypeptide domain from a non-immunoglobulin scaffold.

The term "antibody" herein is used in the broadest sense and specifically includes full-length monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments and derivatives, so long as they exhibit the desired biological activity. Various techniques relevant to the production of antibodies are provided in, e.g., Harlow, et al., ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1988). An "antibody fragment" comprises a portion of a full-length antibody, e.g. antigen-binding or variable regions thereof. Examples of antibody fragments include Fab, Fab', F(ab)₂, F(ab')₂, F(ab)₃, Fv (typically the V_L and V_H domains of a single arm of an antibody), single-chain Fv (scFv), dsFv, Fd fragments (typically the V_H and CH1 domain), and dAb (typically a V_H domain) fragments; V_H, V_L, V_HH, and V-NAR domains; minibodies, diabodies, triabodies, tetrabodies, and kappa bodies (see, e.g., Ill et al., *Protein Eng* 1997;10: 949-57); camel IgG; IgNAR; and multispecific antibody fragments formed from antibody fragments, and one or more isolated CDRs or a functional paratope, where isolated CDRs or antigen-binding residues or polypeptides can be associated or linked together so as to form a functional antibody fragment. Various types of antibody fragments have been described or reviewed in, e.g., Holliger and Hudson, *Nat Biotechnol* 2005; 23, 1126-1136; WO2005040219, and published U.S. Patent Applications 20050238646 and 20020161201.

The term "antibody derivative", as used herein, comprises a full-length antibody or a fragment of an antibody, e.g. comprising at least antigen-binding or variable regions thereof, wherein one or more of the amino acids are chemically modified, e.g., by alkylation, PEGylation, acylation, ester formation or amide formation or the like. This includes, but is not limited to, PEGylated antibodies, cysteine-PEGylated antibodies, and variants thereof.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody that are responsible for antigen binding. The hypervariable region generally comprises amino acid residues from a "complementarity-determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light-chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy-chain variable domain; Kabat et al. 1991) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light-chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy-chain variable domain; Chothia and Lesk, *J. Mol. Biol* 1987;196:901-917). Typically, the numbering of amino acid residues in this region is performed by the method described in Kabat et al., supra. Phrases such as "Kabat position", "variable domain residue numbering as in Kabat" and "according to Kabat" herein refer to this numbering system for heavy chain variable domains or light chain variable domains. Using the Kabat numbering system, the actual linear amino acid sequence of a peptide may contain fewer or additional

amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of CDR H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

By "framework" or "FR" residues as used herein is meant the region of an antibody variable domain exclusive of those regions defined as CDRs. Each antibody variable domain framework can be further subdivided into the contiguous regions separated by the CDRs (FR1, FR2, FR3 and FR4).

By "constant region" as defined herein is meant an antibody-derived constant region that is encoded by one of the light or heavy chain immunoglobulin constant region genes. By "constant light chain" or "light chain constant region" as used herein is meant the region of an antibody encoded by the kappa (C_k) or lambda (C_λ) light chains. The constant light chain typically comprises a single domain, and as defined herein refers to positions 108-214 of C_k , or C_λ , wherein numbering is according to the EU index (Kabat et al., 1991, *Sequences of Proteins of Immunological Interest*, 5th Ed., United States Public Health Service, National Institutes of Health, Bethesda).

By "constant heavy chain" or "heavy chain constant region" as used herein is meant the region of an antibody encoded by the mu, delta, gamma, alpha, or epsilon genes to define the antibody's isotype as IgM, IgD, IgG, IgA, or IgE, respectively. For full length IgG antibodies, the constant heavy chain, as defined herein, refers to the N-terminus of the CH1 domain to the C-terminus of the CH3 domain, thus comprising positions 118-447, wherein numbering is according to the EU index.

By "Fab" or "Fab region" as used herein is meant the polypeptide that comprises the V_H , CH1, V_L , and C_L immunoglobulin domains. Fab may refer to this region in isolation, or this region in the context of a polypeptide, multispecific polypeptide or ABD, or any other embodiments as outlined herein.

By "single-chain Fv" or "scFv" as used herein are meant antibody fragments comprising the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. Methods for producing scFvs are well known in the art. For a review of methods for producing scFvs see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

By "Fv" or "Fv fragment" or "Fv region" as used herein is meant a polypeptide that comprises the V_L and V_H domains of a single antibody.

By "Fc" or "Fc region", as used herein is meant the polypeptide comprising the constant region of an antibody excluding the first constant region immunoglobulin domain.

Thus Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM, Fc may include the J chain. For IgG, Fc comprises immunoglobulin domains $C\gamma_2$ (CH2) and $C\gamma_3$ (CH3) and the hinge between $C\gamma_1$ and $C\gamma_2$. Although the boundaries of the Fc region may vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226, P230 or A231 to its carboxyl-terminus, wherein the numbering is according to the EU index. Fc may refer to this region in isolation, or this region in the context of an Fc polypeptide, as described below. By "Fc polypeptide" or "Fc-derived polypeptide" as used herein is meant a polypeptide that comprises all or part of an Fc region. Fc polypeptides herein include but are not limited to antibodies, Fc fusions and Fc fragments. Also, Fc regions according to the invention include variants containing at least one modification that alters (enhances or diminishes) an Fc associated effector function. Also, Fc regions according to the invention include chimeric Fc regions comprising different portions or domains of different Fc regions, e.g., derived from antibodies of different isotype or species.

By "variable region" as used herein is meant the region of an antibody that comprises one or more Ig domains substantially encoded by any of the V_L (including $V\kappa$ ($V\kappa$) and $V\lambda$) and/or V_H genes that make up the light chain (including κ and λ) and heavy chain immunoglobulin genetic loci respectively. A light or heavy chain variable region (V_L or V_H) consists of a "framework" or "FR" region interrupted by three hypervariable regions referred to as "complementarity determining regions" or "CDRs". The extent of the framework region and CDRs have been precisely defined, for example as in Kabat (see "Sequences of Proteins of Immunological Interest," E. Kabat et al., U.S. Department of Health and Human Services, (1983)), and as in Chothia. The framework regions of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs, which are primarily responsible for binding to an antigen.

The term "specifically binds to" means that an antibody or polypeptide can bind preferably in a competitive binding assay to the binding partner, e.g. NKp46, as assessed using either recombinant forms of the proteins, epitopes therein, or native proteins present on the surface of isolated target cells. Competitive binding assays and other methods for determining specific binding are further described below and are well known in the art.

When an antibody or polypeptide is said to “compete with” a particular monoclonal antibody (e.g. NKp46-1, -2, -4, -6 or -9 in the context of an anti-NKp46 mono- or bi-specific antibody), it means that the antibody or polypeptide competes with the monoclonal antibody in a binding assay using either recombinant target (e.g. NKp46) molecules or surface expressed target (e.g. NKp46) molecules. For example, if a test antibody reduces the binding of NKp46-1, -2, -4, -6 or -9 to a NKp46 polypeptide or NKp46-expressing cell in a binding assay, the antibody is said to “compete” respectively with NKp46-1, -2, -4, -6 or -9.

The term “affinity”, as used herein, means the strength of the binding of an antibody or polypeptide to an epitope. The affinity of an antibody is given by the dissociation constant K_D , defined as $[Ab] \times [Ag] / [Ab-Ag]$, where $[Ab-Ag]$ is the molar concentration of the antibody-antigen complex, $[Ab]$ is the molar concentration of the unbound antibody and $[Ag]$ is the molar concentration of the unbound antigen. The affinity constant K_A is defined by $1/K_D$. Preferred methods for determining the affinity of mAbs can be found in Harlow, et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988), Coligan et al., eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), and Muller, *Meth. Enzymol.* 92:589-601 (1983), which references are entirely incorporated herein by reference. One preferred and standard method well known in the art for determining the affinity of mAbs is the use of surface plasmon resonance (SPR) screening (such as by analysis with a BIAcore™ SPR analytical device).

Within the context of this invention a “determinant” designates a site of interaction or binding on a polypeptide.

The term “epitope” refers to an antigenic determinant, and is the area or region on an antigen to which an antibody or polypeptide binds. A protein epitope may comprise amino acid residues directly involved in the binding as well as amino acid residues which are effectively blocked by the specific antigen binding antibody or peptide, *i.e.*, amino acid residues within the “footprint” of the antibody. It is the simplest form or smallest structural area on a complex antigen molecule that can combine with e.g., an antibody or a receptor. Epitopes can be linear or conformational/structural. The term “linear epitope” is defined as an epitope composed of amino acid residues that are contiguous on the linear sequence of amino acids (primary structure). The term “conformational or structural epitope” is defined as an epitope composed of amino acid residues that are not all contiguous and thus represent separated parts of the linear sequence of amino acids that are brought into proximity to one another by folding of the molecule (secondary, tertiary and/or quaternary structures). A conformational epitope is dependent on the 3-dimensional structure. The term ‘conformational’ is therefore often used interchangeably with ‘structural’. Epitopes may be

identified by different methods known in the art including but not limited to alanine scanning, phage display, X-ray crystallography, array-based oligo-peptide scanning or pepscan analysis, site-directed mutagenesis, high throughput mutagenesis mapping, H/D-Ex Mass Spectroscopy, homology modeling, docking, hydrogen-deuterium exchange, among others. (See e.g., Tong et al., Methods and Protocols for prediction of immunogenic epitopes", *Briefings in Bioinformatics* 8(2):96-108; Gershoni, Jonathan M; Roitburd-Berman, Anna; Siman-Tov, Dror D; Tarnovitski Freund, Natalia; Weiss, Yael (2007). "Epitope Mapping". *BioDrugs* 21 (3): 145–56; and Flanagan, Nina (May 15, 2011); "Mapping Epitopes with H/D-Ex Mass Spec: ExSAR Expands Repertoire of Technology Platform Beyond Protein Characterization", *Genetic Engineering & Biotechnology News* 31 (10).

By "amino acid modification" herein is meant an amino acid substitution, insertion, and/or deletion in a polypeptide sequence. An example of amino acid modification herein is a substitution. By "amino acid modification" herein is meant an amino acid substitution, insertion, and/or deletion in a polypeptide sequence. By "amino acid substitution" or "substitution" herein is meant the replacement of an amino acid at a given position in a protein sequence with another amino acid. For example, the substitution Y50W refers to a variant of a parent polypeptide, in which the tyrosine at position 50 is replaced with tryptophan. A "variant" of a polypeptide refers to a polypeptide having an amino acid sequence that is substantially identical to a reference polypeptide, typically a native or "parent" polypeptide. The polypeptide variant may possess one or more amino acid substitutions, deletions, and/or insertions at certain positions within the native amino acid sequence.

"Conservative" amino acid substitutions are those in which an amino acid residue is replaced with an amino acid residue having a side chain with similar physicochemical properties. Families of amino acid residues having similar side chains are known in the art, and include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

The term "identity" or "identical", when used in a relationship between the sequences of two or more polypeptides, refers to the degree of sequence relatedness between polypeptides, as determined by the number of matches between strings of two or more amino acid residues. "Identity" measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular

mathematical model or computer program (i.e., "algorithms"). Identity of related polypeptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in *Computational Molecular Biology*, Lesk, A. M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D. W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part 1*, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., *SIAM J. Applied Math.* 48, 1073 (1988).

Preferred methods for determining identity are designed to give the largest match between the sequences tested. Methods of determining identity are described in publicly available computer programs. Preferred computer program methods for determining identity between two sequences include the GCG program package, including GAP (Devereux et al., *Nucl. Acid. Res.* 12, 387 (1984); Genetics Computer Group, University of Wisconsin, Madison, Wis.), BLASTP, BLASTN, and FASTA (Altschul et al., *J. Mol. Biol.* 215, 403-410 (1990)). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST Manual, Altschul et al. NCB/NLM/NIH Bethesda, Md. 20894; Altschul et al., supra). The well-known Smith Waterman algorithm may also be used to determine identity.

An "isolated" molecule is a molecule that is the predominant species in the composition wherein it is found with respect to the class of molecules to which it belongs (i.e., it makes up at least about 50% of the type of molecule in the composition and typically will make up at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more of the species of molecule, e.g., peptide, in the composition). Commonly, a composition of a polypeptide will exhibit 98%, 98%, or 99% homogeneity for polypeptides in the context of all present peptide species in the composition or at least with respect to substantially active peptide species in the context of proposed use.

In the context herein, "treatment" or "treating" refers to preventing, alleviating, managing, curing or reducing one or more symptoms or clinically relevant manifestations of a disease or disorder, unless contradicted by context. For example, "treatment" of a patient in whom no symptoms or clinically relevant manifestations of a disease or disorder have been identified is preventive or prophylactic therapy, whereas "treatment" of a patient in whom symptoms or clinically relevant manifestations of a disease or disorder have been identified generally does not constitute preventive or prophylactic therapy.

As used herein, the phrase "NK cells" refers to a sub-population of lymphocytes that is involved in non-conventional immunity. NK cells can be identified by virtue of certain

characteristics and biological properties, such as the expression of specific surface antigens including CD56 and/or NKp46 for human NK cells, the absence of the alpha/beta or gamma/delta TCR complex on the cell surface, the ability to bind to and kill cells that fail to express "self" MHC/HLA antigens by the activation of specific cytolytic machinery, the ability to kill tumor cells or other diseased cells that express a ligand for NK activating receptors, and the ability to release protein molecules called cytokines that stimulate or inhibit the immune response. Any of these characteristics and activities can be used to identify NK cells, using methods well known in the art. Any subpopulation of NK cells will also be encompassed by the term NK cells. Within the context herein "active" NK cells designate biologically active NK cells, including NK cells having the capacity of lysing target cells or enhancing the immune function of other cells. NK cells can be obtained by various techniques known in the art, such as isolation from blood samples, cytophoresis, tissue or cell collections, etc. Useful protocols for assays involving NK cells can be found in *Natural Killer Cells Protocols* (edited by Campbell KS and Colonna M). Humana Press. pp. 219-238 (2000).

The term "internalization", used interchangeably with "intracellular internalization", refers to the molecular, biochemical and cellular events associated with the process of translocating a molecule from the extracellular surface of a cell to the intracellular surface of a cell. The processes responsible for intracellular internalization of molecules are well-known and can involve, *inter alia*, the internalization of extracellular molecules (such as hormones, antibodies, and small organic molecules); membrane-associated molecules (such as cell-surface receptors); and complexes of membrane-associated molecules bound to extracellular molecules (for example, a ligand bound to a transmembrane receptor or an antibody bound to a membrane-associated molecule). Thus, "inducing and/or increasing internalization" refer to events wherein intracellular internalization is initiated and/or the rate and/or extent of intracellular internalization is increased.

As used herein, an agent that has "agonist" activity at NKp46 is an agent that can cause or increase "NKp46 signaling". "NKp46 signaling" refers to an ability of an NKp46 polypeptide to activate or transduce an intracellular signaling pathway. Changes in NKp46 signaling activity can be measured, for example, by assays designed to measure changes in NKp46 signaling pathways, e.g. by monitoring phosphorylation of signal transduction components, assays to measure the association of certain signal transduction components with other proteins or intracellular structures, or in the biochemical activity of components such as kinases, or assays designed to measure expression of reporter genes under control of NKp46-sensitive promoters and enhancers, or indirectly by a downstream effect mediated by the NKp46 polypeptide (e.g. activation of specific cytolytic machinery in NK cells). Reporter

genes can be naturally occurring genes (e.g. monitoring cytokine production) or they can be genes artificially introduced into a cell. Other genes can be placed under the control of such regulatory elements and thus serve to report the level of NKp46 signaling.

“NKp46” refers to a protein or polypeptide encoded by the *Ncr1* gene or by a cDNA prepared from such a gene. Any naturally occurring isoform, allele, ortholog or variant is encompassed by the term NKp46 polypeptide (e.g., an NKp46 polypeptide 90%, 95%, 98% or 99% identical to SEQ ID NO 1, or a contiguous sequence of at least 20, 30, 50, 100 or 200 amino acid residues thereof). The 304 amino acid residue sequence of human NKp46 (isoform a) is shown below:

10	MSSTLPALLC	VGLCLSQRIS	AQQQTLPKPF	IWAEPHFMVP	KEKQVTICCCQ
	GNYGAVEYQL	HFEGSLFAVD	RPKPPERINK	VKFYIPDMNS	RMAGQYSCIY
	RVGELWSEPS	NLLDLVVTEM	YDTPTLSVHP	GPEVISGEKV	TFYCRLDTAT
	SMFLLLKEGR	SSHVQRGY GK	VQAEFPLGPV	TTAHRGTYRC	FGSYNNHAWS
	FPSEPVKLLV	TGDIENTSLA	PEDPTFPADT	WGTYLLTTET	GLQKDHALWD
15	HTAQNLLRMG	LAFLVLVALV	WFLVEDWLSR	KRTRERASRA	STWEGRRRLN

TQTL (SEQ ID NO: 1).

SEQ ID NO: 1 corresponds to NCBI accession number NP_004820, the disclosure of which is incorporated herein by reference. The human NKp46 mRNA sequence is described in NCBI accession number NM_004829, the disclosure of which is incorporated herein by reference.

Producing polypeptides

The antigen binding domains used in the proteins described herein can be readily derived from any of a variety of immunoglobulin or non-immunoglobulin scaffolds, for example affibodies based on the Z-domain of staphylococcal protein A, engineered Kunitz domains, monobodies or adnectins based on the 10th extracellular domain of human fibronectin III, anticalins derived from lipocalins, DARPins (designed ankyrin repeat domains, multimerized LDLR-A module, avimers or cysteine-rich knottin peptides. See, e.g., Gebauer and Skerra (2009) *Current Opinion in Chemical Biology* 13:245–255, the disclosure of which is incorporated herein by reference.

Variable domains are commonly derived from antibodies (immunoglobulin chains), for example in the form of associated V_L and V_H domains found on two polypeptide chains, or a single chain antigen binding domain such as a scFv, a V_H domain, a V_L domain, a dAb, a V-NAR domain or a V_HH domain. In certain advantageous proteins formats disclosed herein that directly enable the use of a wide range of variable regions from Fab or scFv

without substantial further requirements for pairing and/or folding, the an antigen binding domain (e.g., ABD₁ and ABD₂) can also be readily derived from antibodies as a Fab or scFv.

Typically, antibodies are initially obtained by immunization of a non-human animal, e.g., a mouse, rat, guinea pig or rabbit, with an immunogen comprising a polypeptide, or a fragment or derivative thereof, typically an immunogenic fragment, for which it is desired to obtain antibodies (e.g. a human polypeptide). The step of immunizing a non-human mammal with an antigen may be carried out in any manner well known in the art for stimulating the production of antibodies in a mouse (see, for example, E. Harlow and D. Lane, *Antibodies: A Laboratory Manual.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988), the entire disclosure of which is herein incorporated by reference). Human antibodies may also be produced by using, for immunization, transgenic animals that have been engineered to express a human antibody repertoire (Jakobovitz et *Nature* 362 (1993) 255), or by selection of antibody repertoires using phage display methods. For example, a XenoMouse (Abgenix, Fremont, CA) can be used for immunization. A XenoMouse is a murine host that has had its immunoglobulin genes replaced by functional human immunoglobulin genes. Thus, antibodies produced by this mouse or in hybridomas made from the B cells of this mouse, are already humanized. The XenoMouse is described in United States Patent No. 6,162,963, which is herein incorporated in its entirety by reference. Antibodies may also be produced by selection of combinatorial libraries of immunoglobulins, as disclosed for instance in (Ward et al. *Nature*, 341 (1989) p. 544, the entire disclosure of which is herein incorporated by reference). Phage display technology (McCafferty et al (1990) *Nature* 348:552-553) can be used to produce antibodies from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. See, e.g., Griffith et al (1993) *EMBO J.* 12:725-734; US 5,565,332; US 5,573,905; US 5,567,610; and US 5,229,275). When combinatorial libraries comprise variable (V) domain gene repertoires of human origin, selection from combinatorial libraries will yield human antibodies.

Additionally, a wide range of antibodies are available in the scientific and patent literature, including DNA and/or amino acid sequences, or from commercial suppliers. Antibodies will typically be directed to a pre-determined antigen. Examples of antibodies include antibodies that recognize an antigen expressed by a target cell that is to be eliminated, for example a proliferating cell or a cell contributing to a disease pathology. Examples include antibodies that recognize tumor antigens, microbial (e.g. bacterial or parasite) antigens or viral antigens.

Antigen binding domains that bind NKp46 can be derived from the anti-NKp46 antibodies provided herein (see section "CDR Sequences"). Variable regions can be used directly, or can be modified by selecting hypervariable or CDR regions from the NKp46

antibodies and placing them into an appropriate V_L or V_H framework, for example human frameworks. Antigen binding domains that bind NKp46 can also be derived de novo using methods for generating antibodies. Antibodies can be tested for binding to NKp46 polypeptides. In one aspect of any embodiment herein, a polypeptide (e.g. multispecific polypeptide, bispecific or monospecific antibody) that binds to NKp46 will be capable of binding NKp46 expressed on the surface of a cell, e.g. native NKp46 expressed by a NK cell.

Antigen binding domains (ABDs) that bind antigens of interest can be selected based on the desired antigen of interest (e.g. an antigen other than NKp46), and may include for example cancer antigens such as antigens present on tumor cells and/or on immune cells capable of mediating a pro-tumoral effect, e.g. a monocyte or a macrophage, optionally a suppressor T cell, regulatory T cell, or myeloid-derived suppressor cell (for the treatment of cancer); bacterial or viral antigens (for the treatment of infectious disease); or antigens present on pro-inflammatory immune cells, e.g. T cells, neutrophils, macrophages, etc. (for the treatment of inflammatory and/or autoimmune disorder). As used herein, the term "bacterial antigen" includes, but is not limited to, intact, attenuated or killed bacteria, any structural or functional bacterial protein or carbohydrate, or any peptide portion of a bacterial protein of sufficient length (typically about 8 amino acids or longer) to be antigenic. Examples include gram-positive bacterial antigens and gram-negative bacterial antigens. In some embodiments the bacterial antigen is derived from a bacterium selected from the group consisting of *Helicobacter* species, in particular *Helicobacter pylori*; *Borrelia* species, in particular *Borrelia burgdorferi*; *Legionella* species, in particular *Legionella pneumophila*; *Mycobacteria* s species, in particular *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*; *Staphylococcus* species, in particular *Staphylococcus aureus*; *Neisseria* species, in particular *N. gonorrhoeae*, *N. meningitidis*; *Listeria* species, in particular *Listeria monocytogenes*; *Streptococcus* species, in particular *S. pyogenes*, *S. agalactiae*; *S. faecalis*; *S. bovis*, *S. pneumoniae*; anaerobic *Streptococcus* species; pathogenic *Campylobacter* species; *Enterococcus* species; *Haemophilus* species, in particular *Haemophilus influenzae*; *Bacillus* species, in particular *Bacillus anthracis*; *Corynebacterium* species, in particular *Corynebacterium diphtheriae*; *Erysipelothrix* species, in particular *Erysipelothrix rhusiopathiae*; *Clostridium* species, in particular *C. perfringens*, *C. tetani*; *Enterobacter* species, in particular *Enterobacter aerogenes*, *Klebsiella* species, in particular *Klebsiella 1S pneumoniae*, *Pasteurella* species, in particular *Pasteurella multocida*, *Bacteroides* species; *Fusobacterium* species, in particular *Fusobacterium nucleatum*; *Streptobacillus* species, in particular *Streptobacillus moniliformis*; *Treponema* species, in

particular *Treponema pertenu*; *Leptospira*; pathogenic *Escherichia* species; and *Actinomyces* species, in particular *Actinomyces israeli*.

As used herein, the term "viral antigen" includes, but is not limited to, intact, attenuated or killed whole virus, any structural or functional viral protein, or any peptide portion of a viral protein of sufficient length (typically about 8 amino acids or longer) to be antigenic. Sources of a viral antigen include, but are not limited to viruses from the families: Retroviridae (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g., polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Caliciviridae (e.g., strains that cause gastroenteritis); Togaviridae (e.g., equine encephalitis viruses, rubella viruses); Flaviviridae (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g., coronaviruses); Rhabdoviridae (e.g., vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g., Ebola viruses); Paramyxoviridae (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g., influenza viruses); Bunyaviridae (e.g., Hantaan viruses, bunya viruses, phleboviruses and Nairo viruses); Arenaviridae (hemorrhagic fever viruses); Reoviridae (e.g., reoviruses, orbiviruses and rotaviruses); Bornaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g., African swine fever virus); and unclassified viruses (e.g., the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), Hepatitis C; Norwalk and related viruses, and astroviruses). Alternatively, a viral antigen may be produced recombinantly.

As used herein, the terms "cancer antigen" and "tumor antigen" are used interchangeably and refer to antigens (other than NKp46 or CD16) that are differentially expressed by cancer cells or are expressed by non-tumoral cells (e.g. immune cells) having a pro-tumoral effect (e.g. an immunosuppressive effect), and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, or expressed at lower levels or less frequently, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated ras oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting

from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses. Still other cancer antigens can be expressed on immune cells capable of contributing to or mediating a pro-tumoral effect, e.g. cell that contributes to immune evasion, a monocyte or a macrophage, optionally a suppressor T cell, regulatory T cell, or myeloid-derived suppressor cell.

The cancer antigens are usually normal cell surface antigens which are either over-expressed or expressed at abnormal times, or are expressed by a targeted population of cells. Ideally the target antigen is expressed only on proliferative cells (e.g., tumor cells) or pro-tumoral cells (e.g. immune cells having an immunosuppressive effect), however this is rarely observed in practice. As a result, target antigens are in many cases selected on the basis of differential expression between proliferative/disease tissue and healthy tissue. Example of cancer antigens include: Receptor Tyrosine Kinase-like Orphan Receptor 1 (ROR1), Crypto, CD4, CD20, CD30, CD19, CD38, CD47, Glycoprotein NMB, CanAg, Her2 (ErbB2/Neu), a Siglec family member, for example CD22 (Siglec2) or CD33 (Siglec3), CD79, CD138, CD171, PSCA, L1-CAM, PSMA (prostate specific membrane antigen), BCMA, CD52, CD56, CD80, CD70, E-selectin, EphB2, Melanotransferrin, Mud 6 and TMEFF2. Examples of cancer antigens also include Immunoglobulin superfamily (IgSF) such as cytokine receptors, Killer-Ig Like Receptor, CD28 family proteins, for example, Killer-Ig Like Receptor 3DL2 (KIR3DL2), B7-H3, B7-H4, B7-H6, PD-L1, IL-6 receptor. Examples also include MAGE, MART-1/Melan-A, gp100, major histocompatibility complex class I-related chain A and B polypeptides (MICA and MICB), adenosine deaminase-binding protein (ADAbp), cyclophilin b, colorectal associated antigen (CRC)-C017-1A/GA733, protein tyrosine kinase 7(PTK7), receptor protein tyrosine kinase 3 (TYRO-3), nectins (e.g. nectin-4), major histocompatibility complex class I-related chain A and B polypeptides (MICA and MICB), proteins of the UL16-binding protein (ULBP) family, proteins of the retinoic acid early transcript-1 (RAET1) family, carcinoembryonic antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, prostate specific antigen (PSA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens, GAGE-family of tumor antigens, anti-Müllerian hormone Type II receptor, delta-like ligand 4 (DLL4), DR5, ROR1 (also known as Receptor Tyrosine Kinase-Like Orphan Receptor 1 or NTRKR1 (EC 2.7.10.1), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, MUC family, VEGF, VEGF receptors, Angiopoietin-2, PDGF, TGF- α , EGF, EGF receptor, members of the human EGF-like receptor family, e.g., HER-2/neu, HER-3, HER-4 or a heterodimeric receptor comprised of at least one HER subunit, gastrin releasing peptide receptor antigen, Muc-1, CA125, integrin receptors, $\alpha\text{v}\beta\text{3}$ integrins, $\alpha\text{5}\beta\text{1}$ integrins, $\alpha\text{IIb}\beta\text{3}$ -integrins, PDGF beta receptor, SVE-cadherin, IL-8 receptor, hCG, IL-

6 receptor, CSF1R (tumor-associated monocytes and macrophages), α -fetoprotein, E-cadherin, α -catenin, β -catenin and γ -catenin, p120ctn, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotypic, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papillomavirus proteins, imp-1, P1A, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7, and c-erbB-2, although this is not intended to be exhaustive. In one aspect, the antigen of interest is an antigen (e.g. any one of the antigens listed above) capable of undergoing intracellular internalization, for example when bound by a conventional human IgG1 antibody, either in the presence or absence of Fc γ receptor cells. In one aspect, the antigen of interest is a CD19 or CD20 polypeptide; in one aspect, the multispecific protein comprises a V_H and/or V_L, or a scFv or another ABD that binds CD19 or CD20 comprising an amino acid sequence which is at least 60%, 70%, 80%, 85%, 90% or 95% identical to the sequence of the anti-CD19 or anti-CD20 respective V_H, V_L or scFv described in the Examples herein, or comprises the heavy and light chain CDR1, -2 and -3 of the anti-CD19 or anti-CD20 heavy and light chain variable regions disclosed herein. In one aspect, the multispecific protein competes for binding to a human CD19 or CD20 polypeptide with an antibody, or a F5 or T6 protein, comprising the respective anti-CD19 or anti-CD20 V_H, V_L or scFv disclosed in the Examples.

In one embodiment, the ABD that binds an antigen of interest is derived from (e.g. comprises the hypervariable region of, or comprises one, two, three, four, five or six of the CDRs of) a parental antibody that binds an antigen of interest (e.g. a murine antibody, a human antibody) which, when bound to its antigenic target (the antigen of interest on cells), increases or induces down-modulation or intracellular internalization of the antigen of interest. In one embodiment, the antigen of interest is a cancer antigen, e.g. one of the cancer antigens listed above known to internalize (e.g. Immunoglobulin superfamily (IgSF) members, for example cytokine receptor α or β chains, Killer-Ig Like Receptors, CD28 family proteins, B7-H3, B7-H4, B7-H6, KIR3DL2, PTK7, ROR1, L1-CAM, Siglec family members, EGF receptor and EGF-like receptor family members, EGFR, HER-2, integrins, anti-Müllerian hormone Type II receptor, CSF-1R, and others) In one embodiment, the antigen target is a polypeptide present on an immune cell capable of mediating a pro-tumoral effect, e.g. a monocyte or a macrophage, optionally a suppressor T cell, regulatory T cell, or myeloid-derived suppressor cell.

In one embodiment, the ABD binds to a cancer antigen, a viral antigen, a microbial antigen, or an antigen present on an infected cell (e.g. virally infected) or on a pro-inflammatory immune cell. In one embodiment, said antigen is a polypeptide selectively

expressed or overexpressed on a tumor cell, and infected cell or a pro-inflammatory cell. In one embodiment, said antigen is a polypeptide that when inhibited, decreases the proliferation and/or survival of a tumor cell, an infected cell or a pro-inflammatory cell.

The ABDs which are incorporated into the polypeptides can be tested for any desired activity prior to inclusion in a multispecific NKp46-binding protein, for example the ABD can be tested for binding to an antigen of interest.

An ABD derived from an antibody will generally comprise at minimum a hypervariable region sufficient to confer binding activity. It will be appreciated that an ABD may comprise other amino acids or functional domains as may be desired, including but not limited to linker elements (e.g. linker peptides, CH1, C κ or C λ domains, hinges, or fragments thereof). In one example an ABD comprises a scFv, a V_H domain and a V_L domain, or a single domain antibody (nanobody or dAb) such as a V-NAR domain or a V_HH domain. Exemplary antibody formats are further described herein and an ABD can be selected based on the desired format.

In any embodiment, an antigen binding domain can be obtained from a humanized antibody in which residues from a complementary-determining region (CDR) of a human antibody are replaced by residues from a CDR of the original antibody (the parent or donor antibody, e.g. a murine or rat antibody) while maintaining the desired specificity, affinity, and capacity of the original antibody. The CDRs of the parent antibody, some or all of which are encoded by nucleic acids originating in a non-human organism, are grafted in whole or in part into the beta-sheet framework of a human antibody variable region to create an antibody, the specificity of which is determined by the engrafted CDRs. The creation of such antibodies is described in, e.g., WO 92/11018, Jones, 1986, *Nature* 321:522-525, Verhoeyen et al., 1988, *Science* 239:1534-1536. An antigen binding domain can thus have non-human hypervariable regions or CDRs and human frameworks region sequences (optionally with back mutations).

Once appropriate antigen binding domains having desired specificity and/or activity are identified, DNA encoding each of the or ABD can be separately placed, in suitable arrangements, in an appropriate expression vector, together with DNA encoding any elements such as CH1, C κ , CH2 and CH3 domains or portions thereof and any other optional elements (e.g. DNA encoding a hinge-derived or linker elements) for transfection into an appropriate host. ABDs will be arranged in an expression vector, or in separate vectors as a function of which type of polypeptide is to be produced, so as to produce the Fc-polypeptides having the desired domains operably linked to one another. The host is then used for the recombinant production of the multispecific polypeptide.

For example, a polypeptide fusion product can be produced from a vector in which the first of the two ABD is operably linked (e.g. directly, or via a CH1, C κ or C λ constant region and/or hinge region) to the N-terminus of a CH2 domain, and the CH2 domain is operably linked at its C-terminus to the N-terminus a CH3 domain. The second of the two ABD can be on a
5 second polypeptide chain that forms a dimer, e.g. heterodimer, with the polypeptide comprising the first ABD. The polypeptide may comprise a full length and/or dimeric Fc domain.

The multispecific polypeptide can then be produced in an appropriate host cell or by any suitable synthetic process. A host cell chosen for expression of the multispecific polypeptide
10 is an important contributor to the final composition, including, without limitation, the variation in composition of the oligosaccharide moieties decorating the protein in the immunoglobulin CH2 domain. Thus, one aspect of the invention involves the selection of appropriate host cells for use and/or development of a production cell expressing the desired therapeutic protein such that the multispecific polypeptide retains FcRn and CD16 binding. The host cell
15 may be of mammalian origin or may be selected from COS-1, COS-7, HEK293, BHK21, CHO, BSC-1, Hep G2, 653, SP2/0, 293, HeLa, myeloma, lymphoma, yeast, insect or plant cells, or any derivative, immortalized or transformed cell thereof. The host cell may be any suitable species or organism capable of producing N-linked glycosylated polypeptides, e.g. a mammalian host cell capable of producing human or rodent IgG type N-linked glycosylation.

20 Multimeric bispecific proteins such as heterodimers, heterotrimers and tetramers (the latter including for example bispecific antibodies with two heavy chains and two light chains) can be produced according to a variety of formats. The multimeric polypeptide will generally comprise a dimeric Fc domain that is capable of binding to human CD16 or CD16A and optionally other Fc γ receptors, e.g., CD16B, CD32A, CD32B and/or CD64). Fc moieties with
25 substantial FcRn and CD16 (CD16A) binding can be obtained through the use of suitable CH2 and/or CH3 domains, as further described herein. In one embodiment, an Fc moiety is derived from a human IgG1 isotype constant region. In one embodiment, an Fc moiety may be obtained by production of the polypeptide in a host cell or by a process that yields N297-linked glycosylation, e.g. a mammalian cell. In one embodiment, an Fc moiety comprises
30 one or more amino acid modifications, e.g. in the CH2 domain, that increases binding to CD16 or CD16A.

In one example, the protein comprises a first and a second polypeptide chain each comprising a variable domain fused to a human Fc domain (comprising a CH3 domain capable of undergoing preferential CH3-CH3 hetero-dimerization), wherein the first and
35 second chain associate via CH3-CH3 dimerization and the protein comprises a dimeric Fc

domain. The variable domains of each chain can be part of the same or different antigen binding domains.

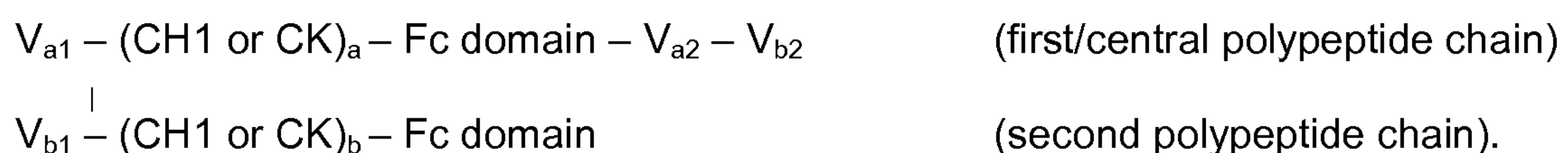
One advantageous way of making multimeric polypeptides is through the assembly of different polypeptide chains that each comprise at least one heavy or light chain variable domain fused to a human CH1 or C κ constant domain (a V-(CH1/C κ) unit), wherein the protein chains undergo CH1-C κ dimerization and are bound to one another by non-covalent bonds and optionally further disulfide bonds formed between respective CH1 and C κ domains. In one embodiment, the invention provides an isolated or purified heterodimeric or heterotrimeric protein that binds to a first and second antigen, wherein the protein comprises at least two or three polypeptide chains, each comprising a V-(CH1/C κ) unit, whereby the chains are bound to one another by non-covalent bonds and optionally further bound via disulfide bonds between CH1 and C κ domains, and still further optionally, whereby the chains are bound by non-covalent bonds between the respective variable regions, CH1 and C κ domains, and CH3 domains of the Fc portion.

In one example, the protein comprises a first and a second polypeptide chain each comprising a variable domain fused to a CH1 or C κ domain (a V-(CH1/C κ) unit), in turn fused at its C-terminus to a human Fc domain (comprising a CH3 domain capable of undergoing CH3-CH3 dimerization), wherein the first and second chain associate via CH1-C κ and CH3-CH3 dimerization and the protein comprises a dimeric Fc domain. The variable domains of each chain can be part of the same or different antigen binding domains.

The variable and constant regions can be selected and configured such that each chain will preferentially associate with its desired complementary partner chain. The resulting multimeric protein will therefore be simple to produce using conventional production methods using recombinant host cells. The choice of which V_H or V_L to associate with a CH1 and C κ in a unit is based on affinity between the units to be paired so as to drive the formation of the desired multimer. The resulting multimer will be bound by non-covalent bonds between complementary V_H and V_L domains, by non-covalent bonds between complementary CH1 and C κ domains, and optionally by further disulfide bonding between complementary CH1 and C κ domains (and optionally further disulfide bonds between complementary hinge domains). V_H-V_L associations are stronger than V_H-V_H or V_L-V_L, consequently, as shown herein, one can place a V_H or a V_L next to either a CH1 or a C κ , and the resulting V-C unit will partner preferably with its V-C counterpart. For example V_H-C κ will pair with V_L-CH1 preferentially over V_H-CH1. Additionally, by including an Fc domain, preferred chain pairing is further improved, as the two Fc-containing chains are bound by non-covalent bonds

between CH3 domains of the Fc domains. The different V-C combinations, optionally further combined with Fc pairing thereby provides tools to make heteromultimeric proteins.

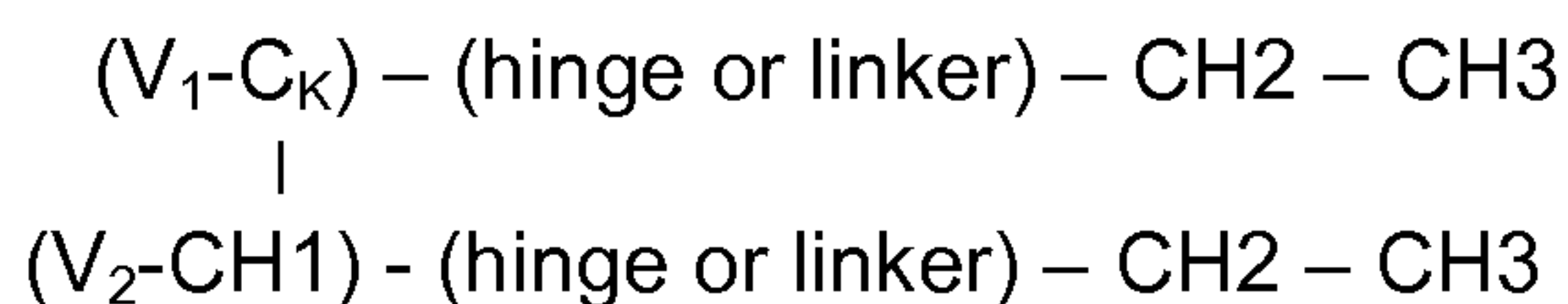
In one example, the multispecific protein comprises a first and a second polypeptide chain each comprising a variable domain fused to a CH1 or C κ domain (a V-(CH1/C κ) unit), in turn fused at its C-terminus to a human Fc domain, wherein the V-(CH1/C κ) unit of the first chain has undergone CH1-C κ dimerization with the V-(CH1/C κ) unit of the second chain thereby forming a first antigen binding domain (ABD₁) and a dimeric Fc domain, wherein one of the polypeptide chains further comprises an antigen binding domain that forms a second antigen binding domain (ABD₂), and wherein the Fc domain binds to a human CD16 polypeptide. In one embodiment, the Fc domain comprises N-linked glycosylation at residue N297 (Kabat EU numbering). In one example, the protein has a domain arrangement:



In one example, the protein comprises three polypeptide chains, each comprising a variable domain fused to a CH1 or C κ domain (a V-(CH1/C κ) unit), wherein a first (central) chain comprises two V-(CH1/C κ) units and a human Fc domain interposed between the units, the second chain comprises one V-(CH1/C κ) unit and a human Fc domain, and the third chain comprises one V-(CH1/C κ) unit, wherein one of the V-(CH1/C κ) units of the central chain has undergone CH1-C κ dimerization with the V-(CH1/C κ) unit of the second chain thereby forming a first antigen binding domain (ABD₁) and a dimeric Fc domain, and wherein the other of the V-(CH1/C κ) units of the central chain has undergone CH1-C κ dimerization with the V-(CH1/C κ) unit of the third chain thereby forming a second antigen binding domain (ABD₂), and wherein the Fc domain binds to a human CD16 polypeptide. In one embodiment, the Fc domain comprises N-linked glycosylation at residue N297 (Kabat EU numbering). In one example, the protein has a domain arrangement:

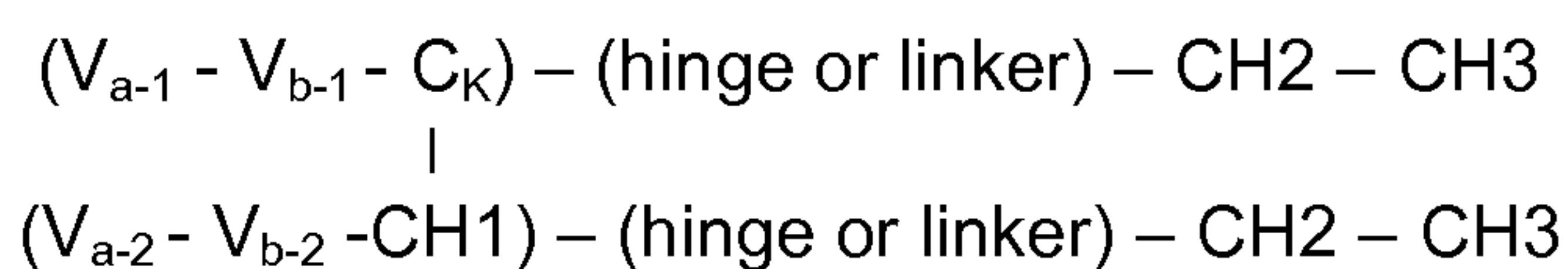


In certain formats, heterodimers are formed in which variable domains adjacent to the CH1 or C κ domain do not require association with the second chain to form an antigen binding domain. For example, through use of single variable domains, or scFv, each chain will contain a functional ABD. Examples based CH1-C κ dimerization with single variable exemplary heterodimer molecules can have a domain arrangement:



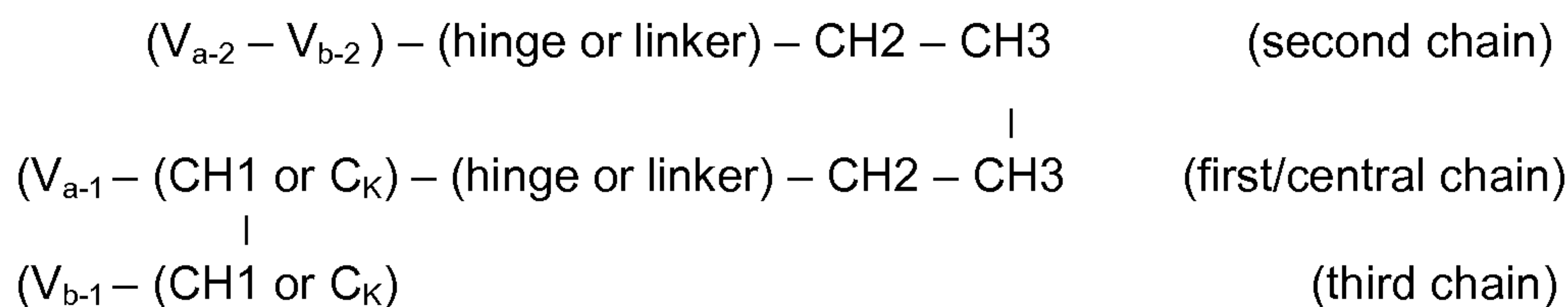
wherein V_1 and V_2 are single variable domains (e.g. V_H domain, a V_L domain, a dAb, a V-NAR domain or a V_{HH} domain), and one of V_1 and V_2 binds NKp46 and the other binds an antigen of interest.

In one embodiment, exemplary heterodimer molecules can have a domain arrangement:



wherein V_{a-1} , V_{b-1} , V_{a-2} and V_{b-2} are each a V_H domain or a V_L domain, and wherein one of V_{a-1} and V_{b-1} is a V_H and the other is a V_L such that V_{a-1} and V_{b-1} form a first antigen binding domain (ABD), wherein one of V_{a-2} and V_{b-2} is a V_H and the other is a V_L such that V_{a-2} and V_{b-2} form a second antigen binding domain, wherein one of the ABD binds NKp46 and the other binds an antigen of interest. In one variant of the foregoing, any of, or each of the V_{a-1} , V_{b-1} , V_{a-2} and V_{b-2} are a scFv (made up of two variable domains). Each pair of V domains can be separated by a linker peptide (e.g. to form a scFv).

In similar approaches, trimers can be constructed. Exemplary heterotrimer molecules can have the following domain arrangement:



wherein the first/central chain and the second chain associate by CH3-CH3 dimerization and the first/central chain and the third chain associate by the CH1 or Ck dimerization, wherein the domains of the first/central chain and the third chain are selected to be complementary to permit the first and third chains to associate by CH1-Ckdimerization, and wherein V_{a-1} , V_{b-1} , V_{a-2} and V_{b-2} are each a V_H domain or a V_L domain, and wherein one of V_{a-1} and V_{b-1} is a V_H and the other is a V_L such that V_{a-1} and V_{b-1} form a first antigen binding domain (ABD), wherein one of V_{a-2} and V_{b-2} is a V_H and the other is a V_L such that V_{a-2} and V_{b-2} form a second antigen binding domain (e.g. an scFv wherein V_{a-2} and V_{b-2} are separated by a linker), wherein one of the ABD binds NKp46 and the other binds an antigen of interest.

In one embodiment, multimeric proteins are constructed based upon two Fc-containing chains (e.g. chains 1 and 2) to create a dimer via CH3-CH3 dimerization and/or hinge dimerization, and a further chain (e.g. chain 3) comprising a V- C_H /Ck unit that

dimerizes with one of chains 1 or 2. Exemplary molecules can have the following domain arrangement:

$V_{a-1} - V_{b-1} - (C_K \text{ or } CH1) - (\text{hinge or linker}) - CH2 - CH3$ Chain 1

5 $V_{a-2} - (C_K \text{ or } CH1) - (\text{hinge or linker}) - CH2 - CH3$ Chain 2

$V_{b-2} - (C_K \text{ or } CH1)$ Chain 3

10 wherein V_{a-1} , V_{b-1} , V_{a-2} and V_{b-2} are each a V_H domain or a V_L domain, and wherein one of V_{a-1} and V_{b-1} is a V_H and the other is a V_L such that V_{a-1} and V_{b-1} form a first antigen binding domain (ABD), wherein one of V_{a-2} and V_{b-2} is a V_H and the other is a V_L such that V_{a-2} and V_{b-2} form a second antigen binding domain.

Exemplary molecules may possess the following domain arrangement:

$V_{a-1} - V_{b-1} - (CH1) - (\text{hinge or linker}) - CH2 - CH3$ Chain 1

15 $V_{a-2} - (CH1) - (\text{hinge or linker}) - CH2 - CH3$ Chain 2

$V_{b-2} - (C_K)$ Chain 3

20 wherein V_{a-1} , V_{b-1} , V_{a-2} and V_{b-2} are each a V_H domain or a V_L domain, and wherein one of V_{a-1} and V_{b-1} is a V_H and the other is a V_L such that V_{a-1} and V_{b-1} form a first antigen binding domain (ABD), wherein one of V_{a-2} and V_{b-2} is a V_H and the other is a V_L such that V_{a-2} and V_{b-2} form a second antigen binding domain. The CH1 and C_K are selected such that chain 1 is capable of associating with chain 2 and chain 2 with chain 3. The protein can be configured such that chains 1 and 2 associate via CH3-CH3 dimerization and chains 2 and 3 associate via CH1- C_K dimerization.

25 Optionally, any of the multispecific proteins of the invention may include, CH3 domains which comprise amino acid substitutions, wherein the CH3 domain interface of the antibody Fc region is mutated to create altered charge polarity across the Fc dimer interface such that co-expression of electrostatically matched Fc chains supports favorable attractive interactions thereby promoting desired Fc heterodimer formation, whereas unfavorable repulsive charge interactions suppress unwanted Fc homodimer formation.

30 Heterodimeric or heterotrimeric polypeptides with two ABDs and a dimeric Fc domain can optionally be produced as one or more chains that each associate with a central chain, e.g. by CH1- C_K heterodimerization. Such multimers may be composed of a central (first) polypeptide chain comprising two immunoglobulin variable domains that are part of separate antigen binding domains (of different antigen specificities), the one or more other chains that provide the additional and/or complementary variable domains, and an Fc domain placed on the central and/or one or more other chains. In one embodiment, the Fc domain is interposed between the two ABDs in the multimeric protein.

In one example, the first (central) polypeptide chain will provide one variable domain that will, together with a complementary variable domain on a second polypeptide chain, form an antigen binding domain specific for one (e.g. a first) antigen of interest. The first (central) polypeptide chain will also provide a second variable domain (e.g., placed on the opposite end of the interposed Fc domain) that will be paired with a complementary variable domain to form an antigen binding domain specific for another (e.g. a second) antigen of interest; the variable domain that is complementary to the second variable domain can be placed on the central polypeptide (e.g. adjacent to the second variable domain in a tandem variable domain construct such as an scFv), or can be placed on a separate polypeptide chain, notably a third polypeptide chain. The second (and third, if present) polypeptide chains will associate with the central polypeptide chain by CH1-C κ heterodimerization, forming non-covalent bonds and optionally further interchain disulfide bonds between complementary CH1 and C κ domains (and optionally interchain disulfide bonds between hinge regions), with a primary multimeric polypeptide being formed so long as CH/CK and V_H/V_K domains are chosen to give rise to a preferred dimerization configuration that results preferentially in the desired V_H-V_L pairings. Remaining unwanted pairings can remain minimal during production and/or are removed during purification steps. In a trimer, or when polypeptides are constructed for preparation of a trimer, there will generally be one polypeptide chain that comprises a non-naturally occurring V_H-C κ or V_K-CH1 domain arrangement.

Examples of the domain arrangements (N- to C-termini) of central polypeptide chains for use in such heterodimeric proteins include any of the following:

V – (CH1 or C κ) – Fc domain – V – V;

and

V – V – (CH1 or C κ) – Fc domain;

and

Fc domain – V – V;

and

V – V – Fc domain;

and

V – V – Fc domain – V – (CH1 or C κ).

For example, the domain arrangements (N- to C-termini) of central polypeptide chains for use in such heterodimeric proteins can include:

V_{a-1} – (CH1 or C κ)_a – Fc domain – V_{a-2} – V_{b-2};

and

V_{a-2} – V_{b-2} – Fc domain – V_{a-1} – (CH1 or C κ)_a

wherein V_{a-1} is a light chain or heavy chain variable domain, and wherein one of V_{a-2} and V_{b-2} is a light chain variable domain and the other is a heavy chain variable domain.

Further examples include:

$V_{a-1} - (CH1 \text{ or } CK)_a - Fc \text{ domain} - V_b$;

5 and

$V_b - Fc \text{ domain} - V_{a-1} - (CH1 \text{ or } CK)_a$

wherein V_b is a single variable domain (e.g. dAb, VhH).

The Fc domain of the central chain may be a full Fc domain (CH2-CH3) or a portion thereof sufficient to confer the desired functionality (e.g. CD16 and optionally FcRn or another Fc receptor binding) when it forms a dimeric Fc with a second chain. A second polypeptide chain will then be configured which will comprise an immunoglobulin variable domain and a CH1 or Ck constant region, e.g., a $(CH1 \text{ or } Ck)_b$ unit, selected so as to permit CH1-Ck heterodimerization with the central polypeptide chain; the immunoglobulin variable domain will be selected so as to complement the variable domain of the central chain that is adjacent to the CH1 or Ck domain, whereby the complementary variable domains form an antigen binding domain for a first antigen of interest.

For example, a second polypeptide chain can comprise a domain arrangement:

$V_{b-1} - (CH1 \text{ or } Ck)_b - Fc \text{ domain}$

such that the $(CH1 \text{ or } Ck)_2$ dimerizes with the $(CH1 \text{ or } Ck)_1$ on the central chain, and the V_{b-1} forms an antigen binding domain together with V_{a-1} of the central chain. If the V_{a-1} of the central chain is a light chain variable domain, then V_{b-1} will be a heavy chain variable domain; and if V_{a-1} of the central chain is a heavy chain variable domain, then V_{b-1} will be a light chain variable domain.

The antigen binding domain for the second antigen of interest can then be formed from V_{a-2} and V_{b-2} which are configured as tandem variable domains on the central chain forming the antigen binding domain for the second antigen of interest (e.g. a heavy chain variable domain (V_H) and a light chain (κ) variable domain (V_K), for example forming an scFv unit). The antigen binding domain for the second antigen of interest can also alternatively be formed from a single variable domain V_b present on the central chain.

The resulting heterodimer can, for example, have the following configuration (see further Examples of such proteins shown as formats 13 and 14 shown in **Figure 2D** and **2E**):

$V_{a-1} - (CH1 \text{ or } Ck)_a - Fc \text{ domain} - V_{a-2} - V_{b-2}$ (first/central polypeptide chain)

$V_{b-1} - (CH1 \text{ or } Ck)_b - Fc \text{ domain}$ (second polypeptide chain)

wherein one of V_{a-1} of the first polypeptide chain and V_{b-1} of the second polypeptide chain is a light chain variable domain and the other is a heavy chain variable domain, and wherein

one of V_{a-2} and V_{b-2} is a light chain variable domain and the other is a heavy chain variable domain.

In one embodiment, the heterodimeric bispecific Fc-derived polypeptide comprises a domain arrangement selected from one of the following, optionally wherein one or both of the hinge domains are replaced by a peptide linker, optionally wherein the Fc domain is fused to anti-NKp46 scFv via a peptide linker):

- or
- (V_K-C_K - hinge) – Fc domain – (anti-NKp46)

|

(V_H-CH1- hinge) – Fc domain
- or
- (V_H-CH1- hinge) – Fc domain – (anti-NKp46)

|

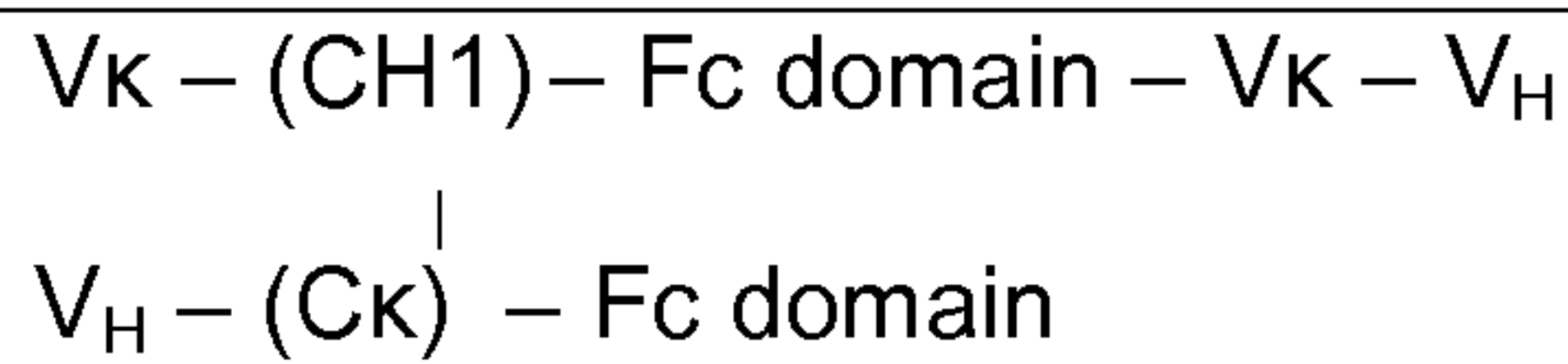
(V_K-C_K- hinge) – Fc domain
- or
- (V_H-CH1- hinge) – Fc domain

|

(V_K-C_K- hinge) – Fc domain – (anti-NKp46)

Other examples of potential domain arrangements for the heterodimeric polypeptides according to the invention include but are not limited to those shown in the table below:

V _K – V _H – Fc domain – V _H – (CH1)	Fc domain – V _K – (C _K)
V _H – V _K – Fc domain – V _H – (CH1)	Fc domain – V _K – (C _K)
V _K – V _H – Fc domain – V _K – (CH1)	Fc domain – V _H – (C _K)
V _H – V _K – Fc domain – V _K – (CH1)	Fc domain – V _H – (C _K)
V _H – (CH1) – Fc domain – V _H – V _K	V _K – (C _K) – Fc domain
V _H – (CH1) – Fc domain – V _K – V _H	V _K – (C _K) – Fc domain
V _K – (CH1) – Fc domain – V _H – V _K	V _H – (C _K) – Fc domain



Heterotrimeric proteins can for example be formed by using a central (first) polypeptide chain comprising a first variable domain (V) fused to a first CH1 or Cκ constant region, a second variable domain (V) fused to a second CH1 or Cκ constant region, and an Fc domain or portion thereof interposed between the first and second variable domains (i.e. the Fc domain is interposed between the first and second (V-(CH1/Cκ) units. For example, a central polypeptide chain for use in a heterotrimeric protein according to the invention can have the domain arrangements (N- to C- termini) as follows:



A second polypeptide chain can then comprise a domain arrangement (N- to C- termini):



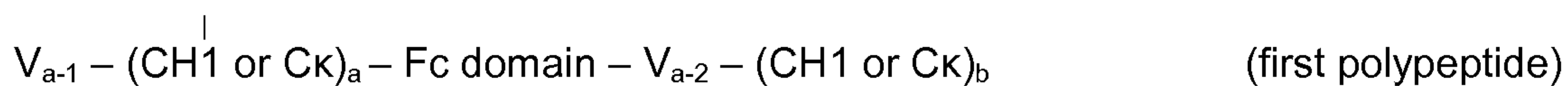
such that the $(CH1 \text{ or } C\kappa)_c$ dimerizes with the $(CH1 \text{ or } C\kappa)_a$ on the central chain, and the V_{a-1} and V_{b-1} form an antigen binding domain.

A third polypeptide chain can then comprise the following domain arrangement (N- to C- termini):



such that the $(CH1 \text{ or } C\kappa)_d$ dimerizes with the $(CH1 \text{ or } C\kappa)_b$ unit on the central chain, and the V_{a-2} and V_{b-2} form an antigen binding domain.

An example of a domain configuration of a resulting heterotrimer with a dimeric Fc domain (also shown as formats 5, 6, 7 and 16 in **Figures 2D** and **2E**) is shown below:



Thus, in a trimeric polypeptide according to the invention, the first polypeptide can have two variable domains that each form an antigen binding domain with a variable domain on a separate polypeptide chain (i.e. the variable domain of the second and third chains), the second polypeptide chain has one variable domain, and the third polypeptide has one variable domain.

A trimeric polypeptide according to the invention may further comprise the following:

(a) a first polypeptide chain comprising a first variable domain (V) fused to a first CH1 or Cκ constant region, a second variable domain (V) fused to a second CH1 or Cκ

constant region, and an Fc domain or portion thereof interposed between the first and second variable domains;

(b) a second polypeptide chain comprising a variable domain fused at its C-terminus to a CH1 or Cκ constant region selected to be complementary to the first CH1 or Cκ constant region of the first polypeptide chain such that the first and second polypeptides form a CH1-Cκ heterodimer, and an Fc domain; and

(c) a third polypeptide chain comprising a variable domain fused (e.g. at its C-terminus) to a CH1 or Cκ constant region, wherein the variable domain and the constant region are selected to be complementary to the second variable domain and second CH1 or Cκ constant region of the first polypeptide chain such that the first and third polypeptides form a CH1-Cκ heterodimer bound by non-covalent and optionally further disulfide bond(s) formed between the CH1 or Cκ constant region of the third polypeptide and the second CH1 or Cκ constant region of the first polypeptide, but not between the CH1 or Cκ constant region of the third polypeptide and the first CH1 or Cκ constant region of the first polypeptide

wherein the first, second and third polypeptides form a CH1-Cκ heterotrimer, and wherein the first variable domain of the first polypeptide chain and the variable domain of the second polypeptide chain form an antigen binding domain specific for a first antigen of interest, and the second variable domain of the first polypeptide chain and the variable domain on the third polypeptide chain form an antigen binding domain specific for a second antigen of interest.

Examples of potential domain arrangements for such trimeric bispecific polypeptides include but are not limited to those shown below:

Vκ- Cκ - Fc domain	(second polypeptide)
V _H - CH1 - Fc domain - V _H - Cκ	(first polypeptide)
Vκ - CH1	(third polypeptide)
V _H - CH1 - Fc domain	(second polypeptide)
Vκ - Cκ - Fc domain - V _H - Cκ	(first polypeptide)
Vκ - CH1	(third polypeptide)
V _H - Cκ - Fc domain	(second polypeptide)
Vκ- CH1 - Fc domain - V _H - CH1	(first polypeptide)
Vκ - Cκ	(third polypeptide)

In further examples, heterotrimers can be constructed with three ABDs and a dimeric Fc. One of the ABDs will bind to NKp46, and the other two ABDs can bind to an antigen of interest, wherein the antigen of interest bound by the two ABDs can be the same antigen or a different antigen. Thus, in one aspect of such an embodiment, the multimeric polypeptide can bind the antigen of interest in a bivalent manner (with two ABDs).

Heterotrimeric proteins can for example be formed by using a central (first) polypeptide chain comprising a first variable domain (V) fused to a first CH1 or Cκ constant region, a second variable domain (V) fused to a second CH1 or Cκ constant region, and an Fc domain or portion thereof interposed between the first and second variable domains (i.e. the Fc domain is interposed between the first and second (V-(CH1/Cκ)) units. For example, a central polypeptide chain for use in a heterotrimeric protein can have the domain arrangements (N- to C- termini) as follows:

$$V_1 - (\text{CH1 or C}\kappa)_a - \text{Fc domain} - V_2 - (\text{CH1 or C}\kappa)_b.$$

A second polypeptide chain can then comprise a domain arrangement (N- to C- termini):

$$V_1 - (\text{CH1 or C}\kappa)_c,$$

or

$$V_1 - (\text{CH1 or C}\kappa)_c - \text{Fc domain}$$

such that the $(\text{CH1 or C}\kappa)_c$ dimerizes with the $(\text{CH1 or C}\kappa)_a$ on the central chain, and the V_{a1} and V_{b1} form an antigen binding domain.

A third polypeptide chain can then comprise a domain arrangement (N- to C- termini):

$$V_2 - (\text{CH1 or C}\kappa)_d - \text{scFv},$$

such that the $(\text{CH1 or C}\kappa)_d$ dimerizes with the $(\text{CH1 or C}\kappa)_b$ unit on the central chain, and the V_{a2} and V_{b2} form an antigen binding domain.

An example of a configuration of a resulting heterotrimer with a dimeric Fc domain (also shown as formats T5 and T6 in **Figure 2F**) has a domain arrangement:

$$V_1 - (\text{CH1 or C}\kappa)_c - \text{Fc domain} \quad (\text{second polypeptide})$$

$$V_1 - (\text{CH1 or C}\kappa)_a - \text{Fc domain} - V_2 - (\text{CH1 or C}\kappa)_b \quad (\text{first polypeptide})$$

$$V_2 - (\text{CH1 or C}\kappa)_d - V_3 - V_3 \quad (\text{third polypeptide})$$

In any of the polypeptide chains herein, a hinge region will typically be present on a polypeptide chain between a CH1 domain and a CH2 domain of an Fc domain, and/or can be present between a Cκ domain and a CH2 domain. A hinge region can optionally be replaced, e.g., by a suitable linker peptide.

In any of the domain arrangements, the Fc domain may comprise a CH2-CH3 unit (a

full length CH2 and CH3 domain or a fragment thereof). In heterodimers or heterotrimers comprising two chains with Fc domains (a dimeric Fc domain), the CH3 domain will be capable of CH3-CH3 dimerization (e.g. it will comprise a wild-type CH3 domain).

In some exemplary configurations, the multispecific protein can be a heterodimer, a heterotrimer or a heterotetramers, wherein the polypeptide chains are engineered for heterodimerization among each other so as to produce the desired protein. In embodiments where the desired chain pairings are not driven by CH1-Ck dimerization, the chains may comprise constant or Fc domains with amino acid modifications (e.g., substitutions) that favor the preferential hetero-dimerization of the two different chains over the homodimerization of two identical chains. In some embodiments, a “knob-into-holes” approach is used in which the CH3 domain interface of the antibody Fc region is mutated so that the antibodies preferentially form heterodimers (further including the attached light chains). These mutations create altered charge polarity across the Fc dimer interface such that co-expression of electrostatically matched Fc chains support favorable attractive interactions thereby promoting desired Fc heterodimer formation, whereas unfavorable repulsive charge interactions suppress unwanted Fc homodimer formation. For example one heavy chain comprises a T366W substitution and the second heavy chain comprises a T366S, L368A and Y407V substitution, see, e.g. Ridgway et al (1996) *Protein Eng.*, 9, pp. 617–621; Atwell (1997) *J. Mol. Biol.*, 270, pp. 26–35; and WO2009/089004, the disclosures of which are incorporated herein by reference. In another approach, one heavy chain comprises a F405L substitution and the second heavy chain comprises a K409R substitution, see, e.g., Labrijn et al. (2013) *Proc. Natl. Acad. Sci. U.S.A.*, 110, pp. 5145–5150. In another approach, one heavy chain comprises T350V, L351Y, F405A, and Y407V substitutions and the second heavy chain comprises T350V, T366S, K392L, and T394W substitutions, see, e.g. Von Kreudenstein et al., (2013) *mAbs* 5:646-654. In another approach, one heavy chain comprises both K409D and K392D substitutions and the second heavy chain comprises both D399K and E356K substitutions, see, e.g. Gunasekaran et al., (2010) *J. Biol. Chem.* 285:19637-19646. In another approach, one heavy chain comprises D221E, P228E and L368E substitutions and the second heavy chain comprises D221R, P228R, and K409R substitutions, see, e.g. Strop et al., (2012) *J. Mol. Biol.* 420: 204-219. In another approach, one heavy chain comprises S364H and F405A substitutions and the second heavy chain comprises Y349T and T394F substitutions, see, e.g. Moore et al., (2011) *mAbs* 3: 546-557. In another approach, one heavy chain comprises a H435R substitution and the second heavy chain optionally may or may not comprise a substitution, see, e.g. US Patent no. 8,586,713. When such hetero-multimeric antibodies have Fc regions derived from a human IgG2 or IgG4, the Fc regions of these antibodies can be engineered to contain amino acid

modifications that permit CD16 binding. In some embodiments, the antibody may comprise mammalian antibody-type N-linked glycosylation at residue N297 (Kabat EU numbering).

In some embodiments the invention also comprises a heterodimeric or heterotrimeric protein that comprises an NKp46-binding ABD and an antigen of interest-binding ABD, in which one or both of the ABDs (e.g., a variable region or other antigen binding domain such as a non-immunoglobulin scaffold) is linked to a constant region, e.g., an Fc domain or portion thereof via a linker, e.g., a flexible polypeptide linker. Optionally, the ABD is placed on a single polypeptide chain (e.g. a tandem variable domain, a V_HH or single V domain, a non-immunoglobulin scaffold).

In some embodiments, one or both of the ABDs is comprised in a V_H and V_L domain that associate with one another to form the ABD. In one embodiment, the V_H and V_L that form an ABD are each within a tandem variable region (a V_H and V_L domain separated by a flexible polypeptide linker).

In some embodiments, one of the ABDs is comprised in a Fab or Fab-like structure, in which a variable domain is linked to a CH1 domain and a complementary variable domain is linked to a complementary C_k (or C_λ) constant domain, wherein the CH1 and C_k (or C_λ) constant domains associate (dimerize). In some embodiments, one of the ABDs is comprised in such a Fab or Fab-like structure and the other ABD is placed on a single polypeptide chain (e.g. a tandem variable domain) and is linked to a constant region, e.g., an Fc domain or portion thereof via a linker, e.g., a flexible polypeptide linker.

In some embodiments, one of the ABDs comprises a Fab or Fab-like structure, in which a variable domain is linked to a CH1 domain and a complementary variable domain is linked to a complementary C_k (or C_λ) constant domain, wherein the CH1 and C_k (or C_λ) constant domains associate to form a heterodimeric protein. For example, a first and second ABD can advantageously comprise or consist of single variable domains (e.g. V_HH domains) having different antigen binding specificities (e.g., V_HH₁ and V_HH₂). Also the V_HH₁ can be fused to a CH1 domain and V_HH₂ can be fused to a C_k or C_λ domain. The V₁ - C_k (or C_λ) chain associates with a V₂-CH1 chain such that a Fab is formed. See, e.g., WO2006/064136 and WO2012/089814 which disclose examples of such antibodies lacking Fc domains, the disclosures of which PCT applications are incorporated herein by reference. The CH1 and/or C_k domains can then be linked to a CH2 domain, optionally via a hinge region (or a linker peptide, e.g., one that has similar functional properties, e.g., dimerization). The CH2 domain(s) is/are then linked to a CH3 domain. The CH2-CH3 domains can thus optionally be embodied as a full-length Fc domain.

In any multispecific protein according to the invention, a hinge region can and generally will be present on a polypeptide chain between a CH1 domain and a CH2 domain,

and/or can be present between a C_k domain and a CH₂ domain. A hinge region can optionally be replaced for example by a suitable linker peptide, e.g. a flexible polypeptide.

The proteins domains described in the present disclosure can optionally be specified as being from N- to C- termini. Protein arrangements of the disclosure for purposes of illustration are shown from N-terminus (on the left) to C-terminus. Domains can be referred to as fused to one another (e.g. a domain can be said to be fused to the C-terminus of the domain on its left, and/or a domain can be said to be fused to the N-terminus of the domain on its right).

The proteins domains described in the present disclosure can be fused to one another directly or via intervening amino acid sequences. For example, a CH₁ or C_k domain can be fused to an Fc domain (or CH₂ or CH₃ domain thereof) via a linker peptide, optionally a hinge region or a fragment thereof. In another example, a V_H or V_k domain can be fused to a CH₃ domain via a linker peptide. V_H and V_L domains linked to another in tandem can and generally will be fused via a linker peptide (e.g. a scFv). V_H and V_L domains linked to an Fc domain will be fused via a linker peptide. Two polypeptide chains will be bound to one another (indicated by “_I”), by non-covalent bonds, and optionally can further be attached via interchain disulfide bonds, formed between cysteine residues within complementary CH₁ and C_k domains.

Linkers

When ABD(s) or a polypeptide chain(s) comprise(s) a tandem variable region (e.g. scFv), two V domains (e.g. a V_H domain and V_L domains are generally linked together by a linker of sufficient length to enable the ABD to fold in such a way as to permit binding to the antigen for which the ABD is intended to bind. Similarly, when an ABD is linked to a constant domain or Fc domain, the linkage may be via a flexible linker (e.g. polypeptide linker) that permits the ABD to be positioned such that it binds to its target antigen and exhibits the desired functionality, e.g. it possesses a sufficient range of motion relative to the rest of the multispecific protein (the Fc domain and/or other ABD) and thereby mediates NKp46 signalling. Examples of linkers include, for example, linkers derived from antibody hinge regions, an amino sequence RTVA, or linkers comprising glycine and serine residues, e.g., the amino acid sequence GEGTSTGS(G₂S)₂GGAD. In another specific embodiment, the V_H domain and V_L domains of a scFv are linked together by the amino acid sequence (G₄S)₃.

Any of the peptide linkers contained in the subject multispecific proteins may comprise a length of at least 4 residues, at least 5 residues, at least 10 residues, at least 15 residues, at least 20 residues, at least 25 residues, at least 30 residues or more. In other embodiments, the linkers comprise a length of between 2-4 residues, between 2-4 residues, between 2-6 residues, between 2-8 residues, between 2-10 residues, between 2-12

residues, between 2-14 residues, between 2-16 residues, between 2-18 residues, between 2- 20 residues, between 2-22 residues, between 2-24 residues, between 2-26 residues, between 2-28 residues, between 2-30 residues, between 2 and 50 residues, or between 10 and 50 residues.

5 An ABD (e.g. an immunoglobulin variable region) can optionally be linked to a constant domain or Fc domain via a flexible linker (e.g. polypeptide linker) that leads to less structural rigidity or stiffness (e.g. between or amongst the ABD and Fc domain) compared to a conventional (e.g. wild-type full length human IgG) antibody. For example, the multispecific protein may have a structure or a flexible linker between the ABD and constant
10 domain or Fc domain that permits an increased range of domain motion compared to the ABD in a conventional (e.g. wild-type full length human IgG) antibody. In particular, the structure or a flexible linker can be configured to confer on the antigen binding sites greater intrachain domain movement compared to antigen binding sites in a conventional human IgG1 antibody. Rigidity or domain motion/interchain domain movement can be determined,
15 e.g., by computer modeling, electron microscopy, spectroscopy such as Nuclear Magnetic Resonance (NMR), X-ray crystallography, or Sedimentation Velocity Analytical ultracentrifugation (AUC) to measure or compare the radius of gyration of proteins comprising the linker or hinge. A test protein or linker may have lower rigidity relative to a comparator protein if the test protein has a value obtained from one of the tests described in
20 the previous sentence differs from the value of the comparator, e.g., an IgG1 antibody or a hinge, by at least 5%, 10%, 25%, 50%, 75%, or 100%. A person of skill in the art would be able to determine from the tests whether a test protein has at lower rigidity to that of another protein, respectively, by interpreting the results of these tests.

25 In one embodiment, the multispecific protein may have a structure or a flexible linker between the ABD and constant domain or Fc domain that permits the NKp46 ABD and the ABD which binds an antigen of interest to have a spacing between said ABDs comprising less than about 80 angstroms, less than about 60 angstroms or ranges from about 40-60 angstroms.

30 In one embodiment, the hinge region will be a fragment of a hinge region (e.g. a truncated hinge region without cysteine residues) or may comprise one or more amino acid modifications which remove (e.g. substitute by another amino acid, or delete) a cysteine residue, optionally both cysteine residues in a hinge region. Removing cysteines can be useful to prevent undesired disulfide bond formation, e.g., the formation of disulfide bridges in a monomeric polypeptide.

In one embodiment, a (poly)peptide linker used to link a CH1 or CK domain to a CH2 or CH3 domain of an Fc domain comprises a fragment of a CH1 domain and/or hinge region. For example, an N-terminal amino acid sequence of CH1 can be fused to a variable domain in order to mimic as closely as possible the natural structure of a wild-type antibody. In one
5 embodiment, the linker comprises an amino acid sequence from a hinge domain or an N-terminal CH1 amino acid. The sequence can be, for example, between 2-4 residues, between 2-4 residues, between 2-6 residues, between 2-8 residues, between 2-10 residues, between 2-12 residues, between 2-14 residues, between 2-16 residues, between 2-18 residues, between 2-20 residues, between 2-22 residues, between 2-24 residues, between
10 2-26 residues, between 2-28 residues, or between 2-30 residues. In one embodiment the linker comprises or consists of the amino acid sequence RTVA.

In one embodiment, the hinge region (or fragment thereof) is derived from a hinge domain of a human IgG1 antibody. For example a hinge domain may comprise the amino acid sequence: T-H-T-C-S-S-C-P-A-P-E-L-L (one letter code), or an amino acid sequence at
15 least 60%, 70%, 80% or 90% identical thereto, optionally wherein one or both cysteines are deleted or substituted by a different amino acid residue.

In one embodiment, the hinge region (or fragment thereof) is derived from a C μ 2-C μ 3 hinge domain of a human IgM antibody. For example a hinge domain may comprise the amino acid sequence: N-A-S-S-M-C-V-P-S-P-A-P-E-L-L (one letter code), or an amino acid
20 sequence at least 60%, 70%, 80% or 90% identical thereto, optionally wherein one or both cysteines are deleted or substituted by a different amino acid residue.

Polypeptide chains that dimerize and associate with one another via non-covalent bonds may or may not additionally be bound by an interchain disulfide bond formed between respective CH1 and C κ domains, and/or between respective hinge domains on the chains.
25 CH1, C κ and/or hinge domains (or other suitable linking amino acid sequences) can optionally be configured such that interchain disulfide bonds are formed between chains such that the desired pairing of chains is favored and undesired or incorrect disulfide bond formation is avoided. For example, when two polypeptide chains to be paired each possess a CH1 or C κ adjacent to a hinge domain, the polypeptide chains can be configured such that
30 the number of available cysteines for interchain disulfide bond formation between respective CH1/C κ -hinge segments is reduced (or is entirely eliminated). For example, the amino acid sequences of respective CH1, C κ and/or hinge domains can be modified to remove cysteine residues in both the CH1/C κ and the hinge domain of a polypeptide; thereby the CH1 and C κ domains of the two chains that dimerize will associate via non-covalent interaction(s).

In another example, the CH1 or C κ domain adjacent (e.g., N-terminal to) a hinge domain comprises a cysteine capable of interchain disulfide bond formation, and the hinge domain which is placed at the C-terminus of the CH1 or C κ comprises a deletion or substitution of one or both cysteines of the hinge (e.g. Cys 239 and Cys 242, as numbered for human IgG1 hinge according to Kabat). In one embodiment, the hinge region (or fragment thereof) comprise the amino acid sequence: T-H-T-S-P-P-S-P-A-P-E-L-L (one letter code), or an amino acid sequence at least 60%, 70%, 80% or 90% identical thereto.

In another example, the CH1 or C κ domain adjacent (e.g., N-terminal to) a hinge domain comprises a deletion or substitution at a cysteine residue capable of interchain disulfide bond formation, and the hinge domain placed at the C-terminus of the CH1 or C κ comprises one or both cysteines of the hinge (e.g. Cys 239 and Cys 242, as numbered for human IgG1 hinge according to Kabat). In one embodiment, the hinge region (or fragment thereof) comprises the amino acid sequence: T-H-T-C-S-S-C-P-A-P-E-L-L (one letter code), or an amino acid sequence at least 60%, 70%, 80% or 90% identical thereto.

In another example, a hinge region is derived from an IgM antibody. In such embodiments, the CH1/CK pairing mimics the C μ 2 domain homodimerization in IgM antibodies. For example, the CH1 or C κ domain adjacent (e.g., N-terminal to) a hinge domain comprises a deletion or substitution at a cysteine capable of interchain disulfide bond formation, and an IgM hinge domain which is placed at the C-terminus of the CH1 or C κ comprises one or both cysteines of the hinge. In one embodiment, the hinge region (or fragment thereof) comprises the amino acid sequence: T-H-T-C-S-S-C-P-A-P-E-L-L (one letter code), or an amino acid sequence at least 60%, 70%, 80% or 90% identical thereto.

Constant regions

Constant region domains can be derived from any suitable human antibody, including, the constant heavy (CH1) and light (C κ) domains, hinge domains, CH2 and CH3 domains. With respect to heavy chain constant domains, "CH1" generally refers to positions 118-220 according to the EU index as in Kabat.

"CH2" generally refers to positions 237-340 according to the EU index as in Kabat, and "CH3" generally refers to positions 341-447 according to the EU index as in Kabat.

A "hinge" or "hinge region" or "antibody hinge region" herein refers to the flexible polypeptide or linker between the first and second constant domains of an antibody. Structurally, the IgG CH1 domain ends at EU position 220, and the IgG CH2 domain begins at residue EU position 237. Thus for an IgG the hinge generally includes positions 221 (D221 in IgG1) to 236 (G236 in IgG1), wherein the numbering is according to the EU index as in Kabat. References to specific amino acid residues within constant region domains

found within the polypeptides shall be, unless otherwise indicated or as otherwise dictated by context, be defined according to Kabat, in the context of an IgG antibody.

CH2 and CH3 domains which may be present in the subject antibodies or multispecific proteins can be derived from any suitable antibody. Such CH2 and CH3 domains can be used as wild-type domains or may serve as the basis for a modified CH2 or CH3 domain. Optionally the CH2 and/or CH3 domain is of human origin or may comprise that of another species (e.g., rodent, rabbit, non-human primate) or may comprise a modified or chimeric CH2 and/or CH3 domain, e.g., one comprising portions or residues from different CH2 or CH3 domains, e.g., from different antibody isotypes or species antibodies .

In embodiments where a multispecific is intended not to bind to human CD16 polypeptide, a CH2 and/or CH3 domain (or Fc domain comprising same) may comprise a modification to decrease or abolish binding to FcγRIIIA (CD16). For example, CH2 mutations in a dimeric Fc domain proteins at residue N297 (Kabat numbering) can eliminate CD16 binding. However the person of skill in the art will appreciate that other configurations can be implemented. For example, substitutions into human IgG1 or IgG2 residues at positions 233-236 and IgG4 residues at positions 327, 330 and 331 were shown to greatly reduce binding to Fcγ receptors and thus ADCC and CDC. Furthermore, Idusogie *et al.* (2000) J. Immunol. 164(8):4178-84 demonstrated that alanine substitution at different positions, including K322, significantly reduced complement activation.

In certain embodiments herein where binding to CD16A is desired, a CH2 and/or CH3 domain (or Fc domain comprising same) may be a wild-type domain or may comprise one or more amino acid modifications (e.g. amino acid substitutions) which increase binding to human CD16 and optionally another receptor such as FcRn. Optionally, the modifications will not substantially decrease or abolish the ability of the Fc-derived polypeptide to bind to neonatal Fc receptor (FcRn), e.g. human FcRn. Typical modifications include modified human IgG1-derived constant regions comprising at least one amino acid modification (e.g. substitution, deletions, insertions), and/or altered types of glycosylation, e.g., hypofucosylation. Such modifications can affect interaction with Fc receptors: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). FcγRI (CD64), FcγRIIA (CD32A) and FcγRIII (CD 16) are activating (i.e., immune system enhancing) receptors while FcγRIIB (CD32B) is an inhibiting (i.e., immune system dampening) receptor. A modification may, for example, increase binding of the Fc domain to FcγRIIIa on effector (e.g. NK) cells and/or decrease binding to FcγRIIB. Examples of modifications are provided in PCT publication no. WO2014/044686, the disclosure of which is incorporated herein by reference. Specific mutations (in IgG1 Fc domains) which affect (enhance) FcγRIIIa or FcRn binding are also set forth below.

Isotype	Species	Modification	Effector Function	Effect of Modification
IgG1	Human	T250Q/M428L	Increased binding to FcRn	Increased half-life
IgG1	Human	1M252Y/S254T/T256E + H433K/N434F	Increased binding to FcRn	Increased half-life
IgG1	Human	E333A	Increased binding to FcγRIIIa	Increased ADCC and CDC
IgG1	Human	S239D/I332E or S239D/A330L/I332E	Increased binding to FcγRIIIa	Increased ADCC
IgG1	Human	P257I/Q311	Increased binding to FcRn	Unchanged half-life
IgG1	Human	S239D/I332E/G236A	Increased FcγRIIIa/FcγRIIb ratio	Increased macrophage phagocytosis

In some embodiments, the multispecific protein comprises a variant Fc region comprise at least one amino acid modification (for example, possessing 1, 2, 3, 4, 5, 6, 7, 8, 9, or more amino acid modifications) in the CH2 and/or CH3 domain of the Fc region, wherein the modification enhances binding to a human CD16 polypeptide. In other embodiments, the multispecific protein comprises at least one amino acid modification (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, or more amino acid modifications) in the CH2 domain of the Fc region from amino acids 237-341, or within the lower hinge-CH2 region that comprises residues 231-341. In some embodiments, the multispecific protein comprises at least two amino acid modifications (for example, 2, 3, 4, 5, 6, 7, 8, 9, or more amino acid modifications), wherein at least one of such modifications is within the CH3 region and at least one such modifications is within the CH2 region. Encompassed also are amino acid modifications in the hinge region. In one embodiment, encompassed are amino acid modifications in the CH1 domain, optionally in the upper hinge region that comprises residues 216-230 (Kabat EU numbering). Any suitable functional combination of Fc modifications can be made, for example any combination of the different Fc modifications which are disclosed in any of

United States Patents Nos. US, 7,632,497; 7,521,542; 7,425,619; 7,416,727; 7,371,826; 7,355,008; 7,335,742; 7,332,581; 7,183,387; 7,122,637; 6,821,505 and 6,737,056; and/or in PCT Publications Nos. WO2011/109400; WO 2008/105886; WO 2008/002933; WO 2007/021841; WO 2007/106707; WO 06/088494; WO 05/115452; WO 05/110474; WO 04/1032269; WO 00/42072; WO 06/088494; WO 07/024249; WO 05/047327; WO 04/099249 and WO 04/063351; and/or in Lazar et al. (2006) *Proc. Nat. Acad. Sci. USA* 103(11): 405-410; Presta, L.G. et al. (2002) *Biochem. Soc. Trans.* 30(4):487-490; Shields, R.L. et al. (2002) *J. Biol. Chem.* 277(30):26733-26740 and Shields, R.L. et al. (2001) *J. Biol. Chem.* 276(9):6591-6604).

In some embodiments, the multispecific protein comprises an Fc domain comprising at least one amino acid modification (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, or more amino acid modifications) relative to a wild-type Fc region, such that the molecule has an enhanced binding affinity for human CD16 relative to the same molecule comprising a wild-type Fc region, optionally wherein the variant Fc region comprises a substitution at any one or more of positions 221, 239, 243, 247, 255, 256, 258, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 300, 301, 303, 305, 307, 308, 309, 310, 311, 312, 316, 320, 322, 326, 329, 330, 332, 331, 332, 333, 334, 335, 337, 338, 339, 340, 359, 360, 370, 373, 376, 378, 392, 396, 399, 402, 404, 416, 419, 421, 430, 434, 435, 437, 438 and/or 439 (Kabat EU numbering).

In one embodiment, the multispecific protein comprises an Fc domain comprising at least one amino acid modification (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, or more amino acid modifications) relative to a wild-type Fc region, such that the molecule has enhanced binding affinity for human CD16 relative to a molecule comprising a wild-type Fc region, optionally wherein the variant Fc region comprises a substitution at any one or more of positions 239, 298, 330, 332, 333 and/or 334 (e.g. S239D, S298A, A330L, I332E, E333A and/or K334A substitutions), optionally wherein the variant Fc region comprises a substitution at residues S239 and I332, e.g. a S239D and I332E substitution (Kabat EU numbering).

In some embodiments, the multispecific protein comprises an Fc domain comprising altered glycosylation patterns that increase binding affinity for human CD16. Such carbohydrate modifications can be accomplished by, for example, by expressing a nucleic acid encoding the multispecific protein in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery are known in the art and can be used as host cells in which to express recombinant antibodies to thereby produce an antibody with altered glycosylation. See, for example, Shields, R.L. et al. (2002) *J. Biol. Chem.* 277:26733-26740; Umana et al. (1999) *Nat. Biotech.* 17:176-1, as well as, European Patent No: EP 1,176,195; PCT Publications WO 06/133148; WO 03/035835; WO 99/54342, each of which is incorporated

herein by reference in its entirety. In one aspect, the multispecific protein contains one or more hypofucosylated constant regions. Such multispecific protein may comprise an amino acid alteration or may not comprise an amino acid alteration and/or may be expressed or synthesized or treated under conditions that result in hypofucosylation. In one aspect, a multispecific protein composition comprises a multispecific protein described herein, wherein at least 20, 30, 40, 50, 60, 75, 85, 90, 95% or substantially all of the antibody species in the composition have a constant region comprising a core carbohydrate structure (e.g. complex, hybrid and high mannose structures) which lacks fucose. In one embodiment, provided is a multispecific protein composition which is free of N-linked glycans comprising a core carbohydrate structure having fucose. The core carbohydrate will preferably be a sugar chain at Asn297.

Optionally, a multispecific protein comprising a dimeric Fc domain can be characterized by having a binding affinity to a human CD16 polypeptide that is within 1-log of that of a conventional human IgG1 antibody, e.g., as assessed by surface plasmon resonance.

In one embodiment, the multispecific protein comprising a dimeric Fc domain engineered to enhance Fc receptor binding can be characterized by having a binding affinity to a human CD16 polypeptide that is at least 1-log greater than that of a conventional or wild-type human IgG1 antibody, e.g., as assessed by surface plasmon resonance.

Optionally a multispecific protein comprising a dimeric Fc domain can be characterized by a K_d for binding (monovalent) to a human CD16 polypeptide of less than 10^{-5} M (10 μ molar), optionally less than 10^{-6} M (1 μ molar), as assessed by surface plasmon resonance (e.g. as in Example 16, SPR measurements performed on a Biacore T100 apparatus (Biacore GE Healthcare), with bispecific antibodies immobilized on a Sensor Chip CM5 and serial dilutions of soluble CD16 polypeptide injected over the immobilized bispecific antibodies).

CDR sequences and epitopes

In some embodiments, the proteins and antibodies herein bind the D1 domain of NKp46, the D2 domain of NKp46, or bind a region spanning the D1 and D2 domains (at the border of the D1 and D2 domains, the D1/D2 junction), of the NKp46 polypeptide of SEQ ID NO: 1. In some embodiments, the multispecific proteins or antibodies according to the invention have an affinity for human NKp46 characterized by a K_D of less than 10^{-8} M, less than 10^{-9} M, or less than 10^{-10} M.

In another embodiment, the inventive antibodies or multispecific proteins bind NKp46 at substantially the same epitope on NKp46 as antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9. In another embodiment, the antibodies at least partially overlaps, or includes at least one residue in the segment or epitope bound by NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9. In one embodiment, all key residues of the

epitope are in a segment corresponding to domain D1 or D2. In one embodiment, the antibody or multispecific protein binds a residue present in the D1 domain as well as a residue present in the D2 domain. In one embodiment, the antibodies bind an epitope comprising 1, 2, 3, 4, 5, 6, 7 or more residues in the segment corresponding to domain D1 or D2 of the NKp46 polypeptide of SEQ ID NO: 1. In one embodiment, the antibodies bind domain D1 and further bind an epitope comprising 1, 2, 3, or 4 of the residues R101, V102, E104 and/or L105.

In another embodiment, the antibodies or multispecific proteins bind Nkp46 at the D1/D2 domain junction and bind an epitope comprising or consisting of 1, 2, 3, 4 or 5 of the residues K41, E42, E119, Y121 and/or Y194.

In another embodiment, the antibodies or multispecific proteins bind domain D2 and bind an epitope comprising 1, 2, 3, or 4 of the residues P132, E133, I135, and/or S136.

The Examples section provided *infra* further describes the construction of a series of mutant human NKp46 polypeptides. In the examples, the binding of anti-NKp46 antibody or multispecific protein to cells transfected with the NKp46 mutants was measured and compared to the ability of anti-NKp46 antibody to bind wild-type NKp46 polypeptide (SEQ ID NO:1). A reduction in binding between an anti-NKp46 antibody or NKp46 binding multispecific protein and a mutant NKp46 polypeptide as described herein means that there is a reduction in binding affinity (e.g., as measured by known methods such FACS testing of cells expressing a particular mutant, or by Biacore testing of binding to mutant polypeptides) and/or a reduction in the total binding capacity of the anti-NKp46 antibody (e.g., as evidenced by a decrease in Bmax in a plot of anti-NKp46 antibody concentration versus polypeptide concentration). A significant reduction in binding indicates that the mutated residue is directly involved in the binding to the anti-NKp46 antibody to NKp46 or is in close proximity to the binding protein when the anti-NKp46 antibody or NKp46 binding multispecific protein is bound to NKp46. An antibody epitope will thus preferably include such residue and may include additional residues adjacent to such residue.

In some embodiments, a significant reduction in binding means that the binding affinity and/or capacity between an anti-NKp46 antibody or NKp46 binding multispecific protein and a mutant NKp46 polypeptide is reduced by greater than 40 %, greater than 50 %, greater than 55 %, greater than 60 %, greater than 65 %, greater than 70 %, greater than 75 %, greater than 80 %, greater than 85 %, greater than 90% or greater than 95% relative to binding between the antibody and a wild type NKp46 polypeptide (e.g., the polypeptide shown in SEQ ID NO:1). In certain embodiments, binding is reduced below detectable limits. In some embodiments, a significant reduction in binding is evidenced when binding of an anti-NKp46 antibody to a mutant NKp46 polypeptide is less than 50% (e.g., less than 45%,

40%, 35%, 30%, 25%, 20%, 15% or 10%) of the binding observed between the anti-NKp46 antibody and a wild-type NKp46 polypeptide (e.g., the polypeptide shown in SEQ ID NO: 1 (or the extracellular domain thereof)). Such binding measurements can be made using a variety of binding assays known in the art. A specific example of one such assay is described in the Example section.

In some embodiments, anti-NKp46 antibodies or NKp46 binding multispecific proteins are provided that exhibit significantly lower binding for a mutant NKp46 polypeptide in which a residue in a wild-type NKp46 polypeptide (e.g., SEQ ID NO:1) is substituted. In the shorthand notation used here, the format is: Wild type residue: Position in polypeptide: Mutant residue, with the numbering of the residues as indicated in SEQ ID NO: 1.

In some embodiments, an anti-NKp46 antibody binds a wild-type NKp46 polypeptide but has decreased binding to a mutant NKp46 polypeptide having a mutation (e.g., an alanine substitution) any one or more of the residues R101, V102, E104 and/or L105 (with reference to SEQ ID NO:1) compared to binding to the wild-type NKp46).

In some embodiments, an anti-NKp46 antibody or NKp46-binding multispecific protein binds a wild-type NKp46 polypeptide but has decreased binding to a mutant NKp46 polypeptide having a mutation (e.g., an alanine substitution) at one or more of residues K41, E42, E119, Y121 and/or Y194 (with reference to SEQ ID NO:1) compared to binding to the wild-type NKp46).

In some embodiments, an anti-NKp46 antibody or NKp46-binding multispecific protein binds a wild-type NKp46 polypeptide but has decreased binding to a mutant NKp46 polypeptide having a mutation (e.g., an alanine substitution) at one or more of residues P132, E133, I135, and/or S136 (with reference to SEQ ID NO:1) compared to binding to the wild-type NKp46).

The amino acid sequence of the heavy chain variable region of antibodies NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 and NKp46-9 are listed herein in Table B (SEQ ID NOS: 3, 5, 7, 9, 11 and 13 respectively), the amino acid sequence of the light chain variable region of antibodies NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 and NKp46-9 are also listed herein in Table B (SEQ ID NOS: 4, 6, 8, 10, 12 and 14 respectively).

In a specific embodiment, the invention provides is an antibody, e.g. a full length monospecific antibody, a multispecific or bispecific antibody, including a bispecific monomeric polypeptide, or a NKp46-binding multispecific protein that binds essentially the same epitope or determinant as monoclonal antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9; optionally the antibody comprises a hypervariable region of antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9. In any of the

embodiments herein, antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 can be characterized by its amino acid sequence and/or nucleic acid sequence encoding it. In one embodiment, the antibody comprises the Fab or F(ab')₂ portion of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9. Also provided is an antibody that comprises the heavy chain variable region of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9. According to one embodiment, an antibody comprises the three CDRs of the heavy chain variable region of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9. Also provided is a polypeptide that further comprises one, two or three of the CDRs of the light chain variable region of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9. Optionally any one or more of said light or heavy chain CDRs may contain one, two, three, four or five or more amino acid modifications (e.g. substitutions, insertions or deletions). Optionally, provided is a multispecific protein or antibody polypeptide where any of the light and/or heavy chain variable regions comprising part or all of an antigen binding region of antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 are fused to an immunoglobulin constant region of the human IgG type.

In another aspect, the invention provides a protein, e.g., an antibody, a full length monospecific antibody, a multispecific or a bispecific protein, or a polypeptide chain or fragment thereof, or an NKp46-binding multispecific protein as well as a nucleic acid encoding any of the foregoing, wherein the protein comprises the heavy chain CDRs of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9, comprising, for the respective antibody: a HCDR1 region comprising an amino acid sequence as set forth in Table A, or a sequence of at least 4, 5, 6, 7, 8, 9 or 10 contiguous amino acids thereof, wherein one or more of these amino acids may be substituted by a different amino acid; a HCDR2 region comprising an amino acid sequence as set forth in Table A, or a sequence of at least 4, 5, 6, 7, 8, 9 or 10 contiguous amino acids thereof, wherein one or more of these amino acids may be substituted by a different amino acid; a HCDR3 region comprising an amino acid sequence as set forth in as set forth in Table A, or a sequence of at least 4, 5, 6, 7, 8, 9 or 10 contiguous amino acids thereof, wherein one or more of these amino acids may be substituted by a different amino acid.

In another aspect, the invention provides a protein, e.g., an antibody, a full length monospecific antibody, a multispecific or a bispecific protein, or a polypeptide chain or fragment thereof, or an NKp46-binding multispecific protein as well as a nucleic acid encoding any of the foregoing, wherein the protein comprises light chain CDRs of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9, comprising, for the respective antibody: a LCDR1 region comprising an amino acid sequence as set forth in Table A, or a sequence of at least 4, 5, 6, 7, 8, 9 or 10 contiguous amino acids thereof, wherein one or more of these

amino acids may be substituted by a different amino acid; a LCDR2 region comprising an amino acid sequence as set forth in Table A, or a sequence of at least 4, 5, 6, 7, 8, 9 or 10 contiguous amino acids thereof, wherein one or more of these amino acids may be substituted by a different amino acid; a LCDR3 region comprising an amino acid sequence as set forth in Table A, or a sequence of at least 4, 5, 6, 7, 8, 9 or 10 contiguous amino acids thereof, wherein one or more of these amino acids may be deleted or substituted by a different amino acid.

In another aspect, the invention provides a multispecific protein or antibody that binds to human NKp46, comprising:

(a) the heavy chain variable region of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 as set forth in Table B, optionally wherein one, two, three or more amino acids may be substituted by a different amino acid;

(b) the light chain variable region NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 as set forth in Table B, optionally wherein one, two, three or more amino acids may be substituted by a different amino acid;

(c) the heavy chain variable region of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 as set forth in Table B, optionally wherein one or more of these amino acids may be substituted by a different amino acid; and the respective light chain variable region of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 as set forth in Table B, optionally wherein one, two, three or more amino acids may be substituted by a different amino acid;

(d) the heavy chain CDR 1, 2 and 3 (HCDR1, HCDR2) amino acid sequence of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 as shown in Table A, optionally wherein one, two, three or more amino acids in a CDR may be substituted by a different amino acid;

(e) the light chain CDR 1, 2 and 3 (LCDR1, LCDR2, LCDR3) amino acid sequence of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 as shown in Table A, optionally wherein one, two, three or more amino acids in a CDR may be substituted by a different amino acid; or

(f) the heavy chain CDR 1, 2 and 3 (HCDR1, HCDR2, HCDR3) amino acid sequence of NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 as shown in Table A, optionally wherein one, two, three or more amino acids in a CDR may be substituted by a different amino acid; and the light chain CDRs 1, 2 and 3 (LCDR1, LCDR2, LCDR3) amino acid sequence of the respective NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 or NKp46-9 antibody as shown in Table A, optionally wherein one, two, three or more amino acids in a CDR may be substituted by a different amino acid.

In one embodiment, the aforementioned CDRs are according to Kabat, e.g. as shown in Table A. In one embodiment, the aforementioned CDRs are according to Chothia numbering, e.g. as shown in Table A. In one embodiment, the aforementioned CDRs are according to IMGT numbering, e.g. as shown in Table A.

5 In another aspect of any of the embodiments herein, any of the CDR1, CDR2 and CDR3 of the heavy and light chains may be characterized by a sequence of at least 4, 5, 6, 7, 8, 9 or 10 contiguous amino acids thereof, and/or as having an amino acid sequence that shares at least 50%, 60%, 70%, 80%, 85%, 90% or 95% sequence identity with the particular CDR or set of CDRs listed in the corresponding SEQ ID NO or Table A.

10 In another aspect, the invention provides an antibody that competes for NKp46 binding with a monoclonal antibody according to (a) to (f), above.

In another aspect, the invention provides a bispecific antibody comprising an antibody that binds human NKp46 according to (a) to (f), above, or an antibody that competes for binding to NKp46 with any of such antibodies, fused (optionally via intervening amino acid sequences) to a monomeric Fc domain, optionally further fused (optionally via intervening amino acid sequences) to a second antigen binding domain (e.g. a scFv, a V_H domain, a V_L domain, a dAb, a V-NAR domain or a V_HH domain). Optionally the second antigen binding domain will bind a cancer antigen, a viral antigen, a parasitic antigen or a bacterial antigen.

15 The sequences of the CDRs, according to IMGT, Kabat and Chothia definitions systems, are summarized in Table A below. The sequences of the variable chains of the antibodies according to the invention are listed in Table B below. In any embodiment herein, a V_L or V_H sequence can be specified or numbered so as to contain or lack a signal peptide or any part thereof.

Table A

mAb	CDR definition	HCDR1		HCDR2		HCDR3	
		SEQ ID	Sequence	SEQ ID	Sequence	SEQ ID	Sequence
NKp46-1	Kabat	15	DYVIN	18	EIYPGSGTNYYNEKFKA	21	RGRYGLYAMD Y
	Chothia	16	GYTFTDY	19	PGSG	22	GRYGLYAMD
	IMGT	17	GYTFTDYV	20	GYTFTDYVIYPGSGTN	23	ARRGRYGLYAMDY
NKp46-2	Kabat	31	SDYAWN	34	YITYSGSTSYNPSLES	36	GGYYGSSWGV FAY
	Chothia	32	GYSITSDY		YSG	37	GGYGSSWGVF A

	IMGT	33	GYSITSDYA	35	ITYSGST	38	ARGGYYGSSW GVFAY
NKp46-3	Kabat	46	EYTMH	49	GISPNIGGTSYNQKFKG	51	RGGSFDDY
	Chothia	47	GYTFTEY		PNIG	52	GGSFDD
	IMGT	48	GYTFTEYT	50	ISPNIGGT	53	ARRGGSFDDY
NKp46-4	Kabat	60	SFTMH	63	YINPSSGYTEYNQKFKD	65	GSSRGFDDY
	Chothia	61	GYTFTSF		PSSG	66	SSRGFDD
	IMGT	62	GYTFTSF	64	INPSSGYT	67	VRGSSRGFDDY
NKp46-6	Kabat	73	SSWMH	76	HIHPNSGISNYNEKFKG	78	GGRFDD
	Chothia	74	GYTFTSS		PNSG		GRFDD
	IMGT	75	GYTFTSSW	77	IHPNSGIS	79	ARGGRFDD
NKp46-9	Kabat	85	SDYAWN	88	YITYSGSTNYPNPSLKS	89	CWDYALYAMD C
	Chothia	86	GYSITSDY		YSG	90	WDYALYAMD
	IMGT	87	GYSITSDYA	35	ITYSGST	91	ARCWDYALYA MDC
Bab281	Kabat	97	NYGMN	100	WINTNTGEPTYAEEFKG	102	DYLYYFDDY
	Chothia	98	GYTFTNY		TNTG	103	YLYYFD
	IMGT	99	GYTFTNYG	101	INTNTGEP	104	ARDYLYYFDDY
mAb	CDR definition	LCDR1		LCDR2		LCDR3	
		SEQ ID	Sequence	SEQ ID	Sequence	SEQ ID	Sequence
NKp46-1	Kabat	24	RASQDISNYLN	27	YTSRLHS	28	QQGNTRPWT
	Chothia	25	SQDISNY		YTS	29	YTSGNTRPW
	IMGT	26	QDISNY		YTS	30	YTSQQGNTRP WT
NKp46-2	Kabat	39	RVSENIYSYLA	42	NAKTLAE	43	QHHYGTPWT
	Chothia	40	SENIYSY		NAK	44	HYGTPW
	IMGT	41	ENIYSY		NAK	45	QHHYGTPWT

NKp46-3	Kabat	54	RASQSISDYLH	57	YASQSIG	58	QNGHSFPLT
	Chothia	55	SQSISDY		YAS	59	GHSFPL
	IMGT	56	QSISDY		YAS		QNGHSFPLT
NKp46-4	Kabat	68	RASENIYSNLA	70	AATNLAD	71	QHFWGTPRT
	Chothia		SENIYSN		AAT	72	FWGTPR
	IMGT	69	ENIYSN		AAT		QHFWGTPRT
NKp46-6	Kabat	80	RASQSISDYLH		YASQSIG	82	QNGHSFLMYT
	Chothia	81	GRFDSQSISDY		YAS	83	GHSFLMY
	IMGT		QSISDY		YAS	84	YASQNGHSFL MYT
NKp46-9	Kabat	92	RTSENIYSYLA	93	NAKTLAE	94	QHHYDTPLT
	Chothia		SENIYSY		NAK	95	NAKHYDTPL
	IMGT		ENIYSY		NAK	96	QHHYDTPLT
Bab281	Kabat	105	KASENVVITYVS	108	GASNRYT	109	GQGYSYPYT
	Chothia	106	SENVVITY		GAS	110	GYSYPY
	IMGT	107	ENVVITY		GAS	111	GQGYSYPYT

Table B

Antibody	SEQ ID NO	Amino acid sequence
NKp46-1 VH	3	QVQLQQSGPELVKPGASVKMSCKASGYTFTDYVINWGKQRSGQG LEWIGEIYPGSGTNYYNEKFKAKATLTADKSSNIAYMQLSSLTSEDS AVYFCARRGRYGLYAMDYWGQGTSVTVSS
NKp46-1 VL	4	DIQMTQTTSSLSASLGDRVTISCRASQDISNYLNWYQQKPDGTVKL

		LIYYTSRLHSGVPSRFSGSGSGTDYSLTINNLEQEDIATYFCQQGNT RPWTFGGGGTKLEIK
NKp46-2 VH	5	EVQLQESGPGLVKPSQSLSLTCTVTGYSITSDYAWNWIWIRQFPGNKL EWMGYITYSGSTSYNPSLESISRITRDTSTNQFFLQLNSVTTEDTAT YYCARGGYYGSSWGVFAYWGQGTLVTVSA
NKp46-2 VL	6	DIQMTQSPASLSASVGETVTITCRVSENIYSYLAWYQQKQKGKSPQL LVYNAKTLEAGVPSRFSGSGSGTQFSLKINSLQPEDFGSYQCQHHY GTPWTFGGGGTKLEIK
NKp46-3 VH	7	EVQLQQSGPELVKPGASVKISCKTSGYTFTEYTMHWVKQSHGKSL EWIGGISPNIGGTSYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSA VYYCARRGGSFDYWGQGTTTLTVSS
NKp46-3 VL	8	DIVMTQSPATLSVTPGDRVSLSCRASQSISDYLHWYQQKSHESPRL LIKYASQSIGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHS FPLTFGAGTKLELK
NKp46-4 VH	9	QVQLQQSAVELARPGASVKMSCKASGYTFTSFTMHWVKQRPGQG LEWIGYINPSSGYTEYNQKFKDKTTLTADKSSSTAYMQLDSLTSDD SAVYYCVRGSSRGFDYWGQGTLVTVSA
NKp46-4 VL	10	DIQMIQSPASLSVSVGETVTITCRASENIYSNLAWFQQKQKGKSPQLL VYAATNLADGVPSRFSGSGSGTQYSLKINSLQSEDFGIYYCQHFV GTPRTFGGGTKLEIK
NKp46-6 VH	11	QVQLQQPGSVLVRPGASVKLSCKASGYTFTSSWMHWAKQRPGQ GLEWIGHIHPNSGISNYNEKFKGKATLTVDTSSTAYVDLSSLTSED SAVYYCARGGRFDDWGAGTTTVTVSS
NKp46-6 VL	12	DIQMTQSPSSLSASLGERVSLTCRASQDIGSSLNLWLQQEPDGTIKR LIYATSSLD SGV PKRFSGSRSGSDYSLTISSEDFVDYYCLQYAS SPWTFGGGGTKLEIK
NKp46-9 VH	13	DVQLQESGPGLVKPSQSLSLTCTVTGYSITSDYAWNWIWIRQFPGNKL EWMGYITYSGSTNYNPSLKSISRITRDTSKNQFFLQLNSVTTEDTAT YYCARCWDYALYAMDCWGQGTSVTVSS
NKp46-9 VL	14	DIQMTQSPASLSASVGETVTITCRTSENIYSYLAWCQQKQKGKSPQL LVYNAKTLEAGVPSRFSGSGSGTHFSLKINSLQPEDFGIYYCQHHY DTPLTFGAGTKLELK

Also provided, as described in the Examples herein, is a multispecific protein or antibody comprising the amino acid sequences of monomeric bispecific polypeptides which respectively comprise an scFv comprising the heavy and light chain CDR1, 2 and 3 of the
5 respective heavy and light chain variable region listed as SEQ ID NOS: 3-14 of antibodies NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6 and NKp46-9, a monomeric Fc domain, and an scFv comprising the heavy and light chain CDR1, 2 and 3 of the heavy and light chain variable region of an anti-CD19 antibody, e.g. any of the anti-CD19 antibodies described in the Examples herein.

10 Once the multispecific protein is produced it can be assessed for biological activity, e.g., antigen binding, ability to elicit target cell lysis and/or specific signaling activities elicited thereby.

In one aspect of any embodiment described herein, the inventive multispecific protein is capable of inducing activation of an NKp46-expressing cell (e.g. an NK cell, a reporter cell)

when the protein is incubated in the presence of the NKp46-expressing cell (e.g. purified NK cells) and a target cell that expresses the antigen of interest).

In one aspect of any embodiment described herein, the inventive multispecific protein is capable of inducing an increase of CD137 present on the cell surface of an NKp46- and/or a CD16-expressing cell (e.g. an NK cell, a reporter cell) when the protein is incubated in the presence of the NKp46- and/or a CD16-expressing cell (e.g. purified NK cells), optionally in the absence of target cells.

In one aspect of any embodiment described herein, the inventive multispecific protein is capable of inducing NKp46 signaling in an NKp46-expressing cell (e.g. an NK cell, a reporter cell) when the protein is incubated in the presence of an NKp46-expressing cell (e.g. purified NK cells) and a target cell that expresses the antigen of interest).

Optionally, NK cell activation or signaling is characterized by the increased expression of a cell surface marker of activation, e.g. CD107, CD69, Sca-1 or Ly-6A/E, KLRG1, etc.

Activity can be measured for example by bringing target cells and NKp46-expressing cells into contact with one another, in presence of the multispecific polypeptide. In one example, the aggregation of target cells and NK cells is measured. In another example, the multispecific protein may, for example, be assessed for the ability to cause a measurable increase in any property or activity known in the art as associated with NK cell activity, respectively, such as marker of cytotoxicity (CD107) or cytokine production (for example IFN- γ or TNF- α), increases in intracellular free calcium levels, the ability to lyse target cells in a redirected killing assay, etc.

In the presence of target cells (target cells expressing the antigen of interest) and NK cells that express NKp46, the multispecific protein will be capable of causing an increase in a property or activity associated with NK cell activity (e.g. activation of NK cell cytotoxicity, CD107 expression, IFN γ production) *in vitro*. For example, a multispecific protein according to the invention can be selected based on its ability to increase an NK cell activity by more than about 20%, preferably by least about 30%, at least about 40%, at least about 50%, or more compared to that achieved with the same effector: target cell ratio with the same NK cells and target cells that are not brought into contact with the multispecific protein, as measured by an assay that detects NK cell activity, e.g., an assay which detects the expression of an NK activation marker or which detects NK cell cytotoxicity, e.g., an assay that detects CD107 or CD69 expression, IFN γ production, or a classical *in vitro* chromium release test of cytotoxicity. Examples of protocols for detecting NK cell activation and cytotoxicity assays are described in the Examples herein, as well as for example, in Pessino

et al, *J. Exp. Med.*, 1998, 188 (5): 953-960; Sivori et al, *Eur J Immunol*, 1999. 29:1656-1666; Brando et al, (2005) *J. Leukoc. Biol.* 78:359-371; El-Sherbiny et al, (2007) *Cancer Research* 67(18):8444-9; and Nolte-'t Hoen et al, (2007) *Blood* 109:670-673). Optionally, a multispecific protein according to the invention can be selected for or characterized by its ability to have greater ability to induce NK cell activity towards target cells, i.e., lysis of target cells compared to a conventional human IgG1 antibody that binds to the same antigen of interest, as measured by an assay of NK cell activity (e.g. an assay that detects NK cell-mediated lysis of target cells that express the antigen of interest).

As shown herein, a multispecific protein according to the invention which possesses an Fc domain that does not bind CD16, does not, substantially induce NKp46 signaling (and/or NK activation that results therefrom) of NK cells when the protein is not bound to the antigen of interest on target cells (e.g. in the absence of the antigen of interest and/or target cells). Thus, the monovalent NKp46 binding component of the multispecific protein does not itself cause NKp46 signalling. Accordingly, in the case of multispecific proteins possessing an Fc domain that binds CD16, such multispecific protein can be assessed for its ability to elicit NKp46 signaling or NKp46-mediated NK cell activation by testing the effect of this multispecific protein on NKp46 expression, by CD16-negative NK cells. The multispecific protein can optionally be characterized as not substantially causing (or increasing) NKp46 signaling by an NKp46-expressing, CD16-negative cell (e.g. a NKp46⁺CD16⁻ NK cell, a reporter cell) when the multispecific protein is incubated with such NKp46-expressing, CD16-negative cells (e.g., purified NK cells or purified reporter cells) in the absence of target cells.

In one aspect of any embodiment herein, a multispecific protein described herein that binds CD16 can for example be characterized by:

(a) being capable of inducing NK cells that express CD16 and NKp46 to lyse target cells, when incubated in the presence of the NK cells and target cells; and

(b) lack of agonist activity at NKp46 when incubated with CD16-negative NK cells, e.g. NKp46-expressing NK cells that do not express CD16, in the absence of target cells. Optionally, the NK cells are purified NK cells.

In one aspect of any embodiment herein, a multispecific protein described herein can for example be characterized by:

(a) agonist activity at NKp46, when incubated in the presence of NKp46-expressing NK cells and target cells; and

(b) lack of agonist activity at NKp46 (e.g. when incubated with CD16-negative NK cells, for example CD16-NKp46⁺ NK cells, or when incubated with NK cells and where in the protein comprises (e.g. by modification) an Fc domain that lacks binding to CD16) in the absence of target cells. Optionally, the NK cells are purified NK cells.

Uses of compounds

In one aspect, provided is the use of any of the compounds defined herein, particularly the inventive multispecific proteins or antibodies and/or cells which express same for the manufacture of a pharmaceutical preparation for the treatment, prevention or diagnosis of a disease in a mammal in need thereof. Provided also are the use any of the compounds defined above as a medicament or an active component or active substance in a medicament. In a further aspect the invention provides methods for preparing a pharmaceutical composition containing a compound as defined herein, to provide a solid or a liquid formulation for administration orally, topically, or by injection. Such a method or process at least comprises the step of mixing the compound with a pharmaceutically acceptable carrier.

In one aspect, provided is a method to treat, prevent or more generally affect a predefined condition in an individual or to detect a certain condition by using or administering a multispecific protein or antibody described herein, or a (pharmaceutical) composition comprising same.

For example, in one aspect, the invention provides a method of restoring or potentiating the activity of NKp46⁺ NK cells (e.g. NKp46⁺CD16⁺ NK cells) in a patient in need thereof (e.g. a patient having a cancer, or a viral or bacterial infection), comprising the step of administering a multispecific protein described herein to said patient. In one embodiment, the method is directed at increasing the activity of NKp46⁺ lymphocytes (e.g. NKp46⁺CD16⁺ NK cells) in patients having a disease in which increased lymphocyte (e.g. NK cell) activity is beneficial or which is caused or characterized by insufficient NK cell activity, such as a cancer, or a viral or microbial/bacterial infection.

In another aspect, the invention provides a method of restoring or potentiating the activity of NKp46⁺ NK cells (e.g. NKp46⁺CD16⁺ NK cells) in a patient in need thereof (e.g. a patient having a cancer, or a viral, parasite or bacterial infection), comprising the step of contacting cells derived from the patient, e.g., immune cells and optionally target cells expressing an antigen of interest with a multispecific protein according to the invention and reinfusing the multispecific protein treated cells into the patient. In one embodiment, this method is directed at increasing the activity of NKp46⁺ lymphocytes (e.g. NKp46⁺CD16⁺ NK cells) in patients having a disease in which increased lymphocyte (e.g. NK cell) activity is beneficial or which is caused or characterized by insufficient NK cell activity, such as a cancer, or a viral or microbial, e.g., bacterial or parasite infection.

In another embodiment the subject multispecific proteins may be used or administered in combination with immune cells, particularly NK cells, derived from a patient

who is to be treated or from a different donor, and these NK cells administered to a patient in need thereof such as a patient having a disease in which increased lymphocyte (e.g. NK cell) activity is beneficial or which is caused or characterized by insufficient NK cell activity, such as a cancer, or a viral or microbial, e.g., bacterial or parasite infection. As NK cells (unlike CAR-T cells) do not express TCRs, these NK cells, even those derived from different donors will not induce a GVHD reaction (see e.g., Glienke et al., "Advantages and applications of CAR-expressing natural killer cells", *Front. Pharmacol.* 6, Art. 21:1-6 (2015); Hermanson and Kaufman, *Front. Immunol.* 6, Art. 195:1-6 (2015))

In one embodiment, the multispecific protein disclosed herein that mediates NK cell activation and/or target cell lysis via multiple activating receptors of effector cells, including NKp46, CD16 and CD137, can be used advantageously for treatment of individuals whose effector cells (e.g. NKp46⁺CD16⁺ NK cells) cells are hypoactive, exhausted or suppressed, for example a patient who has a significant population of effector cells characterized by the expression and/or upregulation of one or multiple inhibitory receptors (e.g. TIM-3, PD1, CD96, TIGIT, etc.).

The multispecific polypeptides described herein can be used to prevent or treat disorders that can be treated with antibodies, such as cancers, solid and non-solid tumors, hematological malignancies, infections such as viral infections, and inflammatory or autoimmune disorders.

In one embodiment, the antigen of interest (the non-NKp46 antigen) is an antigen expressed on the surface of a malignant cell of a type of cancer selected from the group consisting of: carcinoma, including that of the bladder, head and neck, breast, colon, kidney, liver, lung, ovary, prostate, pancreas, stomach, cervix, thyroid and skin, including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hairy cell lymphoma and Burkett's lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyosarcoma; other tumors, including neuroblastoma and glioma; tumors of the central and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyosarcoma, and osteosarcoma; and other tumors, including melanoma, xeroderma pigmentosum, keratoacanthoma, seminoma, thyroid follicular cancer and teratocarcinoma, hematopoietic tumors of lymphoid lineage, for example T-cell and B-cell tumors, including but not limited to T-cell disorders such as T-prolymphocytic leukemia (T-PLL), including of the small cell and cerebriform cell type; large granular lymphocyte

leukemia (LGL) preferably of the T-cell type; Sézary syndrome (SS); Adult T-cell leukemia lymphoma (ATLL); a/d T-NHL hepatosplenic lymphoma; peripheral/post-thymic T cell lymphoma (pleomorphic and immunoblastic subtypes); angio immunoblastic T-cell lymphoma; angiocentric (nasal) T-cell lymphoma; anaplastic (Ki 1+) large cell lymphoma; intestinal T-cell lymphoma; T-lymphoblastic; and lymphoma/leukemia (T-Lbly/T-ALL).

In one embodiment, the inventive multispecific polypeptides described herein can be used to prevent or treat a cancer selected from the group consisting of: carcinoma, including that of the bladder, head and neck, breast, colon, kidney, liver, lung, ovary, prostate, pancreas, stomach, cervix, thyroid and skin, including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hairy cell lymphoma and Burkett's lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyosarcoma; other tumors, including neuroblastoma and glioma; tumors of the central and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyosarcoma, and osteosarcoma; and other tumors, including melanoma, xeroderma pigmentosum, keratoacanthoma, seminoma, thyroid follicular cancer and teratocarcinoma. Other exemplary disorders that can be treated according to the invention include hematopoietic tumors of lymphoid lineage, for example T-cell and B-cell tumors, including but not limited to T-cell disorders such as T-prolymphocytic leukemia (T-PLL), including of the small cell and cerebriform cell type; large granular lymphocyte leukemia (LGL) preferably of the T-cell type; Sézary syndrome (SS); Adult T-cell leukemia lymphoma (ATLL); a/d T-NHL hepatosplenic lymphoma; peripheral/post-thymic T cell lymphoma (pleomorphic and immunoblastic subtypes); angio immunoblastic T-cell lymphoma; angiocentric (nasal) T-cell lymphoma; anaplastic (Ki 1+) large cell lymphoma; intestinal T-cell lymphoma; T-lymphoblastic; and lymphoma/leukaemia (T-Lbly/T-ALL).

In one example, the tumor antigen is an antigen expressed on the surface of a lymphoma cell or a leukemia cell, and the multispecific protein is administered to, and/or used for the treatment of, an individual having a lymphoma or a leukemia. Optionally, the tumor antigen is selected from CD19, CD20, CD22, CD30 or CD33.

In one aspect, the methods of treatment comprise administering to an individual a multispecific protein described herein in a therapeutically effective amount, e.g., for the treatment of a disease as disclosed herein, for example any of the cancers identified above. A therapeutically effective amount may be any amount that has a therapeutic effect in a patient having a disease or disorder (or promotes, enhances, and/or induces such an effect

in at least a substantial proportion of patients with the disease or disorder and substantially similar characteristics as the patient).

In one embodiment, a multispecific protein according to the invention is used to treat a cancer that is responsive to CD137 activation, e.g. a solid tumor or a hematological cancer, including but not limited to breast cancer, sarcoma, glioma, colon carcinoma, myeloma, mastocytoma, melanoma, renal carcinoma, and ovarian cancer. In one embodiment, the multispecific protein according to the invention is used to treat a CD137L-expressing cancer. As shown herein, the strong efficacy in inducing tumor cell lysis of the multispecific protein is hypothesized to in part mediated by the upregulation of CD137 (4-1BB) on the surface of NK cells. The co-activating CD137 protein can bind and recognize CD137 ligand (CD137L, 4-1BBL) on tumor cells, resulting in enhanced NK cell activation and cytotoxicity of CD137L-expressing cells. CD137L has been found to be expressed on a variety of tumors, and is more commonly expressed by malignant tumors, especially in moderate or low-differentiated tumors (see, e.g., Vinay et al., (2012) *Mol. Cancer Ther.* 11(5):1062-1070). CD137L-expressing cancers include, for example, lung squamous cell carcinoma, nasal cavity squamous cell carcinoma, esophageal squamous cell carcinoma, cervical squamous cell carcinoma, colonic adenocarcinoma, rectal adenocarcinoma, gallbladder adenocarcinoma, pancreatic adenocarcinoma and breast adenocarcinoma.

The multispecific protein according to the invention may be used with or without a prior step of detecting the expression of the antigen of interest on target cells in a biological sample obtained from an individual (e.g. a biological sample comprising cancer cells, cancer tissue or cancer-adjacent tissue). In another embodiment, the disclosure provides a method for the treatment or prevention of a cancer in an individual in need thereof, the method comprising:

a) detecting cells (e.g. tumor cells) in a sample from the individual that express an antigen of interest, and

b) upon a determination that cells which express an antigen of interest are comprised in the sample, optionally at a level that is increased compared to a reference level (e.g. corresponding to a healthy individual or an individual not deriving substantial benefit from a protein described herein), administering to the individual a multispecific protein (e.g. a multispecific protein according to the invention) that binds to an antigen of interest, to NKp46 (e.g., monovalently), and to CD16 (e.g., via its Fc domain). Optionally, the antigen of interest is a cancer antigen (e.g. a cancer antigen disclosed herein), optionally a cancer antigen known to be capable of undergoing intracellular internalization when contacted with a full length human IgG1 antibody that binds specifically thereto.

In one embodiment, the invention provides a method for the treatment or prevention

of a cancer in an individual in need thereof, the method comprising:

a) detecting cells (e.g. tumor cells) in a sample from an individual (or within the tumor and/or within adjacent tissue) that express CD137L (CD137 ligand), and

b) upon a determination that cells that express CD137L are comprised in the sample (or within the tumor and/or within adjacent tissue), optionally at a level that is increased compared to a reference level (e.g. corresponding to a healthy individual or an individual not deriving substantial benefit from a protein described herein), administering to the individual a multispecific protein (e.g. a protein of the disclosure) that binds to a cancer antigen, to NKp46 (e.g., monovalently), and to CD16. Optionally, the cancer antigen is a cancer antigen disclosed herein), optionally a cancer antigen known to be capable of undergoing intracellular internalization when contacted with a full length human IgG1 antibody that binds specifically thereto.

In one embodiment, the disclosure provides a method for the treatment or prevention of a disease (e.g. a cancer) in an individual in need thereof, the method comprising:

a) detecting cell surface expression of one or a plurality inhibitory receptors on immune effector cells (e.g. NK cells, T cells) in a sample from the individual (e.g. in circulation or in the tumor environment), and

b) upon a determination of cell surface expression of one or a plurality inhibitory receptors on immune effector cells, optionally at a level that is increased compared to a reference level (e.g. corresponding to a healthy individual, an individual not suffering from immune exhaustion or suppression, or an individual not deriving substantial benefit from a protein described herein), administering to the individual a multispecific protein (e.g. a multispecific protein according to the invention) that binds to an antigen of interest (e.g. a cancer antigen), to NKp46 (e.g., monovalently), and to CD16. Optionally, the cancer antigen is a cancer antigen disclosed herein), optionally a cancer antigen known to be capable of undergoing intracellular internalization when contacted with a full length human IgG1 antibody that binds specifically thereto.

In one embodiment, a multispecific protein according to the invention may be used as a monotherapy (without other therapeutic agents), or in combined treatments with one or more other therapeutic agents, including agents normally utilized for the particular therapeutic purpose for which the antibody is being administered. The additional therapeutic agent or agents will normally be administered in amounts and treatment regimens typically used for that agent in a monotherapy for the particular disease or condition being treated. Such therapeutic agents when used in the treatment of cancer, include, but are not limited to anti-cancer agents and chemotherapeutic agents; in the treatment of infectious disease, include, but are not limited to anti-viral agents and antibiotics. Such other therapeutic agents

may further include other immunomodulatory polypeptides such as Ig-fusion proteins, antibodies, cytokines and the like. In some embodiments the administration of the multispecific protein according to the invention and the other therapeutic agent may elicit an additive or synergistic effect on immunity and/or on therapeutic efficacy.

5 The multispecific proteins can also be included in kits. The kits may optionally further contain any number of polypeptides and/or other compounds, e.g., 1, 2, 3, 4, or any other number of multispecific proteins according to the invention and/or other compounds. It will be appreciated that this description of the contents of the kits is not limiting in any way. For example, the kit may contain other types of therapeutic compounds. Optionally, the kits also
10 include instructions for using the polypeptides, e.g., detailing the herein-described methods such as in the detection or treatment of specific disease conditions.

 The invention also provides pharmaceutical compositions comprising the subject multispecific proteins and optionally other compounds as defined above. A multispecific protein and optionally another compound may be administered in purified form together with
15 a pharmaceutical carrier as a pharmaceutical composition. The form depends on the intended mode of administration and therapeutic or diagnostic application. The pharmaceutical carrier can be any compatible, nontoxic substance suitable to deliver the compounds to the patient. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as (sterile) water or physiologically
20 buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters, alcohol, fats, waxes, and inert solids. A pharmaceutically acceptable carrier may further contain physiologically acceptable compounds that act for example to stabilize or to increase the absorption of the compounds. Such physiologically
25 acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition. Pharmaceutically acceptable adjuvants, buffering agents, dispersing agents, and the like,
30 may also be incorporated into the pharmaceutical compositions. Non-limiting examples of such adjuvants include by way of example inorganic and organic adjuvants such as alum, aluminum phosphate and aluminum hydroxide, squalene, liposomes, lipopolysaccharides, double stranded (ds) RNAs, single stranded(s-s) DNAs, and TLR agonists such as unmethylated CpG's.

35 Multispecific proteins according to the invention can be administered parenterally. Preparations of the compounds for parenteral administration must be sterile. Sterilization is

readily accomplished by filtration through sterile filtration membranes, optionally prior to or following lyophilization and reconstitution. The parenteral route for administration of compounds is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, intramuscular, intraarterial, or intralesional routes. The compounds may be administered continuously by infusion or by bolus injection. A typical composition for intravenous infusion could be made up to contain 100 to 500 ml of sterile 0.9% NaCl or 5% glucose optionally supplemented with a 20% albumin solution and 1 mg to 10 g of the compound, depending on the particular type of compound and its required dosing regimen. Methods for preparing parenterally administrable compositions are well known in the art.

Examples

Example 1:

Generation of anti-huNKp46 antibodies

Balb/c mice were immunized with a recombinant human NKp46 extracellular domain recombinant-Fc protein comprising the extracellular domain of the protein of SEQ ID NO: 1. Mice received one primo-immunization with an emulsion of 50 µg NKp46 protein and Complete Freund Adjuvant, intraperitoneally, a 2nd immunization with an emulsion of 50 µg NKp46 protein and Incomplete Freund Adjuvant, intraperitoneally, and finally a boost with 10 µg NKp46 protein, intravenously. Immune spleen cells were fused 3 days after the boost with X63.Ag8.653 immortalized B cells, and cultured in the presence of irradiated spleen cells.

Primary screen: Supernatant (SN) of growing clones were tested in a primary screen by flow cytometry using a cell line expressing the human NKp46 construct at the cell surface. Briefly, for FACS screening, the presence of reactive antibodies in supernatants was revealed by Goat anti-mouse polyclonal antibody (pAb) labeled with PE.

A panel of antibodies that bound NKp46 was selected, produced and their variable regions sequenced and these antibodies and derivatives thereof further evaluated for their activity in the context of a bispecific molecule.

Example 2:

Identification of a bispecific antibody format that binds FcRn but not FcγR for targeting effector cell receptors

Experiments were conducted with the objective being the development of a new bispecific protein format that places an Fc domain on a polypeptide together with an anti-NKp46 binding domain and an anti-target antigen binding domain. Such bispecific proteins

should bind to NKp46 monovalently via its anti-NKp46 binding domain. The monomeric Fc domain should retain at least partial binding to the human neonatal Fc receptor (FcRn), yet not substantially bind human CD16 and/or other human Fc γ receptors. Consequently, such bispecific proteins should not induce Fc γ -mediated (e.g. CD16-mediated) target cell lysis.

Example 2-1 Construction and binding analysis of Anti-CD19-IgG1-Fcmono-Anti-CD3

Since no anti-NKp46 bispecific antibody has been produced that could indicate whether such a protein could be functional, CD3 was used as a model antigen in place of NKp46 in order to investigate the possible functionality of a new monovalent bispecific protein format prior to targeting NK cells via NKp46.

A bispecific Fc-based on a scFv specific for tumor antigen CD19 (anti-CD19 scFv) and a scFv specific for activating receptor CD3 on a T cell (anti-CD3 scFv) was used to assess FcRn binding and CD19-binding functions of a new monomeric bispecific polypeptide format. The domain arrangement of the final polypeptide is referred to as the "F1" format (the star in the CH2 domain indicates an optional N297S mutation, not included in the polypeptide tested here). (See **Figure 2**)

A bispecific monomeric Fc-containing polypeptide was constructed based on a scFv specific for the tumor antigen CD19 (anti-CD19 scFv) and a scFv specific for an activating receptor CD3 on a T cell (anti-CD3 scFv). The CH3 domain incorporated the mutations (EU numbering) L351K, T366S, P395V, F405R, T407A and K409Y. The polypeptide has domains arranged as follows: anti-CD19-CH2-CH3-anti-CD3. A DNA sequence coding for a CH3/VH linker peptide having the amino acid sequence STGS was also designed in order to insert a specific Sall restriction site at the CH3-VH junction.

This CH3 domain incorporated the mutations (EU numbering) L351K, T366S, P395V, F405R, T407A and K409Y. The selected CH2 domain was a wild-type CH2. DNA and amino acid sequences for the monomeric CH2-CH3 Fc portion and the anti-CD19 are shown below.

The light chain and heavy chain DNA and amino acid sequences corresponding to the anti-CD19 scFv were as follows:

Sequence	SEQ ID NO
Anti-CD19-V _k DNA	113
Anti-CD19-V _k amino acid	114
Anti-CD19-V _H DNA	115
Anti-CD19-V _H amino acid	116

The DNA sequences for the monomeric CH2-CH3 Fc portion and final bispecific IgG1-Fcmono polypeptide (the last K was removed in that construct) is shown in SEQ ID NO: 117. The amino acid sequence encoded thereby is shown in SEQ ID NO: 2. The Anti-CD19-F1-Anti-CD3 complete sequence (mature protein) is shown in SEQ ID NO: 118.

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Cloning and the production of the recombinant proteins

Coding sequences were generated by direct synthesis and/or by PCR. PCR was performed using the PrimeSTAR MAX DNA polymerase (Takara, #R045A) and PCR products were purified from 1% agarose gel using the NucleoSpin gel and PCR clean-up kit (Macherey-Nagel, #740609.250). Once purified the PCR products were quantified prior to the In-Fusion ligation reaction which was performed as described in the manufacturer's protocol (ClonTech, #ST0345). The plasmids were obtained after a miniprep preparation run on an EVO200 (Tecan) using the Nucleospin 96 plasmid kit (Macherey-Nagel, #740625.4). Plasmids were then sequenced for sequence confirmation before to transfecting the CHO cell line.

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CHO cells were grown in the CD-CHO medium (Invitrogen) complemented with phenol red and 6 mM GlutaMax. The day before the transfection, cells were counted and seeded at 175,000 cells/ml. For the transfection, cells (200.000 cells/transfection) were prepared as described in the AMAXA SF cell line kit (AMAXA, #V4XC-2032) and nucleofected using the DS137 protocol with the Nucleofector 4D device. All the transfections were performed using 300 ng of verified plasmids. After transfection, cells were seeded into 24 well plates in pre-warmed culture medium. After 24 hours, hygromycin B was added in the culture medium (200 µg/ml). Protein expression was monitored after one week in culture. Cells expressing the proteins were then sub-cloned to obtain the best producers. Sub-cloning was performed using 96 flat-bottom well plates in which the cells are seeded at one cell per well into 200 µl of culture medium complemented with 200 µg/ml of hygromycin B. Cells were left for three weeks before testing the clone's productivity.

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Recombinant proteins which contain an IgG1-Fc fragment were purified using Protein-A beads (rProteinA Sepharose fast flow, GE Healthcare). Briefly, cell culture supernatants were concentrated, clarified by centrifugation and injected onto Protein-A columns to capture the recombinant Fc containing proteins. Proteins were eluted at acidic pH (citric acid 0.1M pH 3), and the eluate immediately neutralized using TRIS-HCL pH 8.5 and dialyzed against 1X PBS. Recombinant scFvs which contain a "six his" tag were purified by affinity chromatography using Cobalt resin. Other recombinant scFvs were purified by size exclusion chromatography (SEC).

Example 2-2: Binding analysis of Anti-CD19-IgG1-Fcmono-Anti-CD3 to B221, JURKAT, HUT78 and CHO cell lines

Cells were harvested and stained with the cell supernatant of the anti-CD19-F1-anti-CD3 producing cells during 1 h at 4°C. After two washes in staining buffer (PBS1X / BSA 0.2% / EDTA 2mM), cells were stained for 30 min at 4°C with goat anti-human (Fc)-PE antibody (IM0550 Beckman Coulter - 1/200). After two washes, stainings were conducted on a BD FACS Canto II and analyzed using the FlowJo software.

CD3 and CD19 expression were also controlled by flow cytometry: Cells were harvested and stained in PBS1X / BSA 0.2% / EDTA 2mM buffer during 30 min at 4°C using 5µl of the anti-CD3-APC and 5µl of the anti-CD19-FITC antibodies. After two washes, stainings were conducted on a BD FACS Canto II and analyzed using the FlowJo software.

The results of these experiments revealed that the Anti-CD19-F1-Anti-CD3 protein binds to CD3 cell lines (HUT78 and JURKAT cell lines) and to the CD19 cell line (B221 cell line) but not to the CHO cell line which was used as a negative control.

Example 2-3:

T- and B- cell aggregation by purified Anti-CD19-F1-Anti-CD3

Purified Anti-CD19-F1-Anti-CD3 was tested in a T/B cell aggregation assay to evaluate whether the antibody promotes the aggregation of CD19 and CD3 expressing cells.

The results of this assay are shown in **Figure 1**. The top panel shows that Anti-CD19-F1-Anti-CD3 does not cause aggregation in the presence of B221 (CD19) or JURKAT (CD3) cell lines, but it does cause aggregation of cells when both B221 and JURKAT cells are co-incubated, indicating that the bispecific antibody is functional. The lower panel shows the results of the control experiment conducted without antibody.

Example 2-4:

Binding of bispecific monomeric Fc polypeptide to FcRn

Affinity study by Surface Plasmon Resonance (SPR)

Biacore T100 general procedure and reagents

SPR measurements were performed on a Biacore T100 apparatus (Biacore GE Healthcare) at 25°C. In all Biacore experiments Acetate Buffer (50 mM Acetate pH5.6, 150 mM NaCl, 0.1% surfactant p20) and HBS-EP+ (Biacore GE Healthcare) were used as the running buffer and regeneration buffer respectively. Sensorgrams were analyzed with

Biacore T100 Evaluation software. Recombinant mouse FcRn was purchased from R&D Systems.

Immobilization of FcRn

Recombinant FcRn proteins were immobilized covalently to carboxyl groups in the dextran layer on a Sensor Chip CM5. The chip surface was activated with EDC/NHS (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimidehydrochloride and N-hydroxysuccinimide (Biacore GE Healthcare)). FcRn proteins were diluted to 10 µg/ml in coupling buffer (10 mM acetate, pH 5.6) and injected until the appropriate immobilization level was reached (i.e. 2500 RU). Deactivation of the remaining activated groups was performed using 100 mM ethanolamine pH 8 (Biacore GE Healthcare).

Affinity study

Monovalent affinity study was conducted following the Single Cycle Kinetic (SCK) protocol. Five serial dilutions of soluble analytes (antibodies and bi-specific molecules) ranging from 41.5 to 660 nM were injected over the FcRn (without regeneration) and allowed to dissociate for 10 min before regeneration. For each analyte, the entire sensorgram was fitted using the 1:1 SCK binding model.

Results

Anti-CD19-F1-Anti-CD3 having its CH2-CH3 domains placed between two antigen binding domains, particularly two scFvs, was evaluated to assess whether such bispecific monomeric Fc protein could retain binding to FcRn and possess an improved *in vivo* half-life compared to conventional bispecific antibodies. The results of these experiments showed that FcRn binding was retained, the model suggesting a 1:1 ratio (1 FcRn for each monomeric Fc) instead of a 2:1 ratio (2 FcRn for each antibody) for a regular or wild-type IgG.

The binding affinity of this multispecific protein was evaluated using SPR, and was compared to a chimeric full length antibody containing intact human IgG1 constant regions. The monomeric Fc retained significant monomeric binding to FcRn (monomeric Fc: affinity of KD=194 nM; full length antibody with bivalent binding: avidity of KD=15.4 nM).

Example 3:

Construction of Anti-CD19 x anti-NKp46 bispecific monomeric Fc domain polypeptides

It was unknown what activating receptors on NK cells would contribute to the lysis of target cells, and moreover since anti-NKp46 antibodies may block NKp46, it was further unknown whether cytotoxicity could be mediated by NKp46. We therefore investigated whether the bispecific protein format could induce NKp46 triggering, and whether it would

induce NKp46 agonism in the absence of target cells, which could lead to inappropriate NK activation distant from the target and/or decreased overall activity toward target cells.

A new bispecific protein format was developed as a single chain protein which binds to FcRn but not Fc γ R. Additionally, multimeric proteins that comprise two or three polypeptide chains, wherein the Fc domain remains monomeric, were developed that are compatible for use with antibody variable regions that do not maintain binding to their target when converted to scFv format. The latter formats can be used conveniently for antibody screening; by incorporating at least one binding region as a F(ab) structure, any anti-target (e.g. anti-tumor) antibody variable region can be directly expressed in a bispecific construct as the F(ab) format within the bispecific protein and tested, irrespective of whether the antibody would retain binding as an scFv, thereby simplifying screening and enhancing the number of antibodies available. These formats in which the Fc domain remains monomeric have the advantage of maintaining maximum conformational flexibility and as shown *infra* may permit optimal binding to NKp46 or target antigens.

Different constructs were made for use in the preparation of bispecific antibodies using the variable domains from the scFv specific for tumor antigen CD19 described in Example 2-1, and different variable regions from antibodies specific for the NKp46 receptor identified in Example 1. A construct was also made using as the anti-NKp46 the variable regions from a commercially available antibody Bab281 (mIgG1, available commercially from Beckman Coulter, Inc. (Brea, CA, USA) (see also Pessino et al, *J. Exp. Med.*, 1998, 188 (5): 953-960 and Sivori et al, *Eur J Immunol*, 1999. 29:1656-1666) specific for the NKp46 receptor.

In order for the Fc domain to remain monomeric in single chain polypeptides or in multimers in which only one chain had an Fc domain, CH3-CH3 dimerization was prevented through two different strategies: (1) through the use of CH3 domain incorporating specific mutations (EU numbering), i.e., L351K, T366S, P395V, F405R, T407A and K409Y; or (2) through the use of a tandem CH3 domain in which the tandem CH3 domains are separated by a flexible linker associated with one another, which prevents interchain CH3-CH3 dimerization. The DNA and amino acid sequences for the monomeric CH2-CH3 Fc portion containing the above-identified point mutations were the same as in Example 2-1. The DNA and amino acid sequences for the monomeric CH2-CH3-linker-CH3 Fc portion with tandem CH3 domains are shown in **Figures 2A-2D**.

The light chain and heavy chain DNA and amino acid sequences for the anti-CD19 scFv were also the same as in Example 2-1. Proteins were cloned, produced and purified as in Example 2-1. Shown below are the light chain and heavy chain DNA and amino acid sequences for different anti-NKp46 scFvs.

Table 1: Amino acid sequences of different anti-NKp46 scFvs

scFv	scFV sequence (VHVK) / - stop
anti-NKp46	
NKp46-1	STGSQVQLQQSGPELVKPGASVKMSCKASGYTFTDYVINWGKQRSGQ GLEWIGEIYPGSGTNYNNEKFKAKATLTADKSSNIAYMQLSSLTSEDSAV YFCARRGRYGLYAMDYWGQGTSTVTVSSVEGGSGGSGGSGGSGGVDD IQMTQTTSSLSASLGDRVTISCRASQDISNYLNWYQQKPDGTVKLLIYYT SRLHSGVPSRFSGSGSGTGYSLTINNLEQEDIATYFCQQGNTRPWTFG GGTKLEIK- (SEQ ID NO: 119)
NKp46-2	STGSEVQLQESGPGLVKPSQSLTCTVTGYSITSDYAWNWIWQFPGNK LEWMGYITYSGSTSYNPSLESRISITRDTSTNQFFLQLNSVTTEDTATYY CARGGYGSSWGVFAYWGQGTSLTVSAVEGGSGGSGGSGGSGGVDD DIQMTQSPASLSASVGETVTITCRVSENIYSYLAWEYQQKQKSPQLLVY NAKTLAEGVPSRFSGSGSGTQFSLKINSLQPEDFGSYQCQHHYGTPTWT FGGGTKLEIK- (SEQ ID NO: 120)
NKp46-3	STGSEVQLQQSGPELVKPGASVKISCKTSGYTFTTEYTMHWVKQSHGKS LEWIGGISPNIGGTSYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVY YCARRGGSFDYWGQGTSLTVSSVEGGSGGSGGSGGSGGVDDIVMTQ SPATLSVTPGDRVSLSCRASQSISDYLHWYQQKSHESPRLLIKYASQSIG GIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHSFPLTFGAGTKLE LK- (SEQ ID NO: 121)
NKp46-4	STGSQVQLQQSAVELARPGASVKMSCKASGYTFTSFTMHWVKQRPGQ GLEWIGYINPSSGYTEYNQKFKDKTTLTADKSSSTAYMQLDSLTSDDSA VYYCVRGSSRGFDYWGQGTSLTVSAVEGGSGGSGGSGGSGGVDDIQ MIQSPASLSVSVGETVTITCRASENIYSNLAWFQQKQKSPQLLVYAATN LADGVPSRFSGSGSGTQYSLKINSLQSEDFGIYYCQHFWGTPRTFGGG TKLEIK- (SEQ ID NO: 122)
NKp46-6	STGSQVQLQQPGSVLVRPGASVKLSCKASGYTFTSSWMHWAKQRPGQ GLEWIGHIHPNSGISNYNEKFKGKATLTVDTSSTAYVDLSSLTSEDSAV YYCARGGRFDDWGAGTTVTVSSVEGGSGGSGGSGGSGGVDDIVMTQ SPATLSVTPGDRVSLSCRASQSISDYLHWYQQKSHESPRLLIKYASQSIG GIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHSFLMYTFGGGTL EIK- (SEQ ID NO: 123)
NKp46-9	STGSDVQLQESGPGLVKPSQSLTCTVTGYSITSDYAWNWIWQFPGNK LEWMGYITYSGSTNYNPSLKSRIITRDTSKNQFFLQLNSVTTEDTATYY CARCWDYALYAMDCWGQGTSTVTVSSVEGGSGGSGGSGGSGGVDDIQ MTQSPASLSASVGETVTITCRTSENIYSYLAWCQQKQKSPQLLVYNAK TLAEGVPSRFSGSGSGTHFSLKINSLQPEDFGIYYCQHHYDTPLTFGAG TKLELK- (SEQ ID NO: 124)
Bab281	STGSQIQLVQSGPELQKPGETVKISCKASGYTFTNYGMNWVKQAPGKG LKWIMGWINTNTGEPTYAEFEKGRFAFSLETSAAYLQINNLIKNEEDTAT YFCARDYLYYFDYWGQGTSLTVSSVEGGSGGSGGSGGSGGVDDNIVMT QSPKSMMSVGERVTLTCKASENVVTYVSWYQQKPEQSPKLLIYGASN RYTGVPDRFTGSGSATDFTLTISVQAEDLADYHCGQGYSYPYTFGGG TKLEIK- (SEQ ID NO: 125)

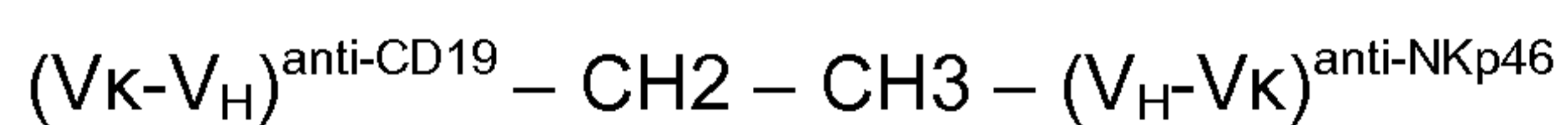
Table 2A: DNA sequences corresponding to different anti-NKp46 scFvs

scFv	anti-	scFV sequences
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NKp46	
NKp46-1	SEQ ID NO: 126
NKp46-2	SEQ ID NO: 127
NKp46-3	SEQ ID NO: 128
NKp46-4	SEQ ID NO: 129
NKp46-6	SEQ ID NO: 130
NKp46-9	SEQ ID NO: 131
Bab281	SEQ ID NO: 132

Format 1 (F1) (Anti-CD19-IgG1-Fcmono-Anti-NKp46 (scFv))

The domain structure of Format 1 (F1) is shown in **Figure 2A**. A bispecific Fc-containing polypeptide was constructed based on a scFv specific for the tumor antigen CD19 (anti-CD19 scFv) and an scFV specific for the NKp46 receptor. The polypeptide is a single chain polypeptide having domains arranged (N- to C- termini) as follows:



A DNA sequence coding for a CH3/VH linker peptide having the amino acid sequence STGS was designed in order to insert a specific Sall restriction site at the CH3-V_H junction. The domain arrangement of the final polypeptide is shown in **Figure 2** (the star in the CH2 domain indicates an optional N297S mutation), where the anti-CD3 scFv is replaced by an anti-NKp46 scFv. The (V_K-V_H) units include a linker between the V_H and V_K domains. Proteins were cloned, produced and purified as in Example 2-1. The amino acid sequences of the bispecific polypeptides (complete sequence (mature protein)) are shown in the corresponding SEQ ID NOS listed in the Table 2B below.

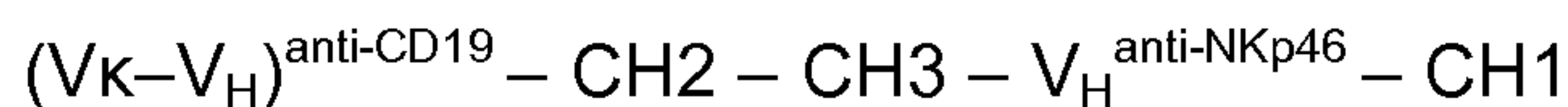
Table 2B

Sequence	SEQ ID NO
CD19-F1-NKp46-1	133
CD19-F1-NKp46-2	134
CD19-F1-NKp46-3	135
CD19-F1-NKp46-4	136
CD19-F1-NKp46-6	137
CD19-F1-NKp46-9	138
CD19-F1-Bab281	139

Format 2 (F2) : CD19-F2-NKp46-3

The domain structure of F2 polypeptides is shown in **Figure 2A**. The DNA and amino acid sequences for the monomeric CH2-CH3 Fc portion were as in Example 2-1 and it similarly contains CH3 domain mutations (the mutations (EU numbering) L351K, T366S, P395V, F405R, T407A and K409Y. The heterodimer is made up of:

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):



and

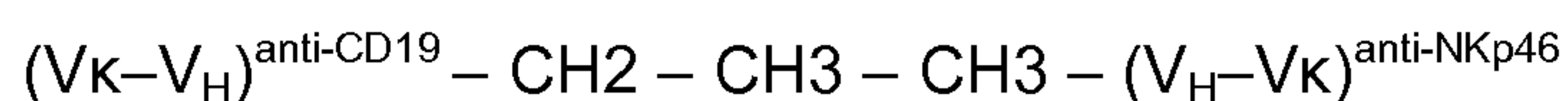
(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): $V_K^{\text{anti-NKp46}} - \text{CK}$.

The (V_K-V_H) unit was made up of a V_H domain, a linker and a V_K unit (i.e. an scFv). As with other formats of the inventive bispecific polypeptides, the DNA sequence coded for a CH3/VH linker peptide having the amino acid sequence STGS designed in order to insert a specific Sall restriction site at the CH3-VH junction. Proteins were cloned, produced and purified as in Example 2-1. The amino acid sequences for the first and second chains of the F2 protein are shown in SEQ ID NO: 140 and 141.

Format 3 (F3): CD19-F3-NKp46-3

The domain structure of F3 polypeptides is shown in **Figure 2A**. The DNA and amino acid sequences for the CH2-CH3 Fc portion comprised a tandem CH3 domain in which the two CH3 domains on the same polypeptide chain associated with one another, thereby preventing dimerization between different bispecific proteins.

The single chain polypeptide has domains arranged (N- to C-termini) as follows:



The (V_K-V_H) units were made up of a V_H domain, a linker and a V_K unit (scFv). Proteins were cloned, produced and purified as in Example 2-1. Bispecific protein was purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a high production yield of 3.4 mg/L and the purified proteins exhibited a simple SEC profile. The amino acid sequence for the F3 protein is shown in SEQ ID NO: 142.

Format 4 (F4): CD19-F4-NKp46-3

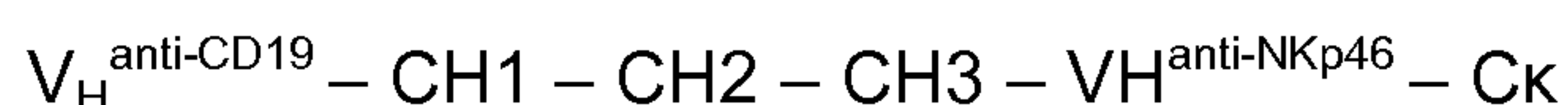
The domain structure of F4 polypeptides is shown in **Figure 2A**. The DNA and amino acid sequences for the CH2-CH3 Fc portion comprised a tandem CH3 domain as in Format F3, and additionally comprise a N297S mutation which prevents N-linked glycosylation and

abolishes FcγR binding. Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins was purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a good production yield of 1mg/L and the purified proteins exhibited a simple SEC profile. The amino acid sequence for the F4 protein with NKp46-3 variable domains is shown in SEQ ID NO: 143.

Format 8 (F8)

The domain structure of F8 polypeptides is shown in **Figure 2B**. The DNA and amino acid sequences for the monomeric CH2-CH3 Fc portion were as in Format F2 and it similarly contains CH3 domain mutations (the mutations (EU numbering) L351K, T366S, P395V, F405R, T407A and K409Y, as well as a N297S mutation which prevents N-linked glycosylation and moreover abolishes FcγR binding. Three variants of F8 proteins were produced: (a) one wherein the cysteine residues in the hinge region were left intact (wild-type, referred to as F8A), (b) a second wherein the cysteine residues in the hinge region were replaced by serine residues (F8B), and (c) a third including a linker sequence GGGSS replacing residues DKTHTCPPCP in the hinge (F8C). Variants F8B and F8C provided production advantages as these versions avoided the formation of homodimers of the central chain. This heterotrimer is made up of;

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):

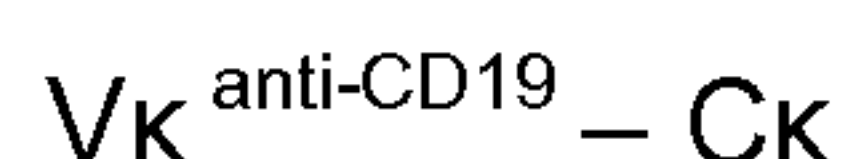


and

(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): $V_K^{\text{anti-NKp46}} - \text{CH1}$

and

(3) a third polypeptide chain having domains arranged as follows (N- to C-termini):



Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins was purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a high production yield of 3.7 mg/L (F8C) and the purified proteins again exhibited a simple SEC profile. The amino acid sequences of the three chains of the F8 protein (C variant) with NKp46-3 variable regions are shown in SEQ ID NOS: 144, 145 and 146.

Format 9 (F9): CD19-F9-NKp46-3

The F9 polypeptide is a trimeric polypeptide having a central polypeptide chain and two polypeptide chains each of which associate with the central chain via CH1-Ck dimerization. The domain structure of the trimeric F9 protein is shown in **Figure 2B**, wherein the bonds between the CH1 and Ck domains are interchain disulfide bonds. The two antigen binding domains have a F(ab) structure permitting the use of these antibodies irrespective of whether they remain functional in a scFv format. The DNA and amino acid sequences for the CH2-CH3 Fc portion comprise a tandem CH3 domain as in Format F4 and comprise a CH2 domain comprising a N297S substitution. Three variants of F9 proteins were produced: (a) a first wherein the cysteine residues in the hinge region left intact (wild-type, referred to as F9A), (b) a second wherein the cysteine residues in the hinge region were replaced by serine residues (F9B), and (c) a third containing a linker sequence GGGSS which replaces residues DKTHTCPPCP in the hinge (F9C). Variants F9B and F9C provided advantages in production by avoiding the formation of homodimers of the central chain. The heterotrimer is made up of:

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):

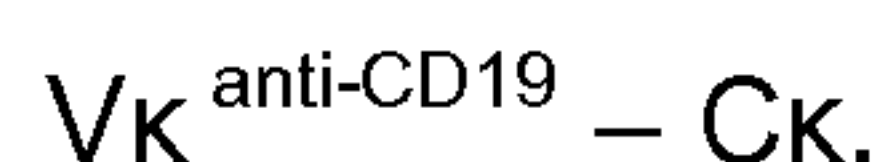


and

(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): $V_K^{\text{anti-NKp46}} - \text{CH1}$

and

(3) a third polypeptide chain having domains arranged as follows (N- to C-termini):



Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins was purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a high production yield of 8.7 mg/L (F9A) and 3.0 mg/L (F9B), and the purified proteins again exhibited a simple SEC profile.

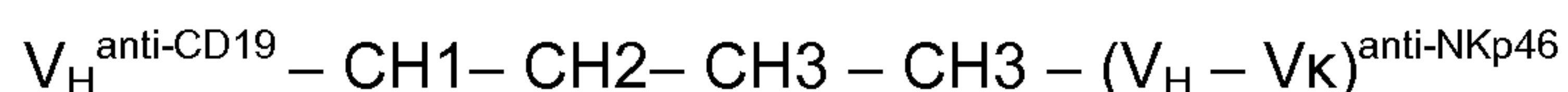
The amino acid sequences of the three chains of the F9 protein variant F9A are shown in the SEQ ID NOS: 147, 148 and 149. The amino acid sequences of the three chains of the F9 protein variant F9B are shown in the SEQ ID NOS: 150, 151 and 152. The amino acid sequences of the three chains of the F9 protein variant F9C are shown in the SEQ ID NOS: 153, 154 and 155.

Format 10 (F10): CD19-F10-NKp46-3

The F10 polypeptide is a dimeric protein having a central polypeptide chain and a second polypeptide chain which associates with the central chain via CH1-Ck dimerization.

The domain structure of the dimeric F10 protein is shown in **Figure 2B** wherein the bonds between the CH1 and Cκ domains are interchain disulfide bonds. One of the two antigen binding domains has a Fab structure, and the other is a scFv. The DNA and amino acid sequences for the CH2-CH3 Fc portion comprise a tandem CH3 domain as shown in Format F4 and comprise a CH2 domain containing a N297S substitution. Three variants of F10 proteins were also produced: (a) a first wherein the cysteine residues in the hinge region were left intact (wild-type, referred to as F10A), (b) a second wherein the cysteine residues in the hinge region were replaced by serine residues (F10B), and (c) a third containing a linker sequence GGGSS replacing residues DKTHTCPPCP in the hinge (F10C). Variants F10B and F10C provided advantages in production as they avoid the formation of homodimers of the central chain. The (Vκ-V_H) unit was made up of a V_H domain, a linker and a Vκ unit (scFv). The heterodimer is made up of:

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):



and

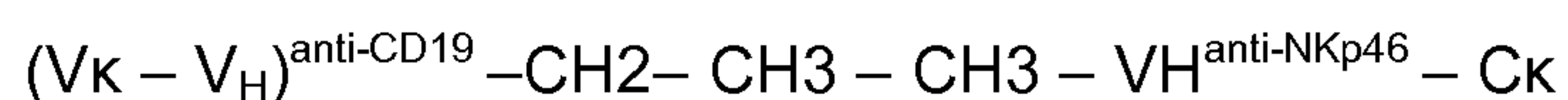
(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): Vκ^{anti-CD19} - Cκ.

These proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins was purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a good production yield of 2 mg/L (F10A) and the purified proteins again exhibited a simple SEC profile. The amino acid sequences of the two chains of the F10A protein variant are shown in SEQ ID NOS: 156 (second chain) and 157 (first chain). The amino acid sequences of the two chains of the F10B protein variant are shown in SEQ ID NOS: 158 (second chain) and 159 (first chain). The amino acid sequences of the two chains of the F10C protein variant are shown in the SEQ ID NOS: 160 (second chain) and 161 (first chain).

Format 11 (F11): CD19-F11-NKp46-3

The domain structure of F11 polypeptides is shown in **Figure 2C**. The heterodimeric protein is similar to F10 except that the structures of the antigen binding domains are reversed. One of the two antigen binding domains has a Fab-like structure, and the other is a scFv. The heterodimer is made up of :

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):



and

(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): $V_K^{\text{anti-NKp46}} - CH1$.

Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins was purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a good production yield of 2 mg/L and the purified proteins similarly exhibited a simple SEC profile. The amino acid sequences of the two chains of the F11 protein are shown in SEQ ID NO: 162 (chain 1) and SEQ ID NO: 163 (chain 2).

Format 12 (F12): CD19-F12-NKp46-3

The domain structure of the dimeric F12 polypeptides is shown in **Figure 2C**, wherein the bonds between the CH1 and Ck domains are disulfide bonds. The heterodimeric protein is similar to F11 but the CH1 and Ck domains within the F(ab) structure are inversed.

The heterodimer is made up of:

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):

$(V_K - V_H)^{\text{anti-CD19}} - CH2 - CH3 - CH3 - V_H^{\text{anti-NKp46}} - CH1$

and

(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): $V_K^{\text{anti-NKp46}} - Ck$.

Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins were purified from the cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a good production yield of 2.8 mg/L and the purified proteins similarly exhibited a simple SEC profile. The amino acid sequences of the two chains of the F12 protein are shown in SEQ ID NO: 164 (chain 1) and SEQ ID NO: 165 (chain 2).

Format 17 (F17): CD19-F17-NKp46-3

The domain structure of the trimeric F17 polypeptides is shown in **Figure 2C**, wherein the bonds between the CH1 and Ck domains are disulfide bonds. The heterodimeric protein is similar to F9 but the V_H and V_K domains, and the CH1 and Ck, domains within the C-terminal F(ab) structure are each respectively inversed with their partner. The heterotrimer is made up of:

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):

$V_H^{\text{anti-CD19}} - \text{CH1} - \text{CH2} - \text{CH3} - \text{CH3} - V_K^{\text{anti-NKp46}} - \text{CH1}$

and

(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): $V_H^{\text{anti-NKp46}} - C_K$

and

(3) a third polypeptide chain having domains arranged as follows (N- to C-termini): $V_K^{\text{anti-CD19}} - C_K$

Additionally, three variants of F17 proteins were produced: (a) a first where the cysteine residues in the hinge region were left intact (wild-type, referred to as F17A), (b) a second wherein the cysteine residues in the hinge region were replaced by serine residues (F10B, and (c) a third containing a linker sequence GGGSS which replaces residues DKTHTCPPCP in the hinge (F17C). Proteins were cloned, produced and purified as in Example 2-1. The amino acid sequences of the three chains of the F17B protein are shown in SEQ ID NOS: 166, 167 and 168.

Example 4:

Bispecific NKp46 antibody formats with dimeric Fc domains

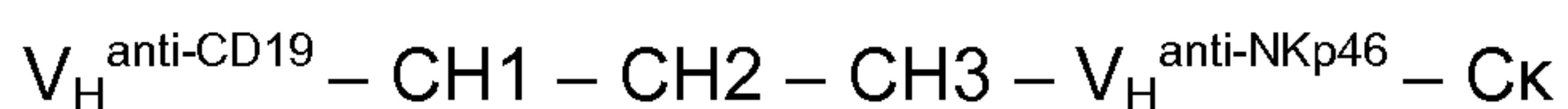
New protein constructions with dimeric Fc domains were developed that share many of the advantages of the monomeric Fc domain proteins of Example 3 but bind to FcRn with greater affinity. Different protein formats were produced that either had low or substantially lack of binding to FcγR (including CD16) or which had binding to FcγRs (including CD16), e.g. the binding affinity to human CD16 was within 1-log of that of wild-type human IgG1 antibodies, as assessed by SPR (e.g. see methods of Example 16. The different polypeptide formats were tested and compared to investigate the functionality of heterodimeric proteins comprising a central chain with a (V_H -(CH1/C_K)-CH2-CH3-) unit or a (V_K -(CH1 or C_K)-CH2-CH3-) unit. One of both of the CH3 domains are fused, optionally via intervening amino acid sequences or domains, to a variable domain(s) (a single variable domain that associates with a variable domain on a separated polypeptide chain, a tandem variable domain (e.g., an scFv), or a single variable domain that is capable of binding antigen as a single variable domain). The two chains associate by CH1-C_K dimerization to form disulfide linked dimers, or if associated with a third chain, to form trimers.

Different constructs were made for use in the preparation of a bispecific antibody using the variable domains DNA and amino acid sequences derived from the scFv specific for tumor antigen CD19 described in Example 2-1 and different variable regions from antibodies specific for NKp46 identified in Example 1. Proteins were cloned, produced and purified as in Example 2-1. Domains structures are shown in **Figures 2A-6D**.

Format 5 (F5): CD19-F5-NKp46-3

The domain structure of the trimeric F5 polypeptide is shown in **Figure 2D**, wherein the interchain bonds between hinge domains (indicated in the figures between CH1/C κ and CH2 domains on a chain) and interchain bonds between the CH1 and C κ domains are interchain disulfide bonds. The heterotrimer is made up of:

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):



and

(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): $V_K^{\text{anti-CD19}} - \text{C}\kappa - \text{CH2} - \text{CH3}$

and

(3) a third polypeptide chain having domains arranged as follows (N- to C-termini):



Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins was purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a high production yield of 37 mg/L and the purified proteins again exhibited a simple SEC profile. The amino acid sequences of the three polypeptide chains are shown in SEQ ID NOS 169 (second chain), 170 (first chain) and 171 (third chain).

Format 6 (F6): CD19-F6-NKp46-3

The domain structure of heterotrimeric F6 polypeptides is shown in **Figure 2D**. The F6 protein is the same as F5, but contains a N297S substitution to avoid N-linked glycosylation. Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins were purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a high production yield of 12 mg/L and the purified proteins exhibited a simple SEC profile. The amino acid sequences of the three polypeptide chains are shown in SEQ ID NOS: 172 (second chain), 173 (first chain) and 174 (third chain).

Format 7 (F7): CD19-F7-NKp46-3

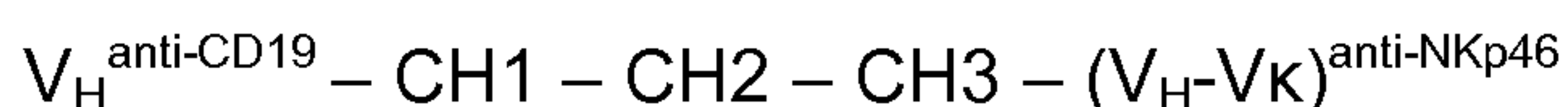
The domain structure of heterotrimeric F7 polypeptides is shown in **Figure 2D**. The F7 protein is the same as F6, except for cysteine to serine substitutions in the CH1 and C κ

domains that are linked at their C-termini to Fc domains, in order to prevent formation of a minor population of dimeric species of the central chain with the $V_K^{\text{anti-NKp46}}$ – CH1 chain. Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins were purified from the cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a high production yield of 11 mg/L and the purified proteins exhibited a simple SEC profile. The amino acid sequences of the three polypeptide chains are shown in SEQ ID NOS: 175 (second chain), 176 (first chain) and 177 (third chain).

Format 13 (F13): CD19-F13-NKp46-3

The domain structure of the dimeric F13 polypeptide is shown in **Figure 2D**, wherein the interchain bonds between hinge domains (indicated between CH1/C κ and CH2 domains on a chain) and interchain bonds between the CH1 and C κ domains are interchain disulfide bonds. The heterodimer is made up of:

(1) a first (central) polypeptide chain having domains arranged as follows (N- to C-termini):



and

(2) a second polypeptide chain having domains arranged as follows (N- to C-termini): $V_K^{\text{anti-CD19}} - C\kappa - \text{CH2} - \text{CH3}$.

The (V_H-V_K) unit was made up of a V_H domain, a linker and a V_K unit (scFv).

Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins were purified from the cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a high production yield of 6.4 mg/L and the purified proteins exhibited a simple SEC profile. The amino acid sequences of the two polypeptide chains are shown in SEQ ID NOS: 178 (second chain) and 179 (first chain).

Format 14 (F14): CD19-F14-NKp46-3

The domain structure of the dimeric F14 polypeptide is shown in **Figure 2E**. The F14 polypeptide is a dimeric polypeptide which shares the structure of the F13 format, but instead of a wild-type Fc domain (CH2-CH3), the F14 bispecific format has CH2 domain mutations N297S to abolish N-linked glycosylation. Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins were purified from cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a high production yield of 2.4 mg/L and the purified proteins exhibited a simple SEC

profile. The amino acid sequences of the two polypeptide chains are shown in SEQ ID NOS: 180 (second chain) and 181 (first chain).

Format 15 (F15): CD19-F15-NKp46-3

The domain structure of the trimeric F15 polypeptides is shown in **Figure 2E**. The F15 polypeptide is a dimeric polypeptide which shares the structure of the F6 format, but differs by inversion of the N-terminal V_H-CH1 and V_K-C_K units between the central and second chains. Proteins were cloned, produced and purified as in Example 2-1. Bispecific proteins were purified from the cell culture supernatant by affinity chromatography using prot-A beads and analyzed and purified by SEC. The protein showed a good production yield of 0.9 mg/L and the purified proteins possessed a simple SEC profile. The amino acid sequences of the three polypeptide chains are shown in SEQ ID NOS: 182 (second chain), 183 (first chain) and 184 (third chain).

Format 16 (F16): CD19-F16-NKp46-3

The domain structure of the trimeric F16 polypeptide is shown in **Figure 2E**. The F16 polypeptide is a dimeric polypeptide which shares the structure of the F6 format, but differs by inversion of the C-terminal V_H-C_K and V_K-CH1 units between the central and second chains. Proteins were cloned, produced and purified as in Example 2-1. The amino acid sequences of the three polypeptide chains are shown in SEQ ID NOS: 185 (second chain), 186 (first chain) and 187 (third chain).

Format T5 (T5)

The domain structure of a trimeric T5 polypeptide is shown in **Figure 2F**. The T5 polypeptide is a trimeric polypeptide which shares the structure of the F5 format, but differs by fusion of an scFv unit at the C-terminus of the third chain (the chain lacking the Fc domain). This protein will therefore have two antigen binding domains for antigens of interest, and one for NKp46, and will bind CD16 via its Fc domain. Proteins were cloned, produced and purified as in Example 2-1. The T5 protein had two antigen binding domains that bind human CD20, originating from different antibodies (and binding to different epitopes on CD20). The first anti-CD20 ABD contained the V_H and V_L of the parent antibody GA101 (GAZYVA®, Gazyvaro®, obinutuzumab, Roche Pharmaceuticals). The second anti-CD20 ABD contained the V_H and V_L of the parent antibody rituximab (Rituxan®, Mabthera®, Roche Pharmaceuticals). The third antigen binding domain binds human NKp46. The amino acid sequences of the three chains of the T5 protein are shown below (Rituximab sequences are

in bold and underlined, anti-GA101 sequences are underlined, anti-NKp46 sequences are in italics).

GA101-T5-Ritux-NKp46

Polypeptide 1 (SEQ ID NO : 188)

QVQLVQSGAEVKKPGSSSVKVSCKASGYAFSYSWINWVRQAPGQGLEWMGRIFPGDGD TDYNGKFKGRVTITADKS
TSTAYMELSSLRSEDTAVYYCARNVFDGYWLVYWGQGT LVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
YFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS
TYRVVSVLT VLVHQLDNLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSV MHEALHNHYTQKSLSLSPGST
GS QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKGKATLTAD
KSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVSARTVAAPSVFIFPPSDEQLKSGTASVVC
LLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSTLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT KSF
NRGEC-

Polypeptide 2 (SEQ ID NO : 189)

DIVMTQTPLSLPVTGPGEPAISCRSSKSLHNSGITYLYWYLQKPGQSPQLLIYQMSNLVSGVPDRFSGSGSGTD
FTLKISRVEAEDVGVYYCAQNLELPYTFGGGTKEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQESVTEQDSKDSYSTLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT KSFNRGEC **CDKTHTC**
PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVS VLT VLVHQLDNLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSV MHEALHNHYTQKSLSLSPGK-

Polypeptide 3: (SEQ ID NO : 190)

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIIYATSNLASGVPVRFSGSGSGTSYSLTIS
RVEAEDAATYYCQQWTSNPPTFGGGTKLEIKASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHGGSSS
EVQLQQSGPELVKPGASVKISCKTSGYTFTEYTMHWVKQSHGKSLEWIGGISPNIGGTSYNQKFKGKATLTVDKS
SSTAYMELRSLTSEDSAVYYCARRGGSFDYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIVMTQSPATLSVTPG
DRVSLSCRASQSISDYLHWYQQKSHESPRLLIKYASQISGISRFSGSGSGSDFTLSINSVEPEDVGVYYCQNG
HSFPLTFGAGTKLELK-

Format T6 (T6)

The domain structure of the trimeric T6 polypeptide is shown in **Figure 2F**. The T6 polypeptide is a trimeric polypeptide which shares the structure of the F6 format, but differs by the fusion of an scFv unit at the C-terminus of the third chain (the chain lacking the Fc domain). This trimeric protein contains two antigen binding domains for antigens of interest, and one for NKp46, and does not bind CD16 via its Fc domain due to the N297 substitution. Proteins were cloned, produced and purified as in Example 2-1. The T6 protein contains two antigen binding domains that bind human CD20. The first anti-CD20 ABD comprises the V_H and V_L of the parent antibody GA101 and the second anti-CD20 ABD comprises the V_H and V_L of rituximab. The amino acid sequences of the three chains of the T6 proteins are shown in SEQ ID NOS : 191, 192 and 193.

Format T9B (T9B)

The domain structure of the trimeric T9B polypeptide is shown in **Figure 2F**. The T9B polypeptide is a trimeric polypeptide which shares the structure of the F9 format (F9B variant), but differs by the fusion of an scFv unit at the C-terminus of the free CH1 domain (on the third chain). This protein contains two antigen binding domains for antigen of interest, and one for NKp46, but will not bind CD16 via its Fc domain due to the monomeric Fc domain and/or the N297 substitution. Trimeric proteins as above described were cloned, produced and purified as in Example 2-1. The T9B protein had two antigen binding domains that bind human CD20. The first anti-CD20 ABD contained the V_H and V_L of the parent antibody GA101 and the second anti-CD20 ABD contained the V_H and V_L of the parent antibody rituximab. The amino acid sequences of the three chains of the T9B proteins are shown below.

GA101-T9B-Ritux-NKp46

Polypeptide 2 : (SEQ ID NO : 195)

DIVMTQTPLSLPVTTPGEPASISCRSSKSLHNSNGITYLYWYLQKPGQSPQLLIYQMSNLVSGVPDRFSGSGSGTD
FTLKISRVEAEDVGVYYCAQNLELPYTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQESVTEQDSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC-

Polypeptide 1: (SEQ ID NO : 194)

QVQLVQSGAEVKKPGSSVKVSCKASGYAFSYSWINWVRQAPGQGLEWMGRIFPGDGD TDYNGKFKGRVTITADKS
TSTAYMELSSLRSEDTAVYYCARNVFDGYWLVYWGQGT LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD
YFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKT
HTSPPSPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYSS
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGGG
GGSGGGGSGGGGSGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGSTGSQVQLQQPGAE LVKPGASVKMSCKASGY
TFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYY
GGDWYFNVWGAGTTVTVSARTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESV
TEQDSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC-

Polypeptide 3 (SEQ ID NO : 196):

QIVLSQSPAILASAPGEKVTMTCRASSSVSYIHW FQQKPGSSPKPWIYATSNLASGVPVRFSGSGSGTSYSLTIS
RVEAEDAATYYCQQWTSNPPTFGGGTKLEIKASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSG
ALTSGVHTFPAVLQSSGLYSLSSV VTPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHGGSSSEVQLQQS
GPELVKPGASVKISCKTSGYTFTEYTMHWVKQSHGKSLEWIGGISPNIGGTSYNQKFKGKATLTVDKSSSTAYME
LRSLTSEDSAVYYCARRGGSFDYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDIVMTQSPATLSVTPGDRVSLSC
RASQISDYLHWYQQKSHESPRLLIKYASQSIGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHSFPLTF
GAGTKLELK-

Format T11 (T1): CD19-T11-NKp46-3

The domain structure of the dimeric T11 polypeptide is shown in **Figure 2F**. The T11 polypeptide is a trimeric polypeptide which shares the structure of the F11 format, but differs by the fusion of an scFv unit at the C-terminus of the free CH1 domain. This dimeric protein contains two antigen binding domains for antigen of interest, and one for NKp46, and does

not bind CD16 via its Fc domain due to the monomeric Fc domain and/or the N297 substitution. Proteins were cloned, produced and purified as in Example 2-1. The T11 protein contains two antigen binding domains that bind human CD20. The first anti-CD20 ABD contained the V_H and V_L of the parent antibody GA101 and the second anti-CD20 ABD contained the V_H and V_L of rituximab. The amino acid sequences of the two chains of the T11 protein are shown below.

GA101-T11-Ritux-NKp46

Polypeptide 1 (SEQ ID NO : 197):

DIVMTQTPLSLPVTTPGEPASISCRSSKSLLSNGITYLYWYLQKPGQSPQLLIYQMSNLVSGVPDRFSGSGSGTD
FTLKISRVEAEDVGVYYCAQNLELPYTFGGGTKVEIKGGGSGGGGSGGGGSQVQLVQSGAEVKKPGSSVKV
ASGYAFSYSWINWVRQAPGQGLEWMGRIFPGDGD TDYNGKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCAR
NVFDGYWLVYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP
AVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTSPSPAPPELLGGPSVFLFPPK
PKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYSSTYRVVSVLTVLHQDWLNGKEYKC
KVSNAKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDS DGSFFLYSKLTVDKSRWQQGNV FSCVMHEALHNHYTQKSLSLSPGSGGGGSGGGGSGGGGSGQPREPQVYT
LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSC
SVMHEALHNHYTQKSLSLSPGSTGSQVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGA
IYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVSARTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY
EKHKVYACEVTHQGLSSPVTKSFNRGEC-

Polypeptide 2 (SEQ ID NO : 198):

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFFQQKPGSSPKPWIIYATSNLASGVPVRFSGSGSGTSYSLTIS
RVEAEDAATYYCQQTWSNPPTFGGGTKLEIKASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHGGSSSEVQLQQS
GPQLVKPGASVKISCKTSGYTFTEYTMHWVKQSHGKSLEWIGGISPNIGGTSYNQKFKGKATLTVDKSSSTAYME
LRSLTSEDSAVYYCARRGGSFYDWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIVMTQSPATLSVTPGDRVSLSC
RASQISDYLHWYQQKSHESPRLLIKYASQSIGIPSRFSGSGSGSDFTLISINSVEPEDVGVYYCQNGHSFPLTF
GAGTKLELK-

Example 5:

NKp46 binding affinity by bispecific proteins detected by Surface Plasmon Resonance (SPR)

Biacore T100 general procedure and reagents

SPR measurements were performed on a Biacore T100 apparatus (Biacore GE Healthcare) at 25°C. In all Biacore experiments HBS-EP+ (Biacore GE Healthcare) and NaOH 10mM served as running buffer and regeneration buffer respectively. Sensorgrams were analyzed with Biacore T100 Evaluation software. Protein-A was purchased from (GE Healthcare). Human NKp46 recombinant proteins were cloned, produced and purified at Innate Pharma.

Immobilization of Protein-A

Protein-A proteins were immobilized covalently to carboxyl groups in the dextran layer on a Sensor Chip CM5. The chip surface was activated with EDC/NHS (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimidehydrochloride and N-hydroxysuccinimide (Biacore GE Healthcare)). Protein-A was diluted to 10 µg/ml in coupling buffer (10 mM acetate, pH 5.6) and injected until the appropriate immobilization level was reached (i.e. 2000 RU). Deactivation of the remaining activated groups was performed using 100 mM ethanolamine pH 8 (Biacore GE Healthcare).

Binding study

The bispecific proteins were first tested in Format F1 as described in Example 2 having different anti-NKp46 variable regions from NKp46-1, NKp46-2, NKp46-3 or NKp46-4 antibodies. Antibodies were next tested as different formats (F3, F4, F5, F6, F9, F10, F11, F13, F14) having the anti-NKp46 variable regions from the NKp46-3 antibody, and were compared to the NKp46-3 antibody as a full-length human IgG1.

Bispecific proteins at 1 µg/mL were captured onto Protein-A chip and recombinant human NKp46 proteins were injected at 5 µg/mL over captured bispecific antibodies. For blank subtraction, cycles were performed again replacing NKp46 proteins with running buffer.

The Bab281 antibody was separately tested for binding to NKp46 by SPR, and additionally by flow cytometry using a cell line expressing the human NKp46 construct on the cell surface. For FACS screening, the presence of reactive antibodies in the supernatants was detected using Goat anti-mouse polyclonal antibody (pAb) labeled with PE. SPC and FACS results showed that the Bab281 based antibody did not bind the NKp46 cell line or to NKp46-Fc proteins. Bab281 lost the ability to bind to its target when presented in the bispecific format.

Affinity study

Monovalent affinity study was conducted following a regular Capture-Kinetic protocol as recommended by the manufacturer (Biacore GE Healthcare kinetic wizard). Seven serial dilutions of human NKp46 recombinant proteins, ranging from 6.25 to 400 nM were sequentially injected over the captured Bi-Specific antibodies and allowed to dissociate for 10 min before regeneration. The entire sensorgram sets were fitted using the 1:1 kinetic binding model.

Results

SPR showed that the bispecific polypeptides of format F1 having the NKp46-1, 2, 3 and 4 scFv binding domains bound to NKp46, while the other bispecific polypeptides having the scFv of different anti-NK46 antibodies did not retain NKp46 binding. The binding

domains that did not retain binding in monomeric bispecific format initially bound to NKp46 but lost the ability to bindNKp46 upon conversion to the bispecific format. All of the bispecific polypeptides of formats F1, F2 F3, F4, F5, F6, F9, F10, F11, F13, and F14 retained binding to NKp46 when using the NKp46-3 variable regions. Monovalent affinities and kinetic association and dissociation rate constants are shown below in Table 3 below.

Table 3

Bispecific mAb	ka (1/Ms)	kd (1/s)	KD (M)
CD19-F1-Bab281	n/a	n/a	n/a (loss of binding)
CD19-F1-NKp46-1	1.23E+05	0.001337	1.09E-08
CD19-F1-NKp46-2	1.62E+05	0.001445	8.93E-09
CD19-F1-NKp46-3	7.05E+04	6.44E-04	9.14E-09
CD19-F1-NKp46-4	1.35E+05	6.53E-04	4.85E-09
CD19-F3-NKp46-3	3.905E+5	0.01117	28E-09
CD19-F4-NKp46-3	3.678E+5	0.01100	30E-09
CD19-F5-NKp46-3	7.555E+4	0.00510	67E-09
CD19-F6-NKp46-3	7.934E+4	0.00503	63E-09
CD19-F9A-NKp46-3	2.070E+5	0.00669	32E-09
CD19-F10A-NKp46-3	2.607E+5	0.00754	29E-09
CD19-F11A-NKp46-3	3.388E+5	0.01044	30E-09
CD19-F13-NKp46-3	8.300E+4	0.00565	68E-09
CD19-F14-NKp46-3	8.826E+4	0.00546	62E-09
NKp46-3 IgG1	2.224E+5	0.00433	20E-09

Example 6:

Engagement of NK cells against Daudi tumor target with Fc-containing NKp46 x CD19 bispecific protein

Bispecific antibodies having a monomeric Fc domain and a domain arrangement according to the single chain F1 or dimeric F2 formats described in Example 3, and a NKp46 binding region based on NKp46-1, NKp46-2, NKp46-3 or NKp46-4 were tested for functional ability to direct NK cells to lyse CD19-positive tumor target cells (Daudi, a well characterized B lymphoblast cell line). The F2 proteins additionally included NKp46-9 variable regions which lost the ability to bindNKp46 in the scFv format but which retained the ability to bindNKp46 in the F(ab)-like format of F2.

Briefly, the cytolytic activity of each of (a) resting human NK cells, and (b) human NK cell line KHYG-1 transfected with human NKp46, was assessed in a classical 4-h ^{51}Cr -release assay in U-bottom 96 well plates. Daudi cells were labelled with ^{51}Cr (50 μCi (1.85 MBq)/ 1×10^6 cells), then mixed with KHYG-1 transfected with hNKp46 at an effector/target ratio equal to 50 for KHYG-1, and 10 (for F1 proteins) or 8.8 (for F2 proteins) for resting NK cells, in the presence of monomeric bi-specific antibodies at different concentrations. After brief centrifugation and 4 hours of incubation at 37°C , samples of supernatant were removed and transferred into a LumaPlate (Perkin Elmer Life Sciences, Boston, MA), and ^{51}Cr release was measured with a TopCount NXT beta detector (PerkinElmer Life Sciences, Boston, MA). All experimental conditions were analyzed in triplicate, and the percentage of specific lysis was determined as follows: $100 \times (\text{mean cpm experimental release} - \text{mean cpm spontaneous release}) / (\text{mean cpm total release} - \text{mean cpm spontaneous release})$. Percentage of total release is obtained by lysis of target cells with 2% Triton X100 (Sigma) and spontaneous release corresponds to target cells in medium (without effectors or Abs).

Results

In the KHYG-1 hNKp46 NK experimental model, each bi-specific antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4 or NKp46-9 induced specific lysis of Daudi cells by human KHYG-1 hNKp46 NK cell line compared to negative controls (Human IgG1 isotype control (IC) and CD19/CD3 bi-specific antibodies), thereby showing that these antibodies induce Daudi target cell lysis by KHYG-1 hNKp46 through CD19/NKp46 cross-linking.

When resting NK cells were used as effectors, each bi-specific antibody NKp46-1, NKp46-2, NKp46-3, NKp46-4 or NKp46-9 again induced specific lysis of Daudi cells by human NK cells compared to the negative control (Human IgG1 isotype control (IC) antibody), thereby showing that these antibodies induce Daudi target cell lysis by human NK cells through CD19/NKp46 cross-linking. Rituximab (RTX, chimeric IgG1) was used as a positive control of ADCC (Antibody-Dependent Cell Cytotoxicity) by resting human NK cells. The maximal response obtained with RTX (at 10 $\mu\text{g/ml}$ in this assay) was 21.6% specific lysis illustrating that the inventive bispecific antibodies have high target cell lysis activity. Results for experiments with resting NK cells are shown in **Figure 3A** for the single chain F1 proteins and **3B** for the dimeric F2 proteins.

Example 7:

Comparison with full length anti-NKp46 mAbs and depleting anti-tumor mAbs: only NKp46 x CD19 bispecific proteins prevent non-specific NK activation

In these experiments bispecific antibodies possessing a specific bispecific format were produced in order to assess whether such bispecific antibodies can mediate NKp46-

mediated NK activation toward cancer target cells without triggering non-specific NK cell activation.

Particularly, NKp46 x CD19 bispecific proteins having an arrangement according to the F2 format described in Example 3 with anti-NKp46 variable domains from NKp46-1, NKp46-2, NKp46-3, NKp46-4 or NKp46-9 were compared to:

(a) full-length monospecific anti-NKp46 antibodies (NKp46-3 as human IgG1), and
(b) the anti-CD19 antibody as a full-length human IgG1 as ADCC inducing antibody control comparator.

The experiments further included as controls: rituximab, an anti-CD20 ADCC inducing antibody control for a target antigen with high expression levels; an anti-CD52 antibody alemtuzumab, a human IgG1 which binds CD52 target present on both targets and NK cells; and a negative control isotype control therapeutic antibody (a human IgG1 that does not bind a target present on the target cells (HUG1-IC).

The different proteins were tested in order to assess their relative functional effects on NK cell activation in the presence of CD19-positive tumor target cells (Daudi cells), in the presence of CD19-negative, CD16-positive target cells (HUT78 T-lymphoma cells), and in the absence of target cells.

Briefly, NK activation was tested by assessing CD69 and CD107 expression on NK cells by flow cytometry. The assay was carried out in 96 U well plates in completed RPMI, 150µL final/well. Effector cells were fresh NK cells purified from donors. Target cells were Daudi (CD19-positive), HUT78 (CD19-negative) or K562 (NK activation control cell line). In addition to K562 positive control, three conditions were tested, as follows:

- > NK cell alone
- > NK cells vs Daudi (CD19⁺)
- > NK cells vs HUT78 (CD19⁻)

Effector :Target (E :T) ratio was 2.5 : 1 (50 000 E : 20 000 T), with an antibody dilution range starting to 10 µg/mL with 1/4 dilution (n=8 concentrations). Antibodies, target cells and effector cells were mixed; spun 1 min at 300g; incubated 4h at 37°C; spun 3 min at 500g; washed twice with Staining Buffer (SB); added 50µL of staining Ab mix; incubated 30 min at 300g; washed twice with SB resuspended pellet with CellFix ; stored overnight at 4°C; and fluorescence was detected with Canto II (HTS).

Results

1. NK cells alone

Results of these experiments are shown in **Figure 4A**. In the absence of target-antigen expressing cells, none of the bispecific anti-NKp46 x anti-CD19 antibodies (including each of the NKp46-1, NKp46-2, NKp46-3, NKp46-4 and NKp46-9 variable regions) activated

NK cells as assessed by CD69 or CD107 expression. The full-length anti-CD19 also did not activate NK cells. However, the full-length anti-NKp46 antibodies did cause detectable activation of NK cells. Alemtuzumab also induced activation of NK cells, at a very high level. The isotype control antibody did not induce activation.

5 2. NK cells vs Daudi (CD19⁺)

Results of these experiments are shown in **Figure 4B**. In the presence of target-antigen expressing cells, each of the bispecific anti-NKp46 x anti-CD19 antibodies (including each of the NKp46-1, NKp46-2, NKp46-3, NKp46-4 and NKp46-9 binding domains) activated NK cells. The full-length anti-CD19 antibody showed at best only very low activation of NK
10 cells. Neither full-length anti-NKp46 antibodies nor alemtuzumab showed a substantial increase in activation beyond what was observed in presence of NK cells alone. The data in **Figure 4** shows that full-length anti-NKp46 antibodies elicited a similar level of baseline activation as was observed in the presence of NK cells alone. Alemtuzumab also induced the activation of NK cells at a similar level of activation to what was observed in the
15 presence of NK cells alone, and at higher antibody concentrations in this setting (ET 2.5 : 1) the activation was greater than with the bispecific anti-NKp46 x anti-CD19 antibody. The isotype control antibody did not induce activation.

3. NK cells vs HUT78 (CD19⁻)

Results of these experiments are shown in **Figure 4C**. In the presence of target-
20 antigen-negative HUT78 cells, none of the bispecific anti-NKp46 x anti-CD19 antibody (including each of the NKp46-1, NKp46-2, NKp46-3, NKp46-4 and NKp46-9 variable regions) activated NK cells. However, the full-length anti-NKp46 antibodies and alemtuzumab caused detectable activation of NK cells at a similar level observed in presence of NK cells alone. Isotype control antibody did not induce activation.

25 The foregoing results indicate that the inventive bispecific anti-NKp46 proteins are able to activate NK cells in a target-cell specific manner, unlike full-length monospecific anti-NKp46 antibodies and further unlike full-length antibodies of depleting IgG isotypes which also activate NK cells in the absence of target cells. The NK cell activation achieved with anti-NKp46 bispecific proteins remarkably was higher than that observed with full length anti-
30 CD19 IgG1 antibodies. Therefore these bispecific antibodies should elicit less non-specific cytotoxicity and may be more potent when used in therapy.

Example 8:

**Comparative efficacy with depleting anti-tumor mAbs: NKp46 x CD19 bispecific
35 proteins at low ET ratio**

These studies aimed to investigate whether bispecific antibodies can mediate NKp46-mediated NK cell activation toward cancer target cells at lower effector: target ratios. The ET ratio used in this Example was 1:1 which is believed to be closer to the setting that would be encountered *in vivo* than the 2.5:1 ET ratio used in Example 7 or the 10:1 ET ratio of Example 6.

NKp46 x CD19 bispecific proteins having an arrangement according to the F2 format described in Example 3 with anti-NKp46 variable domains from NKp46-1, NKp46-2, NKp46-3, NKp46-4 or NKp46-9 were compared to:

- (a) full-length monospecific anti-NKp46 antibodies (NKp46-3 as human IgG1), and
- (b) the anti-CD19 antibody as a full-length human IgG1 as ADCC inducing antibody control comparator.

The experiments further included as controls: rituximab (an anti-CD20 ADCC inducing antibody control for a target antigen with high expression levels); anti-CD52 antibody alemtuzumab (a human IgG1, binds CD52 target present on both targets and NK cells), and negative control isotype control therapeutic antibody (a human IgG1 that does not bind a target present on the target cells (HUG1-IC). The different proteins were tested for their functional effect on NK cell activation as assessed by CD69 or CD107 expression in the presence of CD19-positive tumor target cells (Daudi cells), in the presence of CD19-negative, CD16-positive target cells (HUT78 T-lymphoma cells), and in the absence of target cells. The experiments were carried out as in Example 7 except that the ET ratio was 1:1.

Results

The results of the above experiments are shown in **Figure 5** (**5A**: CD107 and **5B**: CD69). In the presence of target-antigen expressing cells, each of the bispecific anti-NKp46 x anti-CD19 antibodies (respectively including NKp46-1, NKp46-2, NKp46-3, NKp46-4 or NKp46-9 variable regions) activated NK cells in the presence of Daudi cells.

The activation induced by bispecific anti-NKp46 x anti-CD19 antibody in the presence of Daudi cells was far more potent than that elicited by the full-length human IgG1 anti-CD19 antibody. This ADCC inducing antibody had low activity in this setting. Furthermore, in this low E:T ratio setting the activation induced by the bispecific anti-NKp46 x anti-CD19 antibody was as potent as the anti-CD20 antibody rituximab, with a difference being observed only at the highest concentrations that were 10 fold higher than concentrations in which differences were observed at the 2.5:1 ET ratio.

In the absence of target cells or in the presence of target antigen-negative HUT78 cells, full-length anti-NKp46 antibodies and alemtuzumab showed a similar level of baseline activation as was observed in the presence of Daudi cells. Anti-NKp46 x anti-CD19 antibody did not activate NK cells in presence of HUT78 cells.

The foregoing results indicate that the bispecific anti-NKp46 proteins of the invention are able to activate NK cells in a target-cell specific manner and at lower effector: target ratios and are more effective in mediating NK cell activation than traditional human IgG1 antibodies.

5

Example 9:

NKp46 mechanism of action

NKp46 x CD19 bispecific proteins having an arrangement according to the F2, F3, F5 or F6 formats described in Examples 3 or 4 with anti-NKp46 variable domains from NKp46-3 were compared to rituximab (anti-CD20 ADCC inducing antibody), and to a human IgG1 isotype control antibody for their functional ability to direct CD16-/NKp46+ NK cell lines to lyse CD19-positive tumor target cells.

Briefly, the cytolytic activity of the CD16-/NKp46⁺ human NK cell line KHYG-1 was assessed in a classical 4-h ⁵¹Cr-release assay in U-bottom 96 well plates. Daudi or B221 cells were labelled with ⁵¹Cr (50 µCi (1.85 MBq)/1 x 10⁶ cells), then mixed with KHYG-1 at an effector/target ratio equal to 50:1, in the presence of test antibodies at dilution ranges starting from 10⁻⁷ mol/L with 1/5 dilution (n=8 concentrations).

After a brief centrifugation and 4 hours of incubation at 37°C, 50µL of the supernatant were removed and transferred into a LumaPlate (Perkin Elmer Life Sciences, Boston, MA), and ⁵¹Cr release was measured with a TopCount NXT beta detector (PerkinElmer Life Sciences, Boston, MA). All experimental conditions were analyzed in triplicate, and the percentage of specific lysis was determined as follows: 100 x (mean cpm experimental release - mean cpm spontaneous release)/ (mean cpm total release - mean cpm spontaneous release). Percentage of total release is obtained by lysis of target cells with 2% Triton X100 (Sigma) and spontaneous release corresponds to target cells in medium (without effectors or Abs).

Results

The results of the above experiments are shown in **Figure 6A** (KHYG-1 vs Daudi) and **6B** (KHYG-1 vs B221). In the KHYG-1 hNKp46 NK experimental model, each NKp46 x CD19 bispecific protein (Format F2, F3, F5 and F6) induced specific lysis of Daudi or B221 cells by human KHYG-1 hNKp46 NK cell line, while rituximab and the human IgG1 isotype control (IC) antibodies did not.

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Example 10

Anti-KIR3DL2 bispecific proteins

Bispecific proteins targeting human KIR3DL2 (KIR3DL2 x NKp46 bispecific) were constructed as F6 formats and tested for activity. KIR3DL2 (CD158k; killer cell immunoglobulin like receptor, three Ig domains and long cytoplasmic tail 2) is a disulphide-linked homodimer of three-Ig domain molecules of about 140 kD, described in Pende et al. (1996) J. Exp. Med. 184: 505-518, the disclosure of which is incorporated herein by reference. Several allelic variants have been reported for KIR3DL2 polypeptides, each of these are encompassed by the term KIR3DL2. The amino acid sequence of the mature human KIR3DL2 (allele *002) is shown in Genbank accession no. AAB52520. Briefly, the cytolytic activity of NK cells from Buffy coat from donors was assessed in a classic 4-h ⁵¹Cr-release assay in U-bottom 96 well plates. HUT78 tumor cells (CTCL) that express KIR3DL2 were labelled with ⁵¹Cr, then mixed with NK cells at an effector/target ratio equal to 10:1 (25 000:2500), in the presence of test antibodies at dilution ranges starting from 10 µg/mL (or 100 µg/mL) with 1/10 dilution (n=8). Assays were in cRPMI, 150 µL final/well, in triplicates.

Results are shown in **Figure 6C**. Despite its Fc domain not binding to CD16 in this format, the F6 protein structure produced as an NKp46 x KIR3DL2 bispecific protein surprisingly exhibited comparable ability to lyse target cells as a known anti-KIR3DL2 human IgG1 antibody that contained the same variable regions and which binds KIR3DL2 bivalently.

Example 11

Effect of intrachain domain motion within multimeric proteins

It was theorized by the inventors that the ability of NKp46 bispecific proteins to promote NKp46-mediated lysis of target cells may be affected by the distance between the two antigen binding domains in the bispecific protein which may impact the ability of one or both of the NKp46 antigen binding domain and the antigen binding domain which interacts with an antigen of interest to interact with their respective targets. Also, it was further theorized that NKp46 mediated lysis of target cells may be impacted by the structure of the two antigen binding domains and/or their respective conformation, freedom of motion or flexibility which may be impacted by the structure of the two antigen binding domains as well as the manner by which they are associated with each other, e.g., by a linker peptide and its particular length and chemical composition. Particularly, it was theorized that a lytic NKp46-target cell synapse may vary as a function of the size and structure of the bispecific protein. Therefore, the inventors posited that bispecific proteins wherein the antigen binding domains are in a format whereby the antigen binding domains more closely mimics or approximates the conformation, spacing and flexibility of the antigen binding domains of

This was theorized because conformational flexibility, notably intrachain domain motion or movement, may for example affect the effective distance between NKp46 and

antigen-of-interest binding sites, which in turn might have an effect on the NKp46-target cell synapse and the ability of a multimeric bispecific protein to mediate NKp46-mediated signaling and lysis. Based on these suppositions the inventors evaluated the lytic function of multimeric proteins of different bispecific protein formats and which comprise more or less freedom of motion of the antigen binding domains based on the structure of the antigen binding sites and the specific linkers separating these antigen binding sites.

Specifically, different NKp46 x tumor antigen bispecific proteins of different formats such as the F3, F4, F9, F10 and F11 format that bound different tumor antigens were evaluated for their relative ability to induce NKp46-mediated lysis of tumor target cells by KHYG-1 NK cells (NKp46⁺CD16⁻). F5 and F6 bispecific protein formats have distances between the NKp46 binding site and the antigen of interest binding site that are less than that of full-length antibodies. By contrast bispecific proteins targeting human CD19 (CD19 x NKp46 bispecific) in F9 format have binding sites that are spaced farther apart, similar to distances in the two binding sites in conventional full-length antibodies. Bispecific proteins were therefore constructed as F9 formats and compared to F10 and F11 formats. Structurally speaking, format F9, F10 and F11 are very close to one another, however formats F10 and F11 are characterized by one antigen binding domain with a Fab structure and the other antigen binding domain with a tandem variable domain structure (two variable domains separated by a flexible linker). F10 and F11 therefore have greater intrachain domain motion and/or less local steric hindrance, as well as possibly less distance between binding sites than in the F9 proteins.

The cytolytic activity of the CD16-/NKp46⁺ human NK cell line KHYG-1 was assessed in a classical 4-h ⁵¹Cr-release assay in U-bottom 96 well plates. Daudi or B221 cells were labelled with ⁵¹Cr (50 µCi (1.85 MBq)/1 x 10⁶ cells), then mixed with KHYG-1 at an effector/target ratio equal to 50:1, in the presence of test antibodies at dilution range starting from 10⁻⁷ mol/L with 1/5 dilution (n=8 concentrations). The results showed that formats F10 and F11 were both more potent than format F9 in inducing Daudi cell lysis by NK cells. As noted above F9 format proteins have distances between the NKp46 binding site and the antigen of interest binding site which is similar to full-length antibodies or about 80 Å, and the F10 and F11 proteins comprise a single chain domain connected to the Fc by a flexible linker and have substantially less than 80Å between the antigen binding sites (in the case of F10, about 55 Å).

Based thereon we studied the effects of even further shortened distances between the NKp46 and antigen of interest binding domains using other CD19 x NKp46 bispecific proteins. In these experiments F3, F4 protein formats were selected for comparison with protein formats F10 and F11. Each of these proteins have distances between antigen

binding sites of less than 80Å, however, F3 and F4 are shorter than F10 and F11, and F3 and F4 have distances between antigen binding sites that are equivalent to F11 but 25Å less than that of F10. The results of these experiments indicated that the F3, F4, F10 and F11 formats did not significantly differ in their ability to induce Daudi cell lysis by NK cells. These results would suggest that there may be an optimal minimal spacing between the antigen binding domains that improves potency and/or that potency is affected by a combination of the spacing between the antigen binding domains and the flexibility and/or conformation of the antigen binding domains.

Example 12:

Combining NKp46 and CD16 triggering

NKp46 x CD19 bispecific proteins that bind human CD16 having an arrangement according to the F5 format with anti-NKp46 variable domains from NKp46-3 were compared to the same bispecific antibody in a F6 format (which lacks CD16 binding), and to a human IgG1 isotype anti-CD19 antibody, as well as to a human IgG1 isotype control antibody for functional ability to direct purified NK cells to lyse CD19-positive Daudi tumor target cells.

Briefly, the cytolytic activity of fresh human purified NK cells from EFS Buffy Coat was assessed in a classical 4-h ⁵¹Cr-release assay in U-bottom 96 well plates. Daudi or HUT78 cells (negative control cells that do not express CD19) were labelled with ⁵¹Cr and then mixed with NK cells at an effector/target ratio equal to 10:1, in the presence of test antibodies at dilution range starting from 10 µg/ml with 1/10 dilution (n=8 concentrations).

After brief centrifugation and 4 hours of incubation at 37°C, 50µL of supernatant were removed and transferred into a LumaPlate (Perkin Elmer Life Sciences, Boston, MA), and ⁵¹Cr release was measured with a TopCount NXT beta detector (PerkinElmer Life Sciences, Boston, MA). All experimental conditions were analyzed in triplicate, and the percentage of specific lysis was determined as follows: 100 x (mean cpm experimental release - mean cpm spontaneous release) / (mean cpm total release - mean cpm spontaneous release). Percentage of total release is obtained by lysis of target cells with 2% Triton X100 (Sigma) and spontaneous release corresponds to target cells in medium (without effectors or Abs).

The results of these experiments are shown in **Figure 7**. The CD19-F6-NKp46 (bispecific protein in F6 format) whose Fc domain does not bind CD16 due to a N297 substitution was as potent in mediating NK cell lysis of Daudi target cells as the full-length IgG1 anti-CD19 antibody. This result is remarkable especially considering that the control IgG1 anti-CD19 antibody binds CD19 bivalently and further since the anti-CD19 antibody is bound by CD16. The F6 protein was also compared to a protein CD19-F5-NKp46 that was identical to the CD19-F6-NKp46 protein with the exception of an asparagine at Kabat

residue 297. Surprisingly, despite the strong NK activation mediated by CD16 triggering by the CD19-F5-NKp46 (F5 format protein) whose Fc domain binds CD16, the F5 format was far more potent in mediating Daudi target cell lysis than the full-length IgG1 anti-CD19 antibody or the F6 format bispecific protein. This would suggest that NKp46 can enhance target cell lysis even when CD16 is triggered. In fact, at comparable levels of target cell lysis, the CD19-F5-NKp46 was at least 1000 times more potent than the full-length anti-CD19 IgG1. These potency results would suggest that the inventive multispecific NKp46 antibodies should be well suited for use in human therapy, e.g., in treating cancer or infectious diseases.

Example 13:

Mechanisms of action of CD16-binding NKp46 x CD19 bispecific

Lysis of Daudi cells by NKp46 x CD19 bispecific F5 and F6 were compared to a conventional human IgG1 antibody. As a control, lysis was also tested on HUT78 cells that lack CD19; positive control for HUT78 cell lysis was an anti-KIR3DL2 of human IgG1 isotype (HUT78 are KIR3DL2-positive). Cytotoxicity assays were carried out as in Example 10. Flow cytometry staining of NK cell surface markers was carried out as in Example 7.

Results for the cytotoxicity assays are shown in **Figure 8** (Daudi cell in the right hand panel and HUT78 cells in the left hand panel). the CD19-F6-NKp46-3 whose Fc domain does not bind CD16 due to a N297 substitution has as mode of action NKp46 triggering when NK cells encounter the target cell, while the CD19-F5-NKp46-3 bispecific protein demonstrated a far higher potency in mediating cytotoxicity toward Daudi cells. Neither the F5 nor the F6 protein mediated any NK cell cytotoxicity towards HUT78 cells.

The results of flow cytometry staining of NK cell surface markers showed a strong upregulation of CD137 on the surface of NK cells by F5 proteins. These results are shown in **Figure 9** (Left-most panel: NK cells vs. Daudi; middle panel: NK cells vs. HUT78; right-most panel: NK cells alone). The CD19-F5-NKp46-3 whose Fc domain binds CD16 demonstrated the highest CD137 upregulation. The full-length anti-CD19 IgG1 antibody that binds CD16 also elicited CD137 upregulation, but to a far lesser extent than CD19-F5-NKp46-3. The CD19-F6-NKp46-3 which functions via NKp46 but not via CD16 did not elicit any detectable CD137 upregulation. It is hypothesized that the remarkable potency of the F5 format may arise from a particularly strong CD137 upregulation on NK cells which may be mediated by the dual targeting of NKp46 and CD16.

Example 14:

Fc-engineered CD16-binding NKp46 x CD20 bispecific

New bispecific proteins were further constructed in an attempt to generate an agent that could improve on the most potent new generation of Fc enhanced antibodies. In these experiments as the comparison antibody we selected the commercial antibody GA101 (GAZYVA®, Gazyvaro®, obinutuzumab, Roche Pharmaceuticals), which is an Fc-modified human IgG1 antibody having enhanced CD16A binding as a result of hypofucosylated N-linked glycosylation.

NKp46 x CD20 bispecific proteins were produced as proteins without CD16 binding (F6 format), with CD16 binding (F5 format), or as Fc-engineered format based on F5 but comprising two amino acid substitutions in the CH2 domain of the heavy chain that increase binding affinity for human CD16A (referred to as "F5+"). In these constructs the anti-CD20 ABDs comprise the V_H and V_L of GA101.

Lysis of Daudi cells by NKp46 x CD20 bispecific F5, F5+ and F6 antibodies were compared to the commercial antibody GA101 (GAZYVA®). Cytotoxicity assays were carried out as in Example 10.

Results for the cytotoxicity assays are shown in **Figure 10**. As shown therein the GA101-F5+-NKp46-1 bispecific protein demonstrated a far higher potency (approximately 10-fold increase in EC₅₀) in mediating cytotoxicity toward Daudi cells that GA101.

Moreover, when ADCC optimized Fc are used for the bispecific format (F5⁺) a significant difference was observed between F5+-BS lacking the Nkp46 arm (GA101-F5+-IC; black diamond) and F5+-BS co-engaging CD16+Nkp46 (GA101-F5⁺-NKp46-1; black square) confirming the contribution of NKp46 in GA101-F5⁺-NKp46-1 activity. Surprisingly, despite the high affinity of GA101-F5⁺-NKp46-1 for CD16 and the presumable maximum NK-cell mediated lysis, NKp46 nevertheless elicited a substantial further increase in cytotoxic activity. These results would suggest that agents capable of inducing ADCC via CD16, can be improved by further conferring on them the ability to induce NKp46-mediated lysis, and also that the potency of bispecific anti-NKp46 agents can be improved by enhancing affinity for CD16 via Fc engineering.

Example 15:

Binding of different bispecific formats to FcRn

The affinity of different antibody formats for human FcRn was studied by Surface Plasmon Resonance (SPR) by immobilizing recombinant FcRn proteins covalently to carboxyl groups in the dextran layer on a Sensor Chip CM5, as described in Example 2-6.

A chimeric full length anti-CD19 antibody having intact human IgG1 constant regions and NKp46 x CD19 bispecific proteins having an arrangement according to the F3, F4, F5, F6, F9, F10, F11, F13 or F14 formats described in Examples 3 or 4 with anti-NKp46 variable

domains from NKp46-3 (NKp46-2 for F2) were tested; for each analyte, the entire sensorgram was fitted using the steady state or 1:1 SCK binding model.

The results of these experiments are shown in Table 4 below. The bispecific proteins having dimeric Fc domains (formats F5, F6, F13, F14) bound to FcRn with affinity similar to that of the full-length IgG1 antibody. The bispecific proteins with monomeric Fc domains (F3, F4, F9, F10, F11) also displayed binding affinity to FcRn, however with lower affinity than the bispecific proteins having dimeric Fc domains.

Table 4

Antibody/Bispecific	SPR method	KD nM
Human IgG1/K Anti-CD19	SCK / Two state reaction	7.8
CD19-F5-NKp46-3	SCK / Two state reaction	2.6
CD19-F6- NKp46-3	SCK / Two state reaction	6.0
CD19-F13- NKp46-3	SCK / Two state reaction	15.2
CD19-F14- NKp46-3	SCK / Two state reaction	14.0
CD19-F3- NKp46-3	Steady State	474.4
CD19-F4- NKp46-3	Steady State	711.7
CD19-F9A- NKp46-3	Steady State	858.5
CD19-F10A- NKp46-3	Steady State	432.8
CD19-F11- NKp46-3	Steady State	595.5

Example 16:

Binding to FcγR

Different multimeric Fc proteins were evaluated to assess whether such bispecific monomeric Fc proteins could retain binding to Fcγ receptors.

SPR measurements were performed on a Biacore T100 apparatus (Biacore GE Healthcare) at 25°C. In all Biacore experiments HBS-EP+ (Biacore GE Healthcare) and 10 mM NaOH, 500mM NaCl served as running buffer and regeneration buffer respectively. Sensorgrams were analyzed with Biacore T100 Evaluation software. Recombinant human FcR's (CD64, CD32a, CD32b, CD16a and CD16b) were cloned, produced and purified.

F5 and F6 bispecific antibodies CD19-F5-NKp46-3 or CD19-F6-NKp46-3 were immobilized covalently to carboxyl groups in the dextran layer on a Sensor Chip CM5. The chip surface was activated with EDC/NHS (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimidehydrochloride and N-hydroxysuccinimide (Biacore GE Healthcare)). Bispecific antibodies were diluted to 10 µg/ml in coupling buffer (10 mM acetate, pH 5.6) and injected

until the appropriate immobilization level was reached (i.e. 800 to 900 RU). Deactivation of the remaining activated groups was performed using 100 mM ethanolamine pH 8 (Biacore GE Healthcare).

Monovalent affinity study was assessed following a classical kinetic wizard (as recommended by the manufacturer). Serial dilutions of soluble analytes (FcRs) ranging from 0.7 to 60 nM for CD64 and from 60 to 5000 nM for all the other FcRs were injected over the immobilized bispecific antibodies and allowed to dissociate for 10 min before regeneration. The entire sensorgram sets were fitted using the 1:1 kinetic binding model for CD64 and with the Steady State Affinity model for all the other FcRs.

The results showed that while full length wild type human IgG1 bound to all cynomolgus and human Fc γ receptors, the CD19-F6-NKp46-3 bi-specific antibodies did not bind to any of the receptors. The CD19-F5-NKp46-3, on the other hand, bound to each of the human receptors CD64 (KD=0.7 nM), CD32a (KD=846 nM), CD32b (KD=1850 nM), CD16a (KD=1098 nM) and CD16b (KD=2426 nM). Conventional human anti-IgG1 antibodies have comparable binding to these Fc receptors (KD shown in the table below).

Human Fc γ receptor	CD19-F5-NKp46-3 KD (nM)	Full length human IgG1 antibody KD (nM)
CD64	0.7	0.24
CD32a	846	379
CD32b	1850	1180
CD16a	1098	630
CD16b	2426	2410

Example 17:

Epitope mapping of anti-NKp46 antibodies

A. Competition Assays

Competition assays were conducted by Surface Plasmon Resonance (SPR according to the methods described below.

SPR measurements were performed on a Biacore T100 apparatus (Biacore GE Healthcare) at 25°C. In all Biacore experiments HBS-EP+ (Biacore GE Healthcare) and NaOH 10mM NaCl 500 mM served as running buffer and regeneration buffer respectively. Sensorgrams were analyzed with Biacore T100 Evaluation software. Anti-6xHis tag antibody was purchased from QIAGEN. Human 6xHis tagged NKp46 recombinant proteins (NKp46-His) were cloned, produced and purified at Innate Pharma.

Anti-His antibodies were immobilized covalently to carboxyl groups in the dextran layer on a Sensor Chip CM5. The chip surface was activated with EDC/NHS (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimidehydrochloride and N-hydroxysuccinimide (Biacore GE Healthcare)). Protein-A and anti-His antibodies were diluted to 10 µg/ml in coupling buffer (10 mM acetate, pH 5.6) and injected until the appropriate immobilization level was reached (i.e. 2000 to 2500 RU). Deactivation of the remaining activated groups was performed using 100 mM ethanolamine pH 8 (Biacore GE Healthcare).

Parental regular human IgG1 chimeric antibodies having NKp46 binding region corresponding to NKp46-1, NKp46-2, NKp46-3 or NKp46-4 were used for the competition study which has been performed using an Anti-6xHis tag antibody chip. Bispecific antibodies having NKp46 binding region based on NKp46-1, NKp46-2, NKp46-3 or NKp46-4 at 1 µg/mL were captured onto Protein-A chip and recombinant human NKp46 proteins were injected at 5 µg/mL together with a second test bispecific antibody of the NKp46-1, NKp46-2, NKp46-3 or NKp46-4 group.

The results demonstrated that none of NKp46-1, NKp46-2, NKp46-3 or NKp46-4 competed with one another for binding to NKp46. Accordingly these antibodies each bind or interact with a different NKp46epitope.

B. Binding to NKp46 mutants

In order to define the epitopes of these anti-NKp46 antibodies, we designed NKp46 mutants defined by one, two or three substitutions of amino acids exposed at the molecular surface over the 2 domains of NKp46. This approach led to the generation of 42 mutants which were transfected in Hek-293T cells, as shown in the table below. The targeted amino acid mutations in Table 5 below are shown both according to the numbering of SEQ ID NO: 1 (also corresponding to the numbering used in Jaron-Mendelson et al. (2012) *J. Immunol.* 88(12):6165-74.

Table 5

Mutant	Substitution (Numbering according to:Jaron-Mendelson and SEQ ID NO 1)		
1	P40A	K43S	Q44A
2	K41S	E42A	E119A
3	P86A	D87A	
4	N89A	R91A	
5	K80A	K82A	
5bis	E34A	T46A	
6	R101A	V102A	
7	N52A	Y53A	

8	V56A	P75A	E76A
9	R77A	I78A	
10	S97A	I99A	
10bis	Q59A	H61A	
11	L66A	V69A	
12	E108A		
13	N111A	L112A	
14	D114A		
15	T125A	R145S	D147A
16	S127A	Y143A	
17	H129A	K139A	
18	K170A	V172A	
19	I135A	S136A	
19bis	T182A	R185A	
20	R160A		
21	K207A		
22	M152A	R166A	
23	N195A	N196A	
Stalk1	D213A	I214A	T217A
Stalk2	F226A	T233A	
Stalk3	L236A	T240A	
Supp1	F30A	W32A	
Supp2	F62A	F67A	
Supp3	E63A	Q95A	
Supp4	R71A	K73A	
Supp5	Y84A		
Supp6	E104A	L105A	
Supp7	Y121A	Y194A	
Supp8	P132A	E133A	
Supp9	S151A	Y168A	
Supp10	S162A	H163A	
Supp11	E174A	P176A	
Supp12	P179A	H184A	
Supp13	R189A	E204A	P205A

C. Generation of mutants

NKp46 mutants were generated by PCR. The sequences amplified were run on agarose gel and purified using the Macherey Nagel PCR Clean-Up Gel Extraction kit. Two or three purified PCR products generated for each mutant were then ligated into an expression vector, with the ClonTech InFusion system. The vectors containing the mutated sequences were prepared as Miniprep and sequenced. After sequencing, the vectors containing the mutated sequences were prepared as Midiprep using the Promega PureYield™ Plasmid

Midiprep System. HEK293T cells were grown in DMEM medium (Invitrogen), transfected with vectors using Invitrogen's Lipofectamine 2000 and incubated at 37°C in a CO₂ incubator for 24 hours prior to testing for transgene expression.

5 D. Flow cytometry analysis of anti-NKp46 binding to the HEK293T transfected cells

All the anti-NKp46 antibodies were tested for their binding to each mutant by flow cytometry. A first experiment was performed to identify antibodies that lose their binding to one or several mutants at a particular concentration (10 µg/ml). To confirm the loss of binding, titration of antibodies was done using antibodies for which binding seemed to be
10 affected by the NKp46 mutations (1 – 0,1 – 0,01 – 0,001 µg/ml).

E. Results

Antibody NKp46-1 had decreased binding to the mutant 2 (having a mutation at residues K41, E42 and E119) (numbering in NKp46 wild-type) compared to wild-type NK46. Similarly, NKp46-1 also had decreased binding to the supplementary mutant Supp7 (having
15 a mutation at residues Y121 and Y194).

Antibody NKp46-3 had decreased binding to the mutant 19 (having a mutation at residues I135, and S136). Similarly, NKp46-1 also had decreased binding to the supplementary mutant Supp8 (having a mutation at residues P132 and E133).

Antibody NKp46-4 had decreased binding to the mutant 6 (having a mutation at residues R101, and V102). Similarly, NKp46-1 also had decreased binding to the
20 supplementary mutant Supp6 having a mutation at residues E104 and L105.

Using these methods we identified the epitopes for anti-NKp46 antibodies NKp46-1, NKp46-3 and NKp46-4. We determined that the epitopes of NKp46-4, NKp46-3 and NKp46-1 are on NKp46 D1 domain, D2 domain and D1/D2 junction, respectively. R101, V102, E104
25 and L105 are essential residues for NKp46-4 binding and defined a part of NKp46-4 epitope. The epitope of NKp46-1 epitope includes K41, E42, E119, Y121 and Y194 residues. The epitope of NKp46-3 includes P132, E133, I135, and S136 residues.

Example 18:

30 **Improved product profile and yield of different bispecific formats compared to existing formats**

Blinatumomab and two bispecific antibodies having NKp46 and CD19 binding regions based on F1 to F17 formats and NKp46-3, and blinatumomab, respectively were cloned and produced under format 6 (F6), DART™ and BiTE™ formats following the same

protocol and using the same expression system. F6, DART™ and BiTE™ bispecific proteins were purified from cell culture supernatant by affinity chromatography using prot-A beads for F6 or Ni-NTA beads for DART™ and BiTE™. Purified proteins were further analyzed and purified by SEC. DART™ and BiTE™ showed a very low production yield compared to F6 and the purified proteins have a very complex SEC profile. DART™ and BiTE™ are barely detectable by SDS-PAGE after Coomassie staining in the expected SEC fractions (3 and 4 for BiTE™ and 4 and 5 for DART™), whereas the F6 format showed a clear and simple SEC and SDS-PAGE profiles with a major peak (fraction 3) containing the multimeric bispecific proteins. The major peak for the F6 format corresponded to about 30% of the total proteins. These results are consistent for those seen with the F1 to F17 proteins (data not shown) indicating that the Fc domain (or Fc-derived domain) present in those formats facilitates the production and improves the quality and solubility of bispecific proteins.

Moreover, the Fc domains present in proteins F1 to F17 have the advantage of being suitable for usage in affinity chromatography without the need for the incorporation of peptide tags. This is desirable as such tags are undesirable in a therapeutic product as they may potentially elicit undesired immunogenicity. By contrast, DART™ and BiTE™ antibodies cannot be purified using protein A, whereas F1 to F17 antibodies are all bound by protein A. Table 6 below shows the productivity of different formats.

Table 6

Format	SEC	SDS PAGE		Final « productivity » yield
		Reduced	Non Reduced	
F3	2 peaks	√	√	3.4mg/L
F4	2 peaks	√	√	1mg/L
F5	√	√	√	37mg/L
F6	√	√	√	12mg/L
F7	√	√	√	11mg/L
F8C	√	√	√	3.7mg/L
F9A	√	√	√	8.7mg/L
F9B	√	√	√	3.0mg/L
F10A	√	√	√	2.0mg/L
F11	√	√	√	2.0mg/L
F12	√	√	√	2.8mg/L
F13	√	√	√	6.4mg/L
F14	√	√	√	2.4mg/L
F15	√	√	√	0.9mg/L
BiTe™	-	-	-	-

DART™	-	-	-	-
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All headings and sub-headings are used herein for convenience only and should not be construed as limiting the invention in any way. Any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. Unless otherwise stated, all exact values provided herein are representative of corresponding approximate values (e. g., all exact exemplary values provided with respect to a particular factor or measurement can be considered to also provide a corresponding approximate measurement, modified by “about,” where appropriate). All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context.

The use of any and all examples, or exemplary language (e.g., “such as”) provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise indicated. No language in the specification should be construed as indicating any element is essential to the practice of the invention unless as much is explicitly stated.

The description herein of any aspect or embodiment of the invention using terms such as reference to an element or elements is intended to provide support for a similar aspect or embodiment of the invention that “consists of,” “consists essentially of” or “substantially comprises” that particular element or elements, unless otherwise stated or clearly contradicted by context (e.g., a composition described herein as comprising a particular element should be understood as also describing a composition consisting of that element, unless otherwise stated or clearly contradicted by context).

This invention includes all modifications and equivalents of the subject matter recited in the aspects or claims presented herein to the maximum extent permitted by applicable law.

All publications and patent applications cited in this specification are herein incorporated by reference in their entireties as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to

one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

1. A multispecific antigen binding protein comprising a first antigen binding domain ("ABD"), a second ABD, and a CD16A binding polypeptide, optionally an Fc polypeptide or portion thereof capable of binding human CD16A which Fc polypeptide optionally is modified to enhance CD16A binding, wherein one of the first or second ABDs binds to a human NKp46 polypeptide and the other binds an antigen of interest, wherein said antigen of interest optionally comprises an antigen expressed by a tumor cell or an infectious agent, and the multispecific protein is capable of directing an NKp46-expressing NK cell to lyse a target cell expressing the antigen of interest, wherein said lysis of the target cell is mediated by NKp46-signaling.

2. The composition of claim 1, wherein the multispecific protein binds the NKp46 polypeptide monovalently.

3. The multispecific antigen binding protein of claim 1, wherein lysis of the target cell is mediated by a combination of Nkp46 signaling and CD16-mediated antibody-dependent cell-mediated cytotoxicity ("ADCC").

4. The multispecific antigen binding protein of claims 1, 2 or 3 wherein NKp46 signaling mediated lysis and CD16-mediated lysis results in a synergistic enhancement in the lysis of target cells.

5. The multispecific antigen binding protein of any of the foregoing claims which upregulates CD137 expression on NK cells.

6. The multispecific antigen binding protein of any of the foregoing claims which mediates lysis of target cells by a combination of NKp46-signaling, CD137-signaling and CD16A-mediated ADCC.

7. The multispecific antigen binding protein of any of the foregoing claims which comprises an Fc polypeptide containing at least one modification which enhances CD16A binding relative to the corresponding wild-type Fc region.

8. The multispecific antigen binding protein of any of the foregoing claims wherein the CD16A binding polypeptide binds CD16A monovalently or bivalently.

9. The multispecific antigen binding protein of any of the foregoing claims, wherein the ABD which binds to an antigen of interest comprises a monovalent or bivalent antibody or antibody fragment, optionally a Fab or a V_H and V_L domain optionally separated by a linker, which linker further optionally comprises a linear or cyclic peptide optionally entirely or predominantly comprised of serine and glycine residues, a peptide ligand or receptor polypeptide or fragment thereof that specifically binds to another antigen of interest.

10. The multispecific antigen binding protein of any of the foregoing claims, comprising an NKp46 ABD and an ABD which binds an antigen of interest, wherein the spacing between said ABDs comprises less than 80 angstroms, less than 60 angstroms or ranges from about 40-60 angstroms.

11. The multispecific antigen binding protein of any of the foregoing claims, comprising an NKp46 ABD and an ABD which binds an antigen of interest, wherein the spacing between said ABDs comprises about 55 angstroms.

12. The multispecific antigen binding protein of any of the foregoing claims, wherein said protein is a heterodimeric, or heterotrimeric protein.

13. The multispecific antigen binding protein of any of the foregoing claims, wherein each antigen binding domain comprises a V_H and/or V_L domain.

14. The multispecific antigen binding protein of any of the foregoing claims, wherein at least one antigen binding domain comprises a non-immunoglobulin scaffold.

15. The multispecific antigen binding protein of any of the foregoing claims, wherein Fc domain is a dimeric Fc domain.

16. The multispecific antigen binding protein of any of the foregoing claims, wherein at least one of the antigen binding sites is configured to have greater intrachain domain movement compared to antigen binding sites in a conventional human IgG1 antibody.

17. The multispecific antigen binding protein of any of the foregoing claims, wherein said protein comprises at least one polypeptide chain that comprises an antigen

binding domain bound to a constant domain or Fc domain by a flexible linker, optionally a flexible polypeptide linker.

18. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen binding domain that binds to a human NKp46 polypeptide comprises a V_H and a V_L , wherein each of the V_H and V_L are positioned within a tandem variable region comprising a V_H and V_L separated by a polypeptide linker.

19. The multispecific antigen binding protein of any of the foregoing claims, wherein the protein comprises a first and a second polypeptide chain each comprising a V_H and a V_L separated by a polypeptide linker.

20. The multispecific antigen binding protein of claim 19, wherein the V_H and the V_L of the first polypeptide chain associate to form an antigen binding domain that binds NKp46, and the V_H and the V_L of the second polypeptide chain associate to form an antigen binding domain that binds the antigen of interest.

21. The multispecific antigen binding protein of claim 19, wherein the V_H of the first polypeptide chain and the V_L of the second polypeptide chain associate to form an antigen binding domain that binds NKp46 and the V_H of the second polypeptide chain and the V_L of the first polypeptide chain associate to form an antigen binding domain that binds the antigen of interest.

22. The multispecific antigen binding protein of any of the foregoing claims, comprising an NKp46 binding ABD which comprises a Fab or comprises a V_H and V_L domain separated by a linker.

23. The multispecific antigen binding protein of any of the foregoing claims, comprising an ABD which binds to Nkp46 and an ABD which binds an antigen of interest which each comprise a Fab or a V_H and V_L domain separated by a linker.

24. The multispecific antigen binding protein of any of the foregoing claims, comprising an ABD which binds to Nkp46 which comprises a Fab and an ABD which binds an antigen of interest which comprises a V_H and V_L domain separated by a linker.

25. The multispecific antigen binding protein of any of the foregoing claims, comprising an ABD which binds to Nkp46 which comprises a V_H and V_L domain separated by a linker and an ABD which binds to the antigen of interest which comprises a Fab.

26. The multispecific antigen binding protein of any of the foregoing claims, wherein the Fc domain is a dimeric Fc domain comprising N-linked glycosylation at amino acid residue N297 (Kabat EU numbering).

27. The multispecific antigen binding protein of any of the foregoing claims, wherein the multispecific protein further comprises a third or further antigen binding domain that binds to an antigen of interest other than a NKp46 polypeptide.

28. The multispecific antigen binding protein of any of the foregoing claims, wherein the multispecific polypeptide binds to a human Fcγ receptor with an affinity for monovalent binding, as assessed by surface plasmon resonance, that is substantially equivalent to that of a full length wild type human IgG1 antibody.

29. The multispecific antigen binding protein of any of the foregoing claims, wherein the protein, immobilized on a surface, binds a soluble human CD16 with a KD for monovalent binding that is no more than 2000 nM, optionally 1300 nM, optionally, 1100 nM, as determined by surface plasmon resonance using Biacore.

30. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen of interest is a cell surface polypeptide capable of undergoing intracellular internalization when bound by a full-length IgG1 antibody or is a cell surface polypeptide which is not capable of undergoing intracellular internalization when bound by a full-length IgG1 antibody.

31. The multispecific antigen binding protein of any of the foregoing claims, wherein the multispecific protein binds the antigen of interest monovalently.

32. The multispecific antigen binding protein of any of the foregoing claims, wherein the multispecific protein does not substantially increase or induce intracellular internalization of the antigen of interest on target cells.

33. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen of interest is a cancer antigen.

34. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen of interest is expressed by an infectious agent.

35. The multispecific antigen binding protein of any of the foregoing claims, wherein the Fc domain or portion thereof is capable of binding to human FcRn.

36. The multispecific antigen binding protein of any of the foregoing claims, wherein the multispecific protein competes for binding to a NKp46 polypeptide with any one or any combination of monoclonal antibodies NKp46-1, NKp46-2, NKp46-3, NKp46-4, NKp46-6.

37. The multispecific antigen binding protein of any of the foregoing claims, wherein the multispecific protein has decreased binding to a mutant NKp46 polypeptide selected from the group consisting of:

a. a mutant NKp46 polypeptide having a mutation at residues R101, V102, E104 and/or L105 compared to binding to the wild-type NKp46;

b. a mutant NKp46 polypeptide having a mutation any one or more of the residues K41, E42, E119, Y121 and/or Y194 compared to binding to the wild-type NKp46; and

c. a mutant NKp46 polypeptide having a mutation any one or more of the residues P132, E133, I135, and/or S136 compared to binding to the wild-type NKp46.

38. A multimeric protein comprising: (a) a first antigen binding domain that binds to human NKp46; (b) a second antigen binding domain that binds a polypeptide expressed on a target cell; and (c) a dimeric human Fc domain or a portion thereof that comprises N-linked glycosylation at residue N297 (Kabat EU numbering), wherein the multispecific polypeptide is capable of binding to human neonatal Fc receptor (FcRn) and CD16A.

39. The multispecific antigen binding protein of claim 37, wherein the Fc domain comprises one or more amino acid modifications that increase binding affinity for a human CD16A polypeptide.

40. The multispecific antigen binding protein of any of the foregoing claims, wherein the protein comprises a first (central) polypeptide chain providing one variable domain that will, together with a complementary variable domain on a second polypeptide chain, forms an antigen binding domain specific for one antigen of interest, and the first

(central) polypeptide chain also provides a second variable domain that is paired with a complementary variable domain to form an antigen binding domain specific for another antigen of interest, wherein the variable domain that is complementary to the second variable domain can be placed on the central polypeptide or can be placed on a separate polypeptide chain.

41. The multispecific antigen binding protein of any of the foregoing claims, wherein the protein is a hetero-multimeric protein comprising at least two different polypeptide chains that each comprise at least one heavy or light chain variable domain fused to a human CH1 or C κ constant domain (a V-(CH1/C κ) unit), and an Fc domain, wherein the protein chains undergo CH1-C κ dimerization and are bound to one another by disulfide bonds formed between respective CH1 and C κ domain(s), and by non-covalent bonds between the CH3 domains of the respective Fc portion.

42. The multispecific antigen binding protein of any of the foregoing claims, wherein the protein is an isolated heterodimeric polypeptide comprising:

(a) a first polypeptide chain comprising, from N- to C- terminus, a second variable domain and third variable domain, a Fc domain or portion thereof, a first variable domain (V), and a CH1 or C κ constant region; and

(b) a second polypeptide chain comprising, from N- to C- terminus, a first variable domain (V), a CH1 or C κ constant region, and an Fc domain or portion thereof, wherein the CH1 or C κ constant region is selected to be complementary to the CH1 or C κ constant region of the first polypeptide chain such that the first and second polypeptides form a CH1-C κ heterodimer in which the first variable domain of the first polypeptide chain and the first variable domain of the second polypeptide together form an antigen binding domain; and wherein the second variable domain and third variable domain together form an antigen binding domain.

43. The multispecific antigen binding protein of any of the foregoing claims, wherein the protein is an isolated heterodimeric polypeptide comprising:

(a) a first polypeptide chain comprising, from N- to C- terminus, a first variable domain (V), a CH1 or C κ constant region, a Fc domain or portion thereof, a second variable domain and a third variable domain; and

(b) a second polypeptide chain comprising, from N- to C- terminus, a first variable domain (V), a CH1 or C κ constant region, and a Fc domain or portion thereof, wherein the CH1 or C κ constant region is selected to be complementary to the CH1 or C κ constant

region of the first polypeptide chain such that the first and second polypeptides form a CH1-CK heterodimer in which the first variable domain of the first polypeptide chain and the first variable domain of the second polypeptide form an antigen binding domain; and wherein the second variable domain and third variable domain forms an antigen binding domain.

44. The multispecific antigen binding protein of any of claims 1-41 or 43, wherein the protein is a heterodimer and comprises:

(a) a first polypeptide having a domain arrangement:

$V_{a-1} - (CH1 \text{ or } CK)_a - Fc \text{ domain} - V_{a-2} - V_{b-2}$,

and

(b) a second polypeptide chain having a domain arrangement:

$V_{b-1} - (CH1 \text{ or } CK)_b - Fc \text{ domain}$

wherein one of V_{a-1} and V_{b-1} is a light chain variable domain and the other is a heavy chain variable domain, one of V_{a-2} and V_{b-2} is a light chain variable domain and the other is a heavy chain variable domain;

wherein $(CH1 \text{ or } CK)_b$ dimerizes with the $(CH1 \text{ or } CK)_a$ on the central chain, and the V_{b-1} forms an antigen binding domain together with V_{a-1} of the central chain, and wherein V_{a-2} and V_{b-2} together form an antigen binding domain.

45. The multispecific antigen binding protein of claim 44, wherein V_{a-2} and V_{b-2} together form an antigen binding domain that binds NKp46.

46. The multispecific antigen binding protein of any of claims 1-45, wherein the protein is a heterodimer and comprises a domain arrangement:

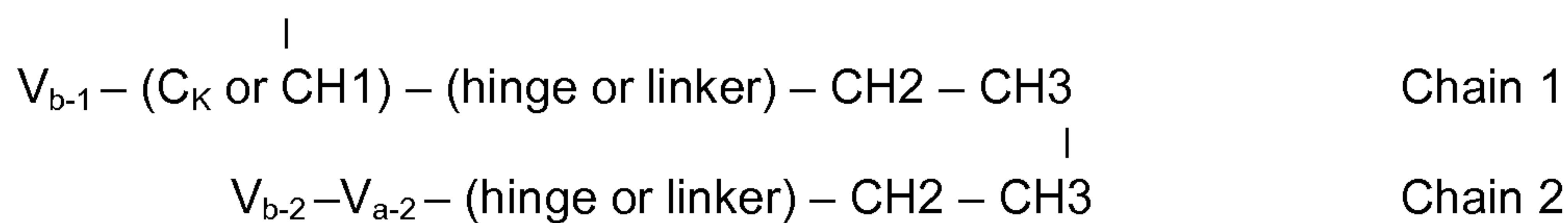
$$\begin{array}{c} (V_{a-1} - V_{b-1} - C_K) - (\text{hinge or linker}) - CH2 - CH3 \\ | \\ (V_{a-2} - V_{b-2} - CH1) - (\text{hinge or linker}) - CH2 - CH3 \end{array}$$

wherein V_{a-1} , V_{b-1} , V_{a-2} and V_{b-2} are each a V_H domain or a V_L domain, and wherein one of V_{a-1} and V_{b-1} is a V_H and the other is a V_L such that V_{a-1} and V_{b-1} form a first antigen binding domain (ABD), wherein one of V_{a-2} and V_{b-2} is a V_H and the other is a V_L such that V_{a-2} and V_{b-2} form a second antigen binding domain, wherein one of the ABD binds NKp46 and the other binds an antigen of interest.

47. The multispecific antigen binding protein of any of claims 1-40, wherein the protein is a heterotrimer comprising a domain arrangement:

$V_{a-1} - (C_K \text{ or } CH1)$

Chain 3



wherein V_{a-1} , V_{b-1} , V_{a-2} and V_{b-2} are each a V_H domain or a V_L domain, and wherein one of V_{a-1} and V_{b-1} is a V_H and the other is a V_L such that V_{a-1} and V_{b-1} form a first antigen binding domain (ABD), wherein one of V_{a-2} and V_{b-2} is a V_H and the other is a V_L such that V_{a-2} and V_{b-2} form a second antigen binding domain, wherein chain 1 and 2 associate by CH3-CH3 dimerization and CH1 and CK are selected such that chain 3 is capable of associating with chain 1.

48. The multispecific antigen binding protein of claim 46 or 47, wherein chain 2 comprises a domain arrangement:



49. The multispecific antigen binding protein of any of the foregoing claims, wherein the protein is an isolated heterodimeric polypeptide comprising at least one of the formats shown in Figure 2A-F or comprises a BiTe or DART.

50. The multispecific antigen binding protein of any of the foregoing claims, wherein a CH1 or C_K on a polypeptide chain is separated from an adjacent CH2 or Fc domain by a hinge domain, optionally wherein the hinge domain comprises a cysteine residue capable of forming interchain disulfide bonds, optionally wherein the hinge region is modified to delete or substitute a cysteine residue, optionally wherein the hinge region is derived from an IgM isotype antibody.

51. The multispecific antigen binding protein of any of the foregoing claims, wherein the polypeptide expressed on a target cell is a cancer antigen, a viral antigen or a bacterial antigen.

52. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen binding domain that binds NKp46 binds to the D2 domain of the NKp46 polypeptide.

53. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen binding domain that binds NKp46 binds to the D1 domain of the NKp46 polypeptide.

54. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen binding domain that binds NKp46 binds to the region intervening the D1 and D2 domains of the NKp46 polypeptide.

55. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen binding domain that binds NKp46 comprises:

(a) a heavy chain comprising CDR 1, 2 and 3 of the heavy chain variable region of SEQ ID NO: 3 and a light chain comprising CDR 1, 2 and 3 of the light chain variable region of SEQ ID NO: 4;

(b) a heavy chain comprising CDR 1, 2 and 3 of the heavy chain variable region of SEQ ID NO: 5 and a light chain comprising CDR 1, 2 and 3 of the light chain variable region of SEQ ID NO: 6;

(c) a heavy chain comprising CDR 1, 2 and 3 of the heavy chain variable region of SEQ ID NO: 7 and a light chain comprising CDR 1, 2 and 3 of the light chain variable region of SEQ ID NO: 8;

(d) a heavy chain comprising CDR 1, 2 and 3 of the heavy chain variable region of SEQ ID NO: 9 and a light chain comprising CDR 1, 2 and 3 of the light chain variable region of SEQ ID NO: 10;

(e) a heavy chain comprising CDR 1, 2 and 3 of the heavy chain variable region of SEQ ID NO: 11 and a light chain comprising CDR 1, 2 and 3 of the light chain variable region of SEQ ID NO: 12; or

(f) a heavy chain comprising CDR 1, 2 and 3 of the heavy chain variable region of SEQ ID NO: 13 and a light chain comprising CDR 1, 2 and 3 of the light chain variable region of SEQ ID NO: 14.

56. The multispecific antigen binding protein of any of the foregoing claims, wherein the antibody or antigen binding domain comprises framework residues from a human framework region and/or is humanized.

57. The multispecific antigen binding protein of any of the foregoing claims, wherein the antigen of interest is a cell surface polypeptide capable of undergoing intracellular internalization when bound by a full-length IgG1 antibody.

58. The multispecific antigen binding protein of any of the foregoing claims, wherein the multispecific protein does not substantially increase or induce intracellular internalization of the antigen of interest on target cells.

59. A pharmaceutical composition comprising a multispecific antigen binding protein of any one of the above claims, and a pharmaceutically acceptable carrier or adjuvant.

60. The pharmaceutical composition of claim 59 which comprises another active agent.

61. A composition comprising a multispecific antigen binding protein of any one of the above claims, and NK and/or target cells that express an antigen of interest.

62. The composition of claim 61, wherein the composition comprises bone marrow or blood, e.g., from a recipient with a hematological cancer.

63. A cell which expresses a multispecific antigen binding protein according to any one of the above claims.

64. Use of a polypeptide or composition of any one of the above claims as a medicament for the treatment of disease.

65. The use of claim 64 which is effected *in vivo*.

66. The use of claim 64 which is effected *in vitro*.

67. The *in vitro* use of claim 66 which comprises incubating a composition comprising NK and/or target cells from a subject with a disease wherein target cells expressing an antigen of interest are desirably eradicated with an effective amount of a multispecific antigen binding protein according to any one of the above claims.

68. A method of treating a disease in a subject comprising administering to the subject a multispecific antigen binding protein, cell or composition according to any of claims 1-63.

69. A method of increasing the expression of CD137 on the surface of NKp46⁺CD16⁺ NK cells in a subject having a disease, the method comprising administering to the subject a composition according to any of claims 1-62.

70. The method or use of claims 64-69, wherein the disease is a cancer, infectious disease or an inflammatory or autoimmune disease.

71. The method or use of claims 64-70, wherein the disease is a cancer characterized by cells that express CD137L (CD137 ligand).

72. A method of increasing the expression of CD137 on the surface of NK cells *in vitro*, the method comprising bringing NKp46⁺CD16⁺ NK cells into contact with a composition according to any of claims 1-62.

73. The use or method of any of claims 64-72 which includes the use of another therapeutic agent, optionally a chemotherapeutic, anticancer agent, receptor agonist or receptor antagonist, or another biologic, e.g., an antibody or antibody fusion protein, which optionally elicits an additive or synergistic effect on the lysis of target cells expressing the antigen of interest.

74. The use or method of claim 73 wherein the other therapeutic agent comprises a CD137 or 4-1BB agonist, e.g., an anti-CD137 agonist antibody or fusion protein, a PD-1 antagonist, or a PD-L1 antagonist, a CTLA-4 antagonist or another immune checkpoint blocking antibody.

75. A method of making a heterodimeric protein, comprising:
a) providing a first nucleic acid encoding at least a first polypeptide chain according to any of claims 40-46 or 48-58;
b) providing a second nucleic acid encoding at least a second polypeptide chain according to any of claims 40-46 or 48-58; and
c) expressing said first and second nucleic acids in a host cell to produce a protein comprising said first and second polypeptide chains, respectively; loading the protein produced onto an affinity purification support, optionally a Protein-A support, and recovering a heterodimeric protein that binds NKp46, CD16 and an antigen of interest.

76. A method of making a heterotrimeric protein, comprising:
(a) providing a first nucleic acid encoding a first polypeptide chain according to any of claims 47 to 58;
(b) providing a second nucleic acid encoding a second polypeptide chain according to any of claims 47 to 58;
(c) providing a third nucleic acid comprising a third polypeptide chain according to any of claims 47 to 58; and

(d) expressing said first, second and third nucleic acids in a host cell to produce a protein comprising said first, second and third polypeptide chains, respectively; loading the protein produced onto an affinity purification support, optionally a Protein-A support, and recovering a heterotrimeric protein.

77. A method for identifying or evaluating a polypeptide, comprising the steps of:

- (a) providing nucleic acid(s) encoding the polypeptides of a protein of any of claims 1-58;
- (b) expressing said nucleic acids in a host cell to produce said proteins, respectively; and recovering said protein; and
- (c) evaluating the protein produced for a biological activity of interest.

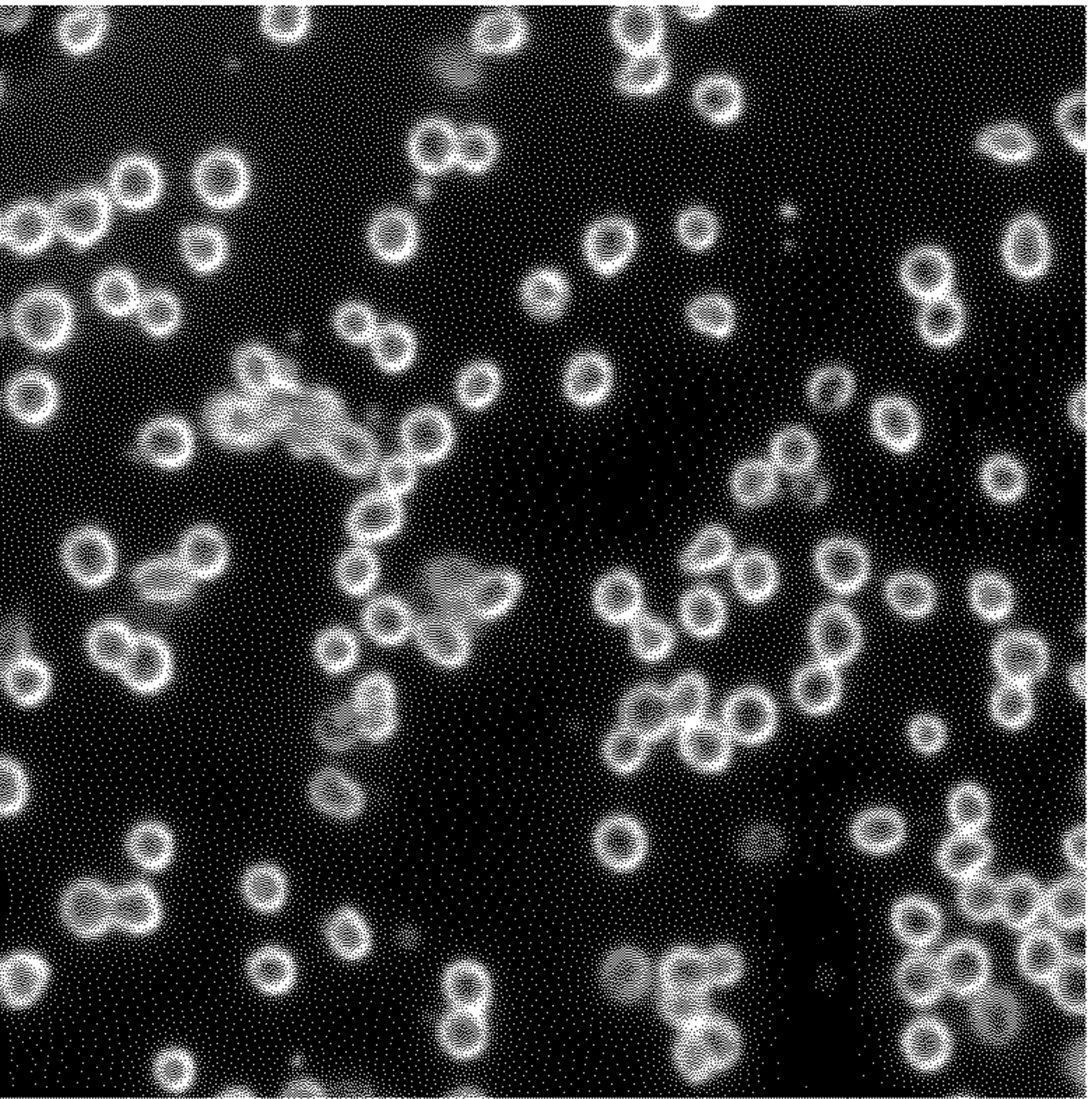
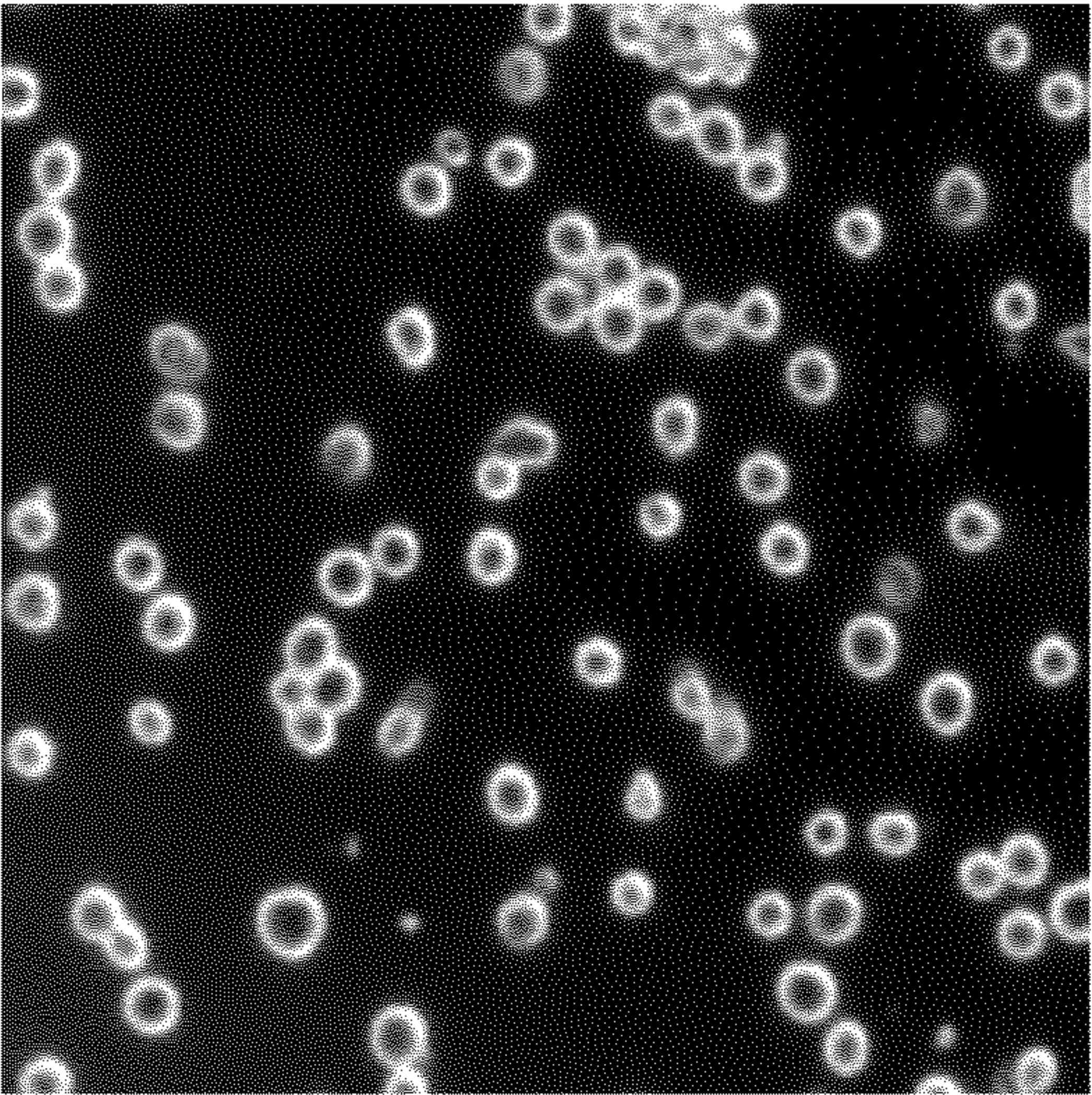
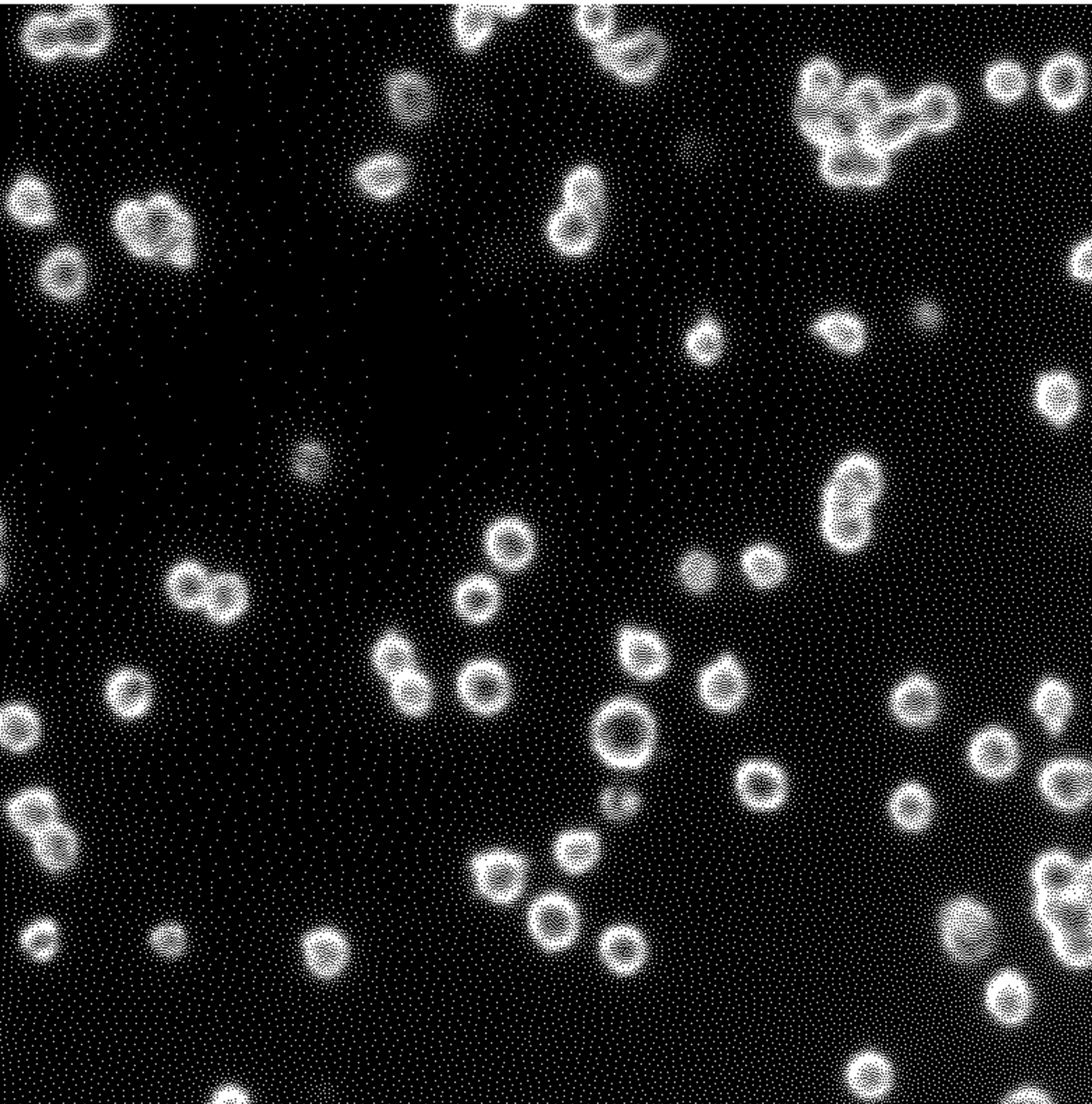
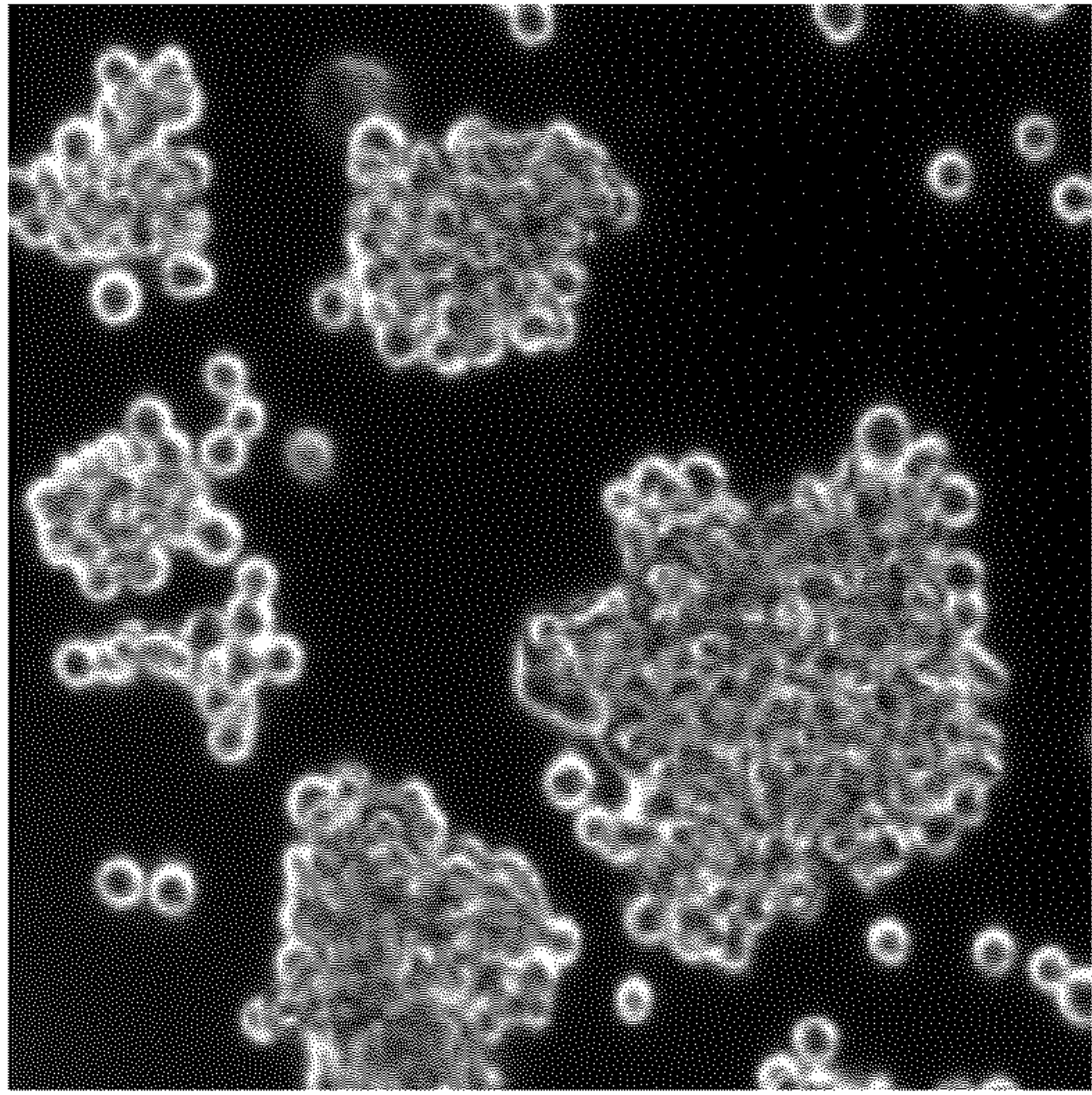
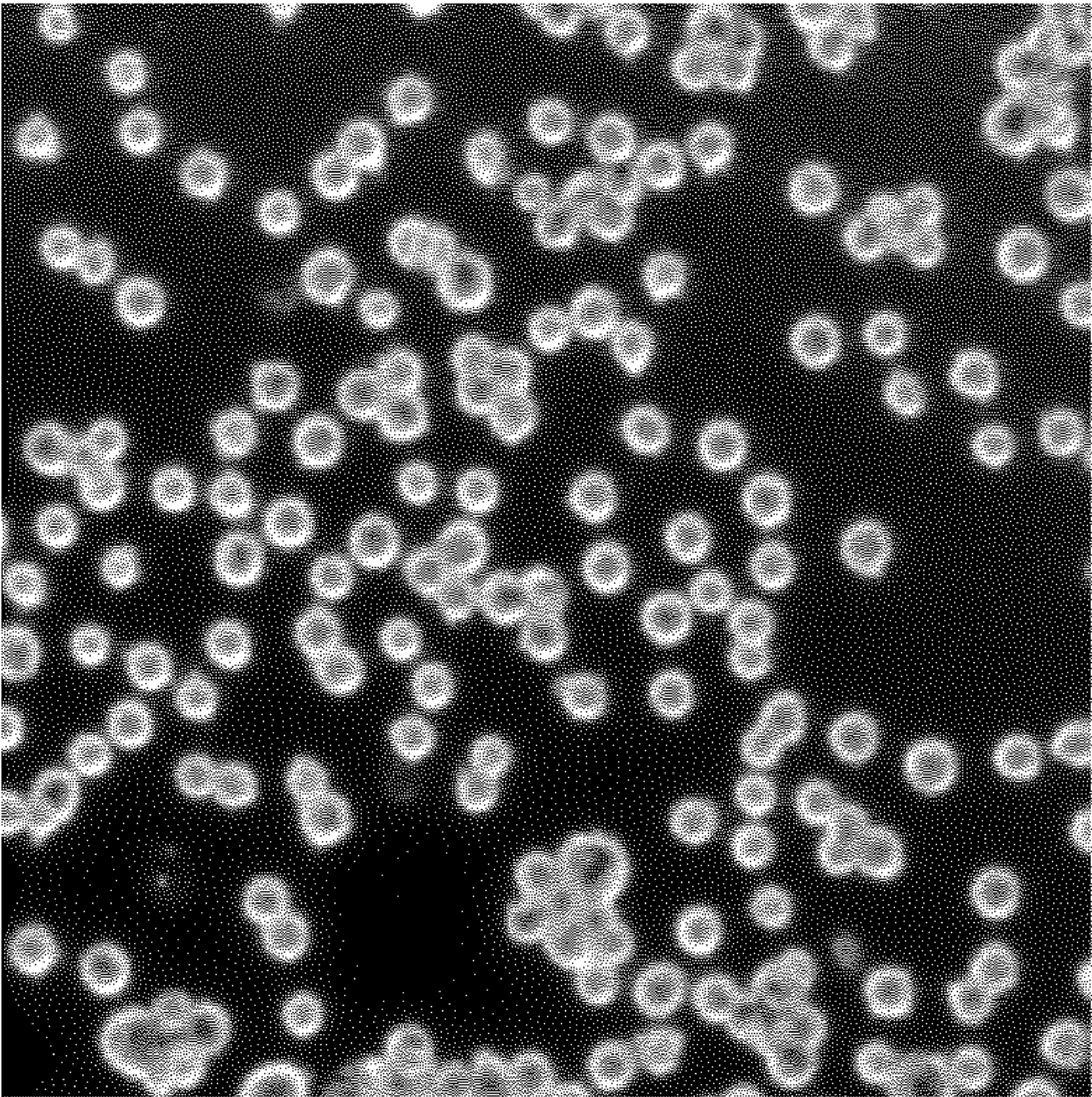
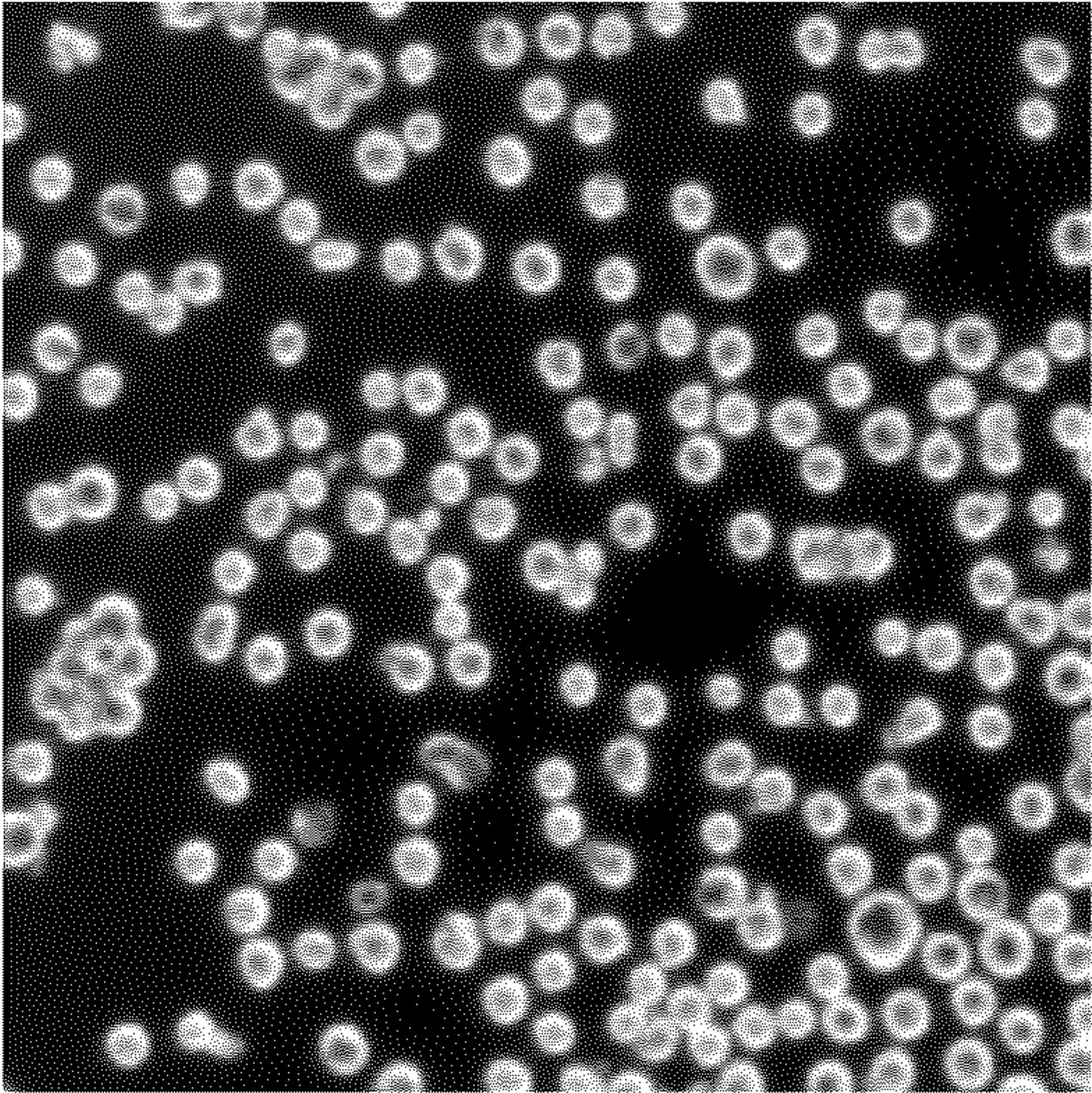
78. The method of claim 77, wherein evaluating the polypeptide comprises: testing the ability of the polypeptide to increase the expression of CD137 on the surface of NKp46⁺CD16⁺ NK cells, when incubated with such effector cells in the presence of target cells (that express antigen of interest).

Figure 1

Jurkat

B221 + Jurkat (50/50)

B221



Anti-CD19-
monoFc-IgG1-
Anti-CD3
(1 µg/ml)

None

Figure 2A

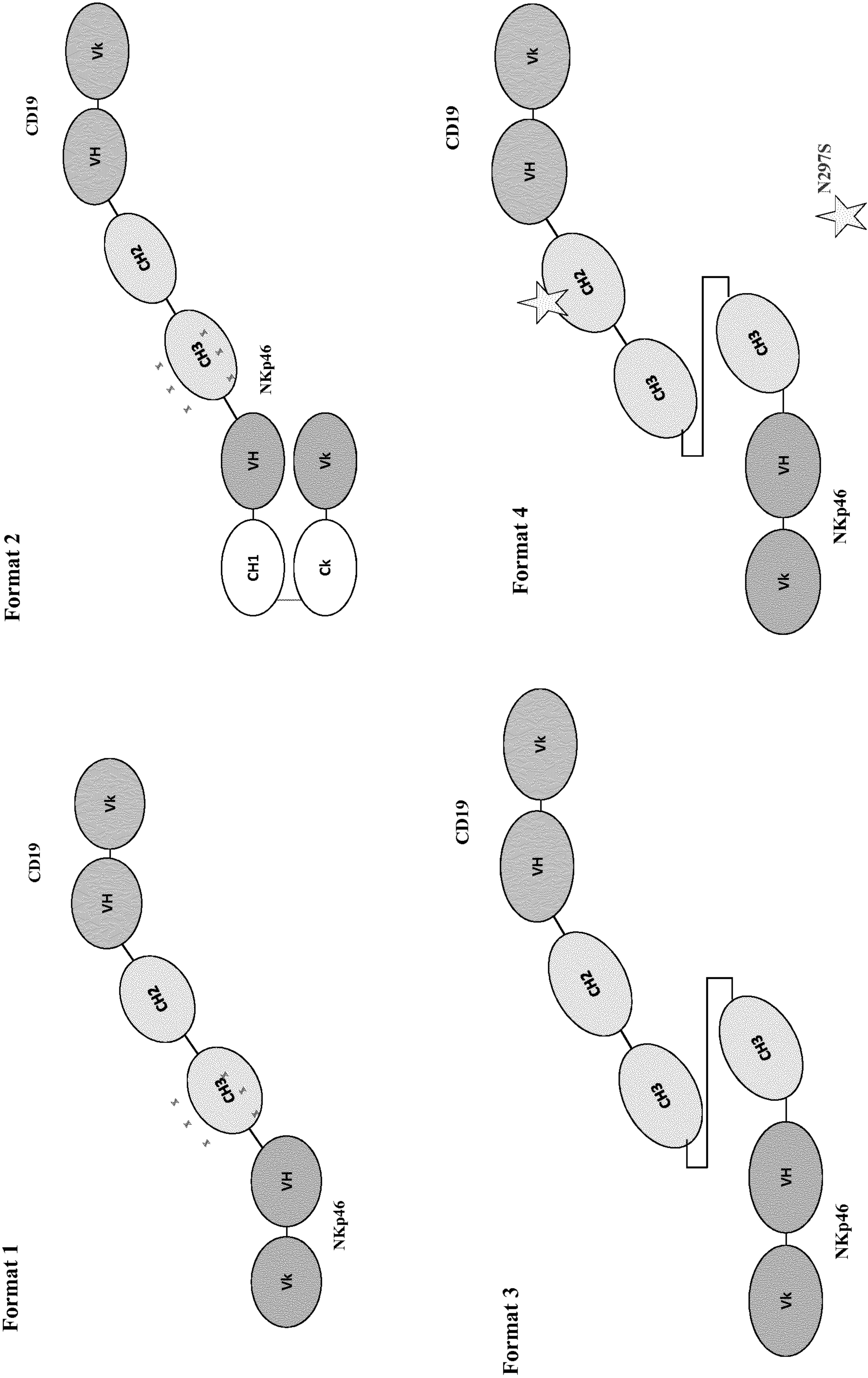


Figure 2B

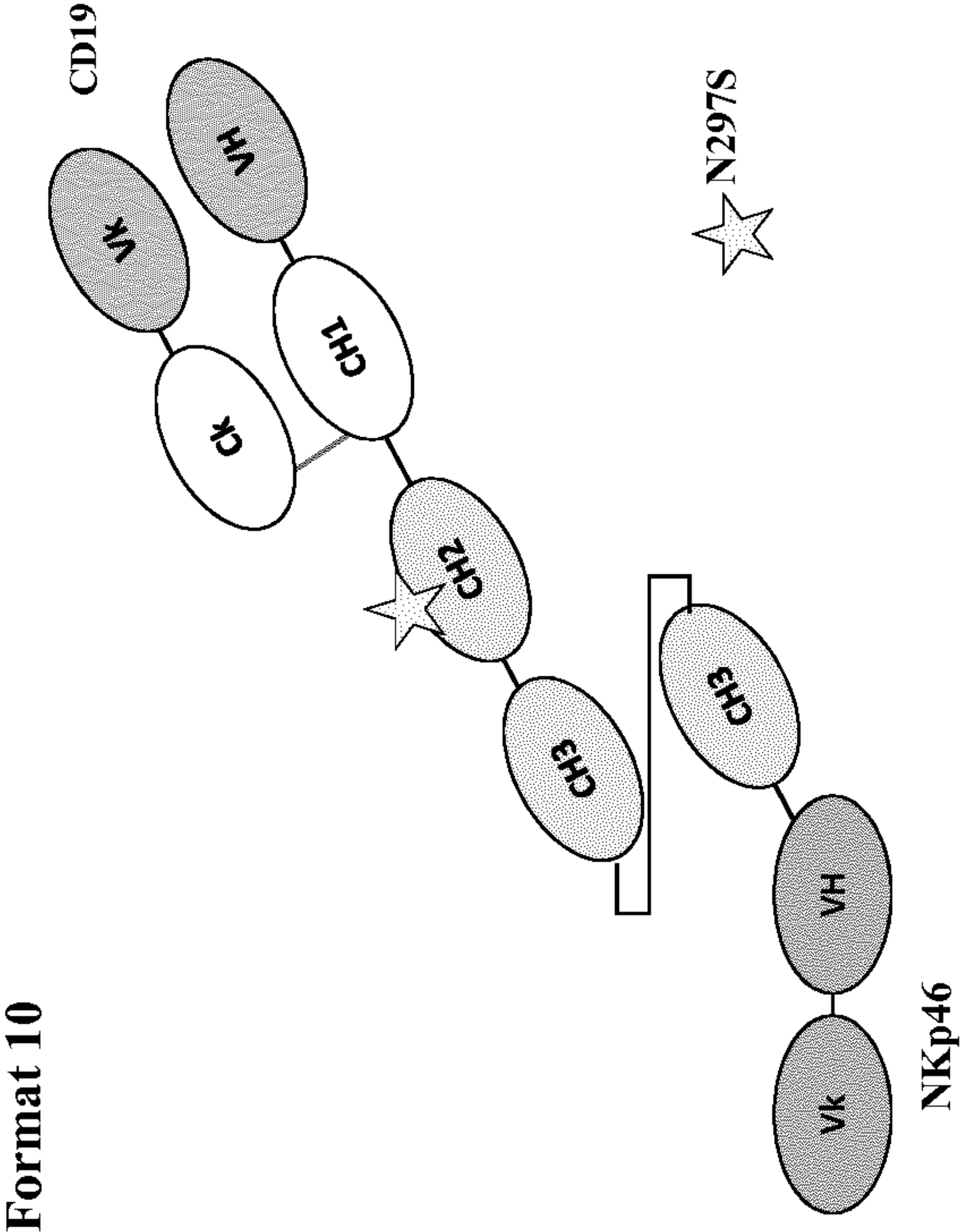
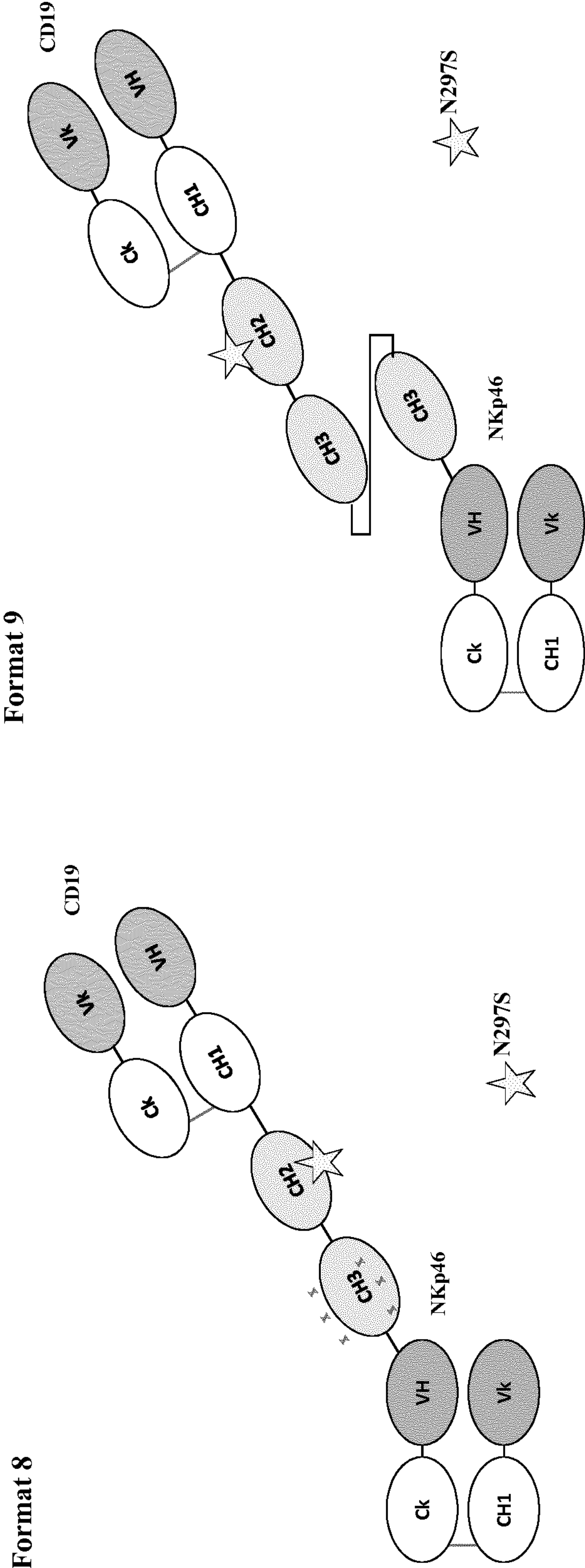


Figure 2C

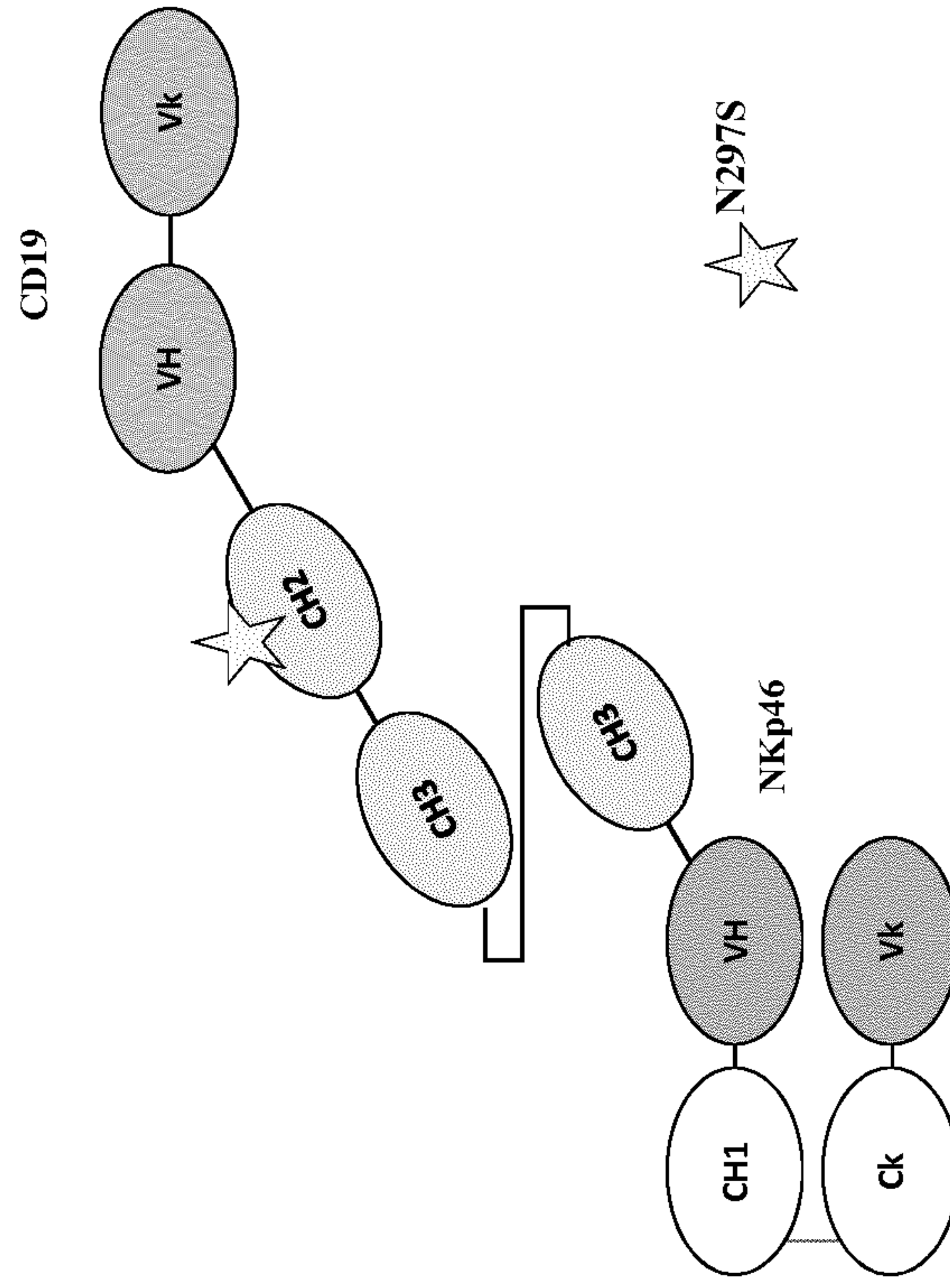
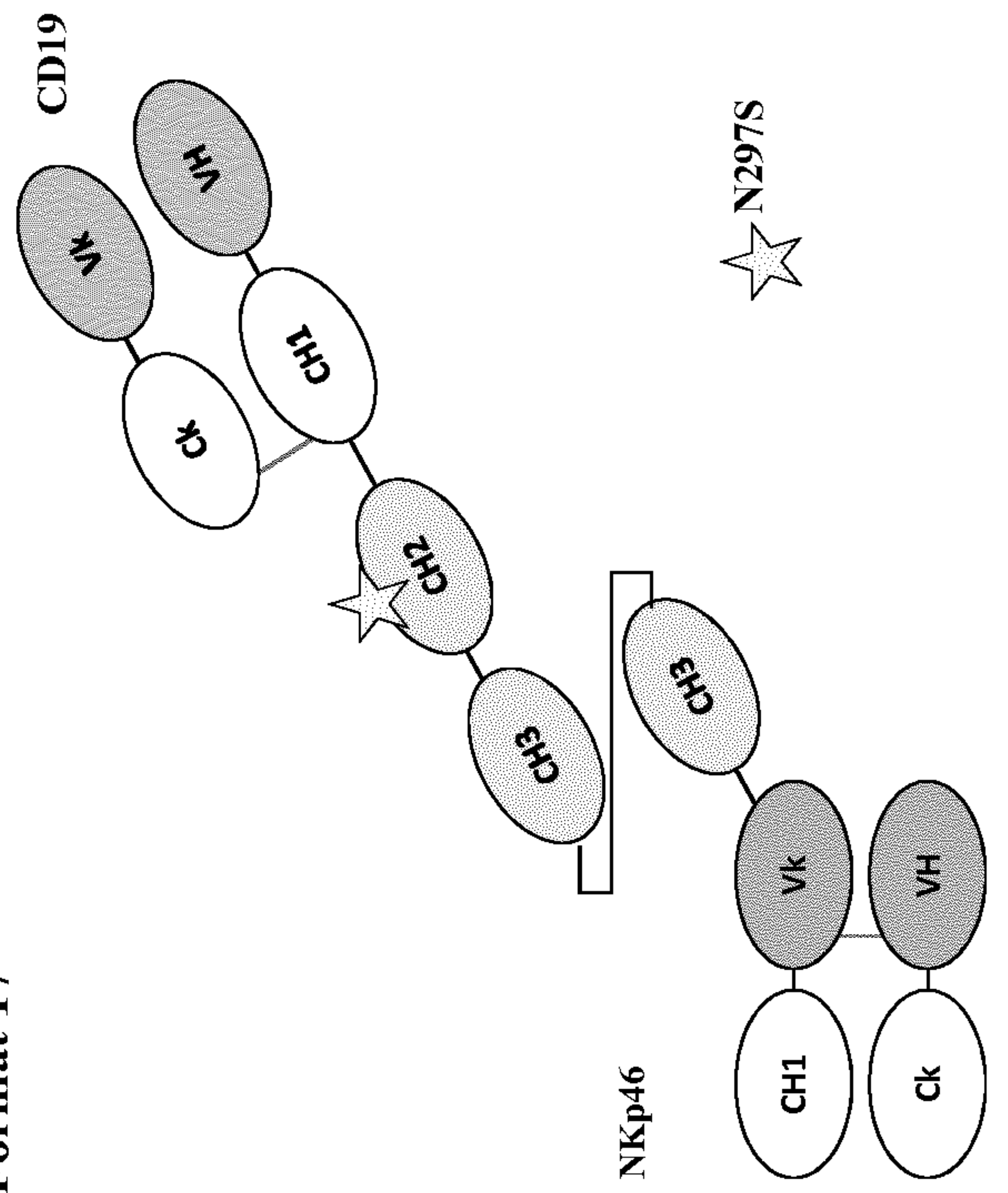
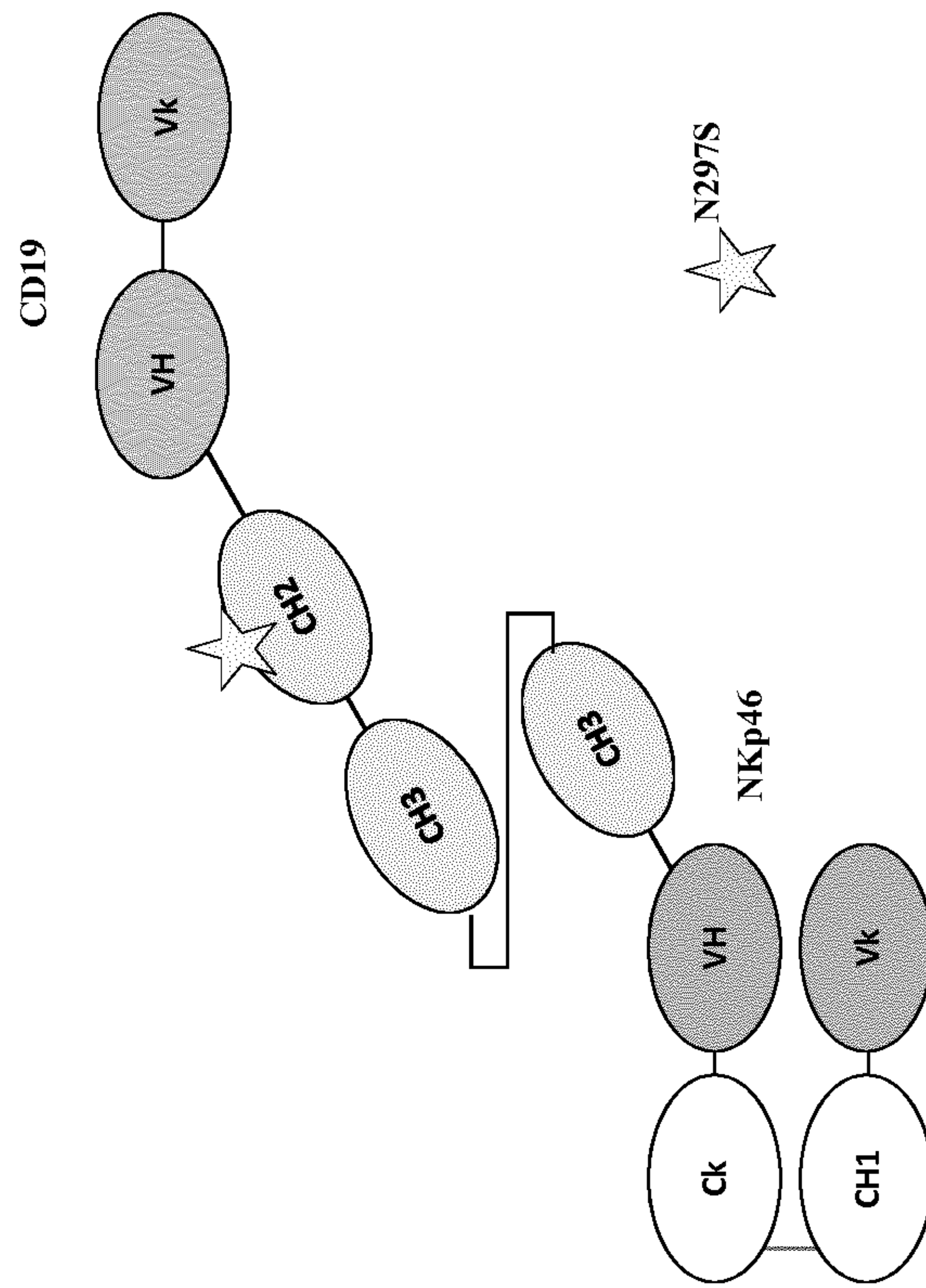
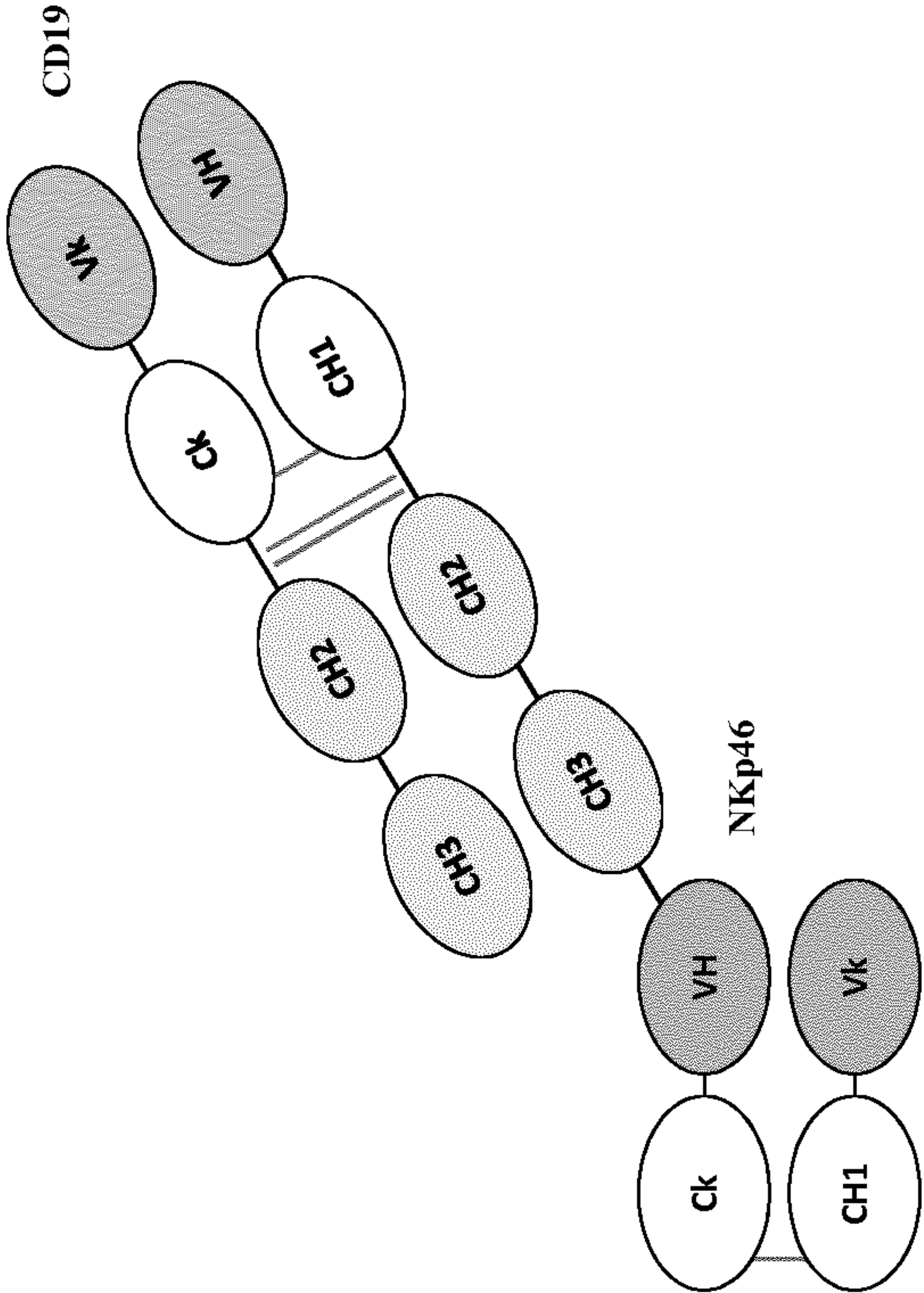
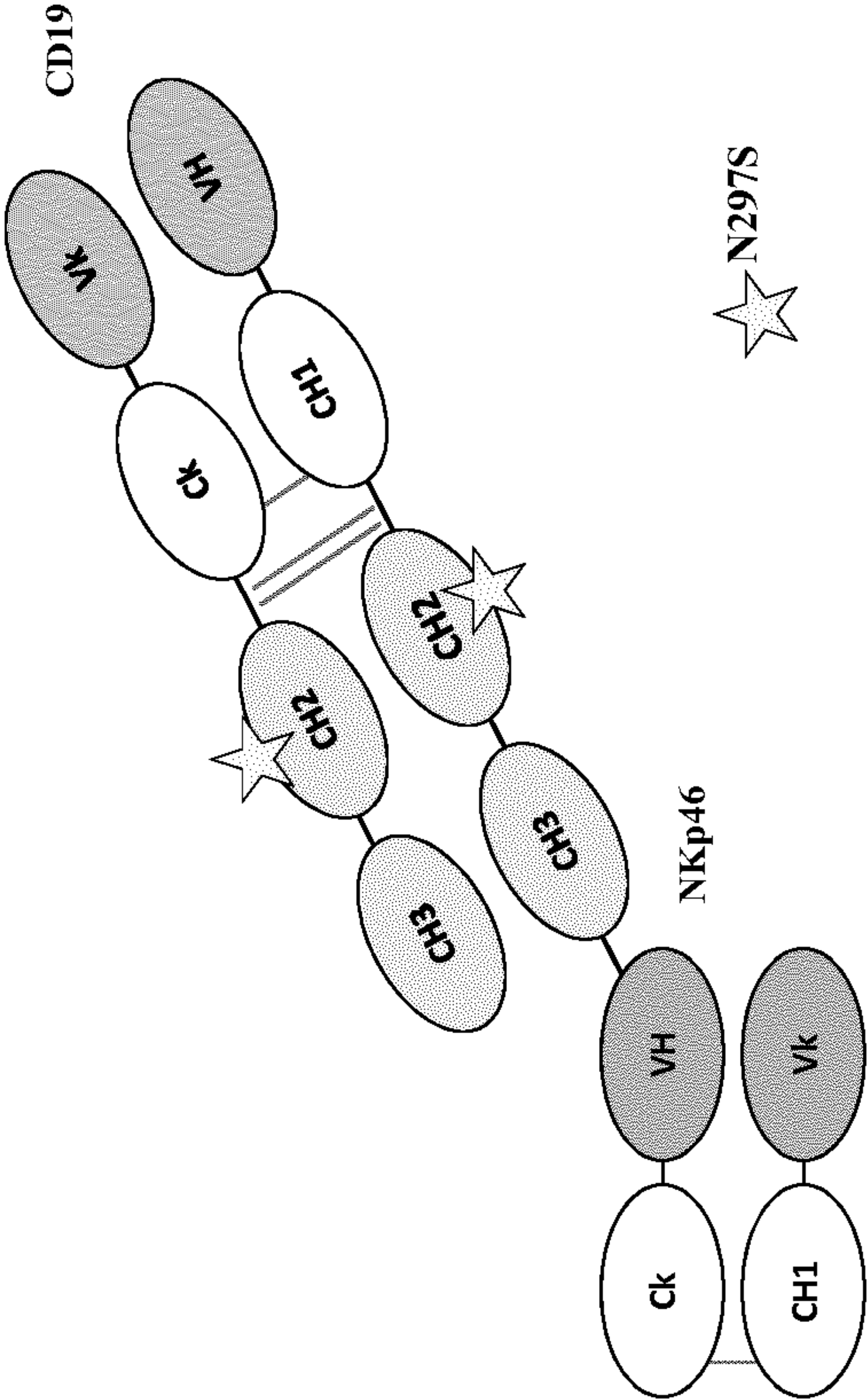


Figure 2D

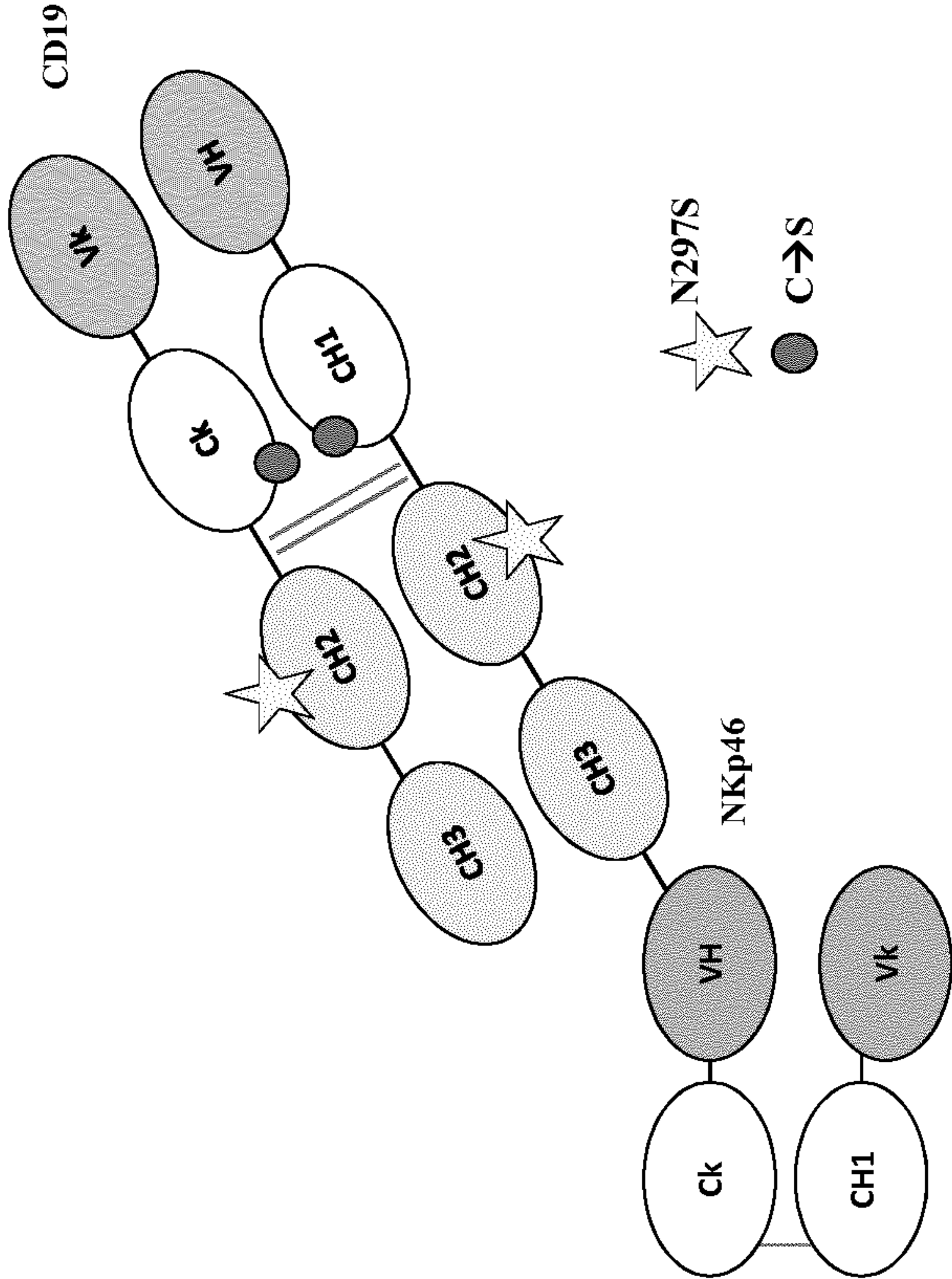
Format 5



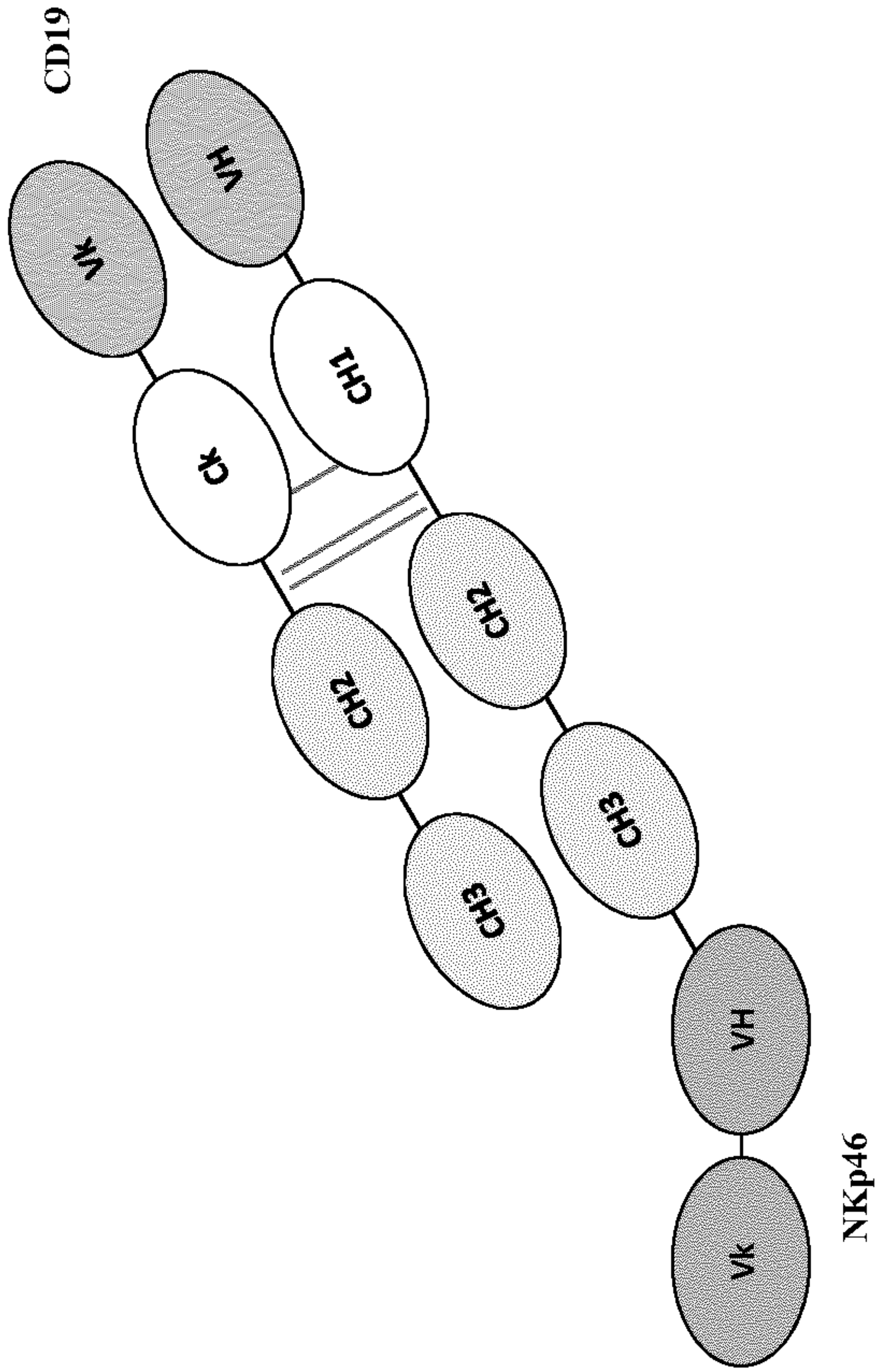
Format 6



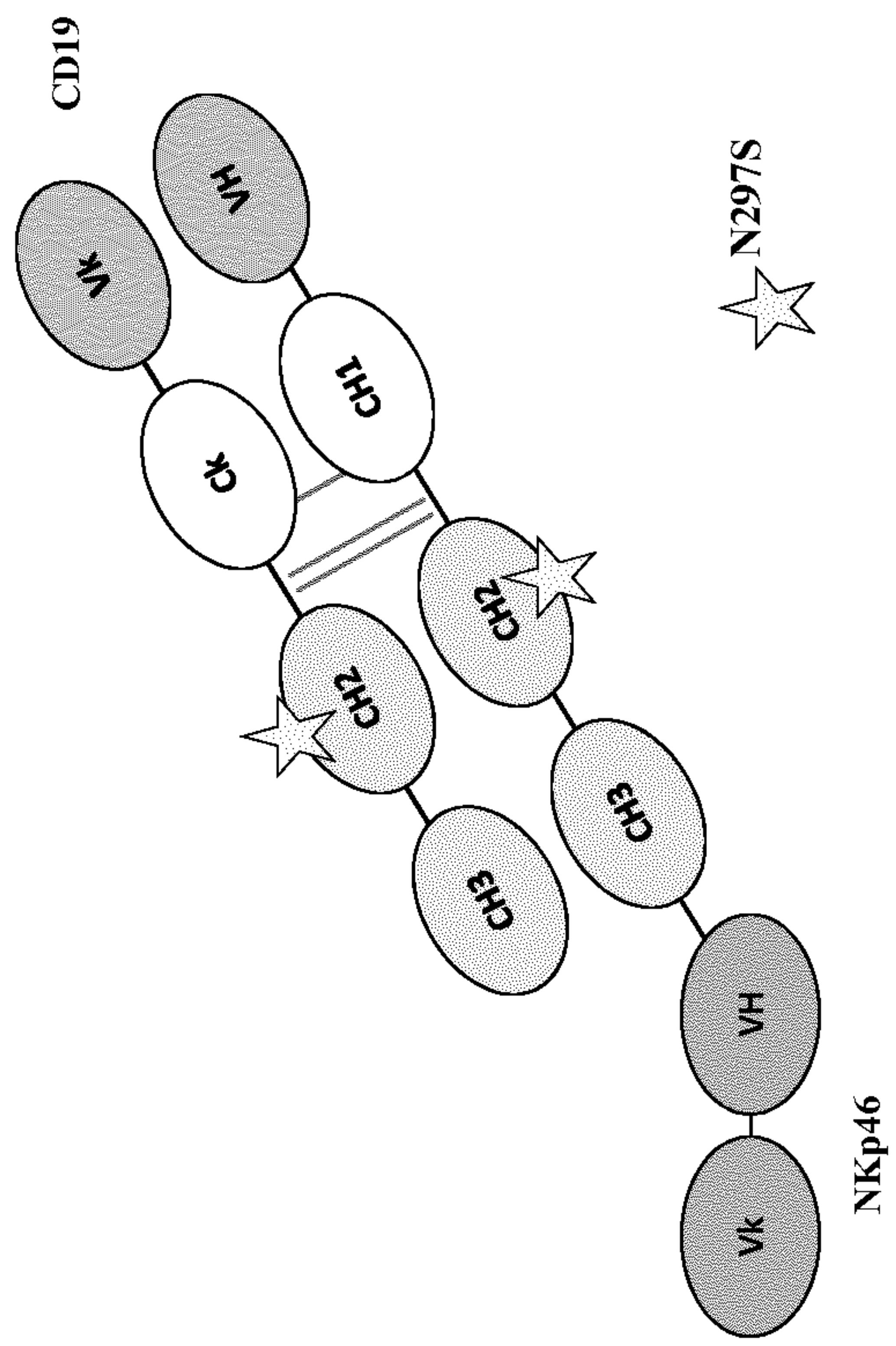
Format 7



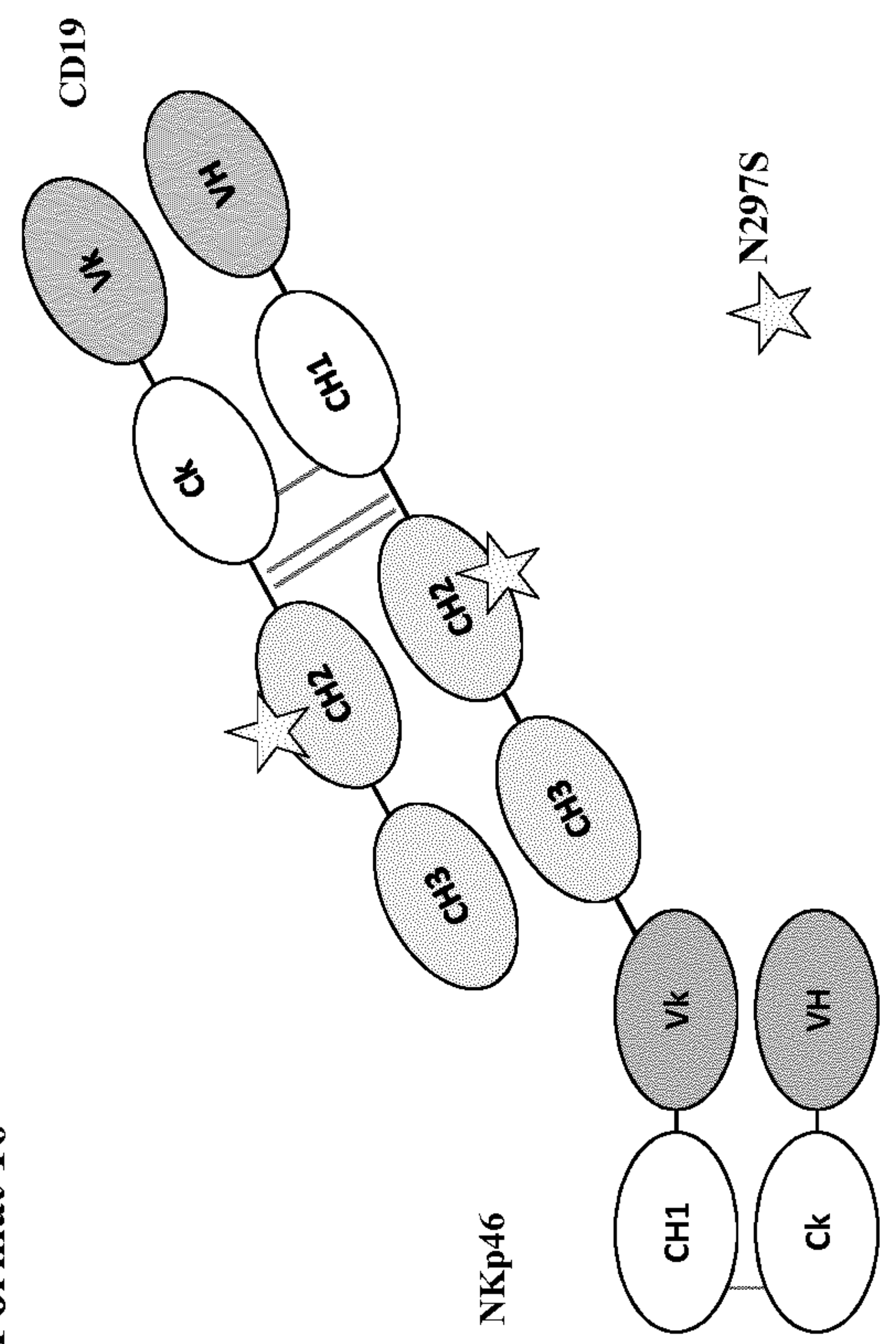
Format 13



Format 14



Format 16



Format 16

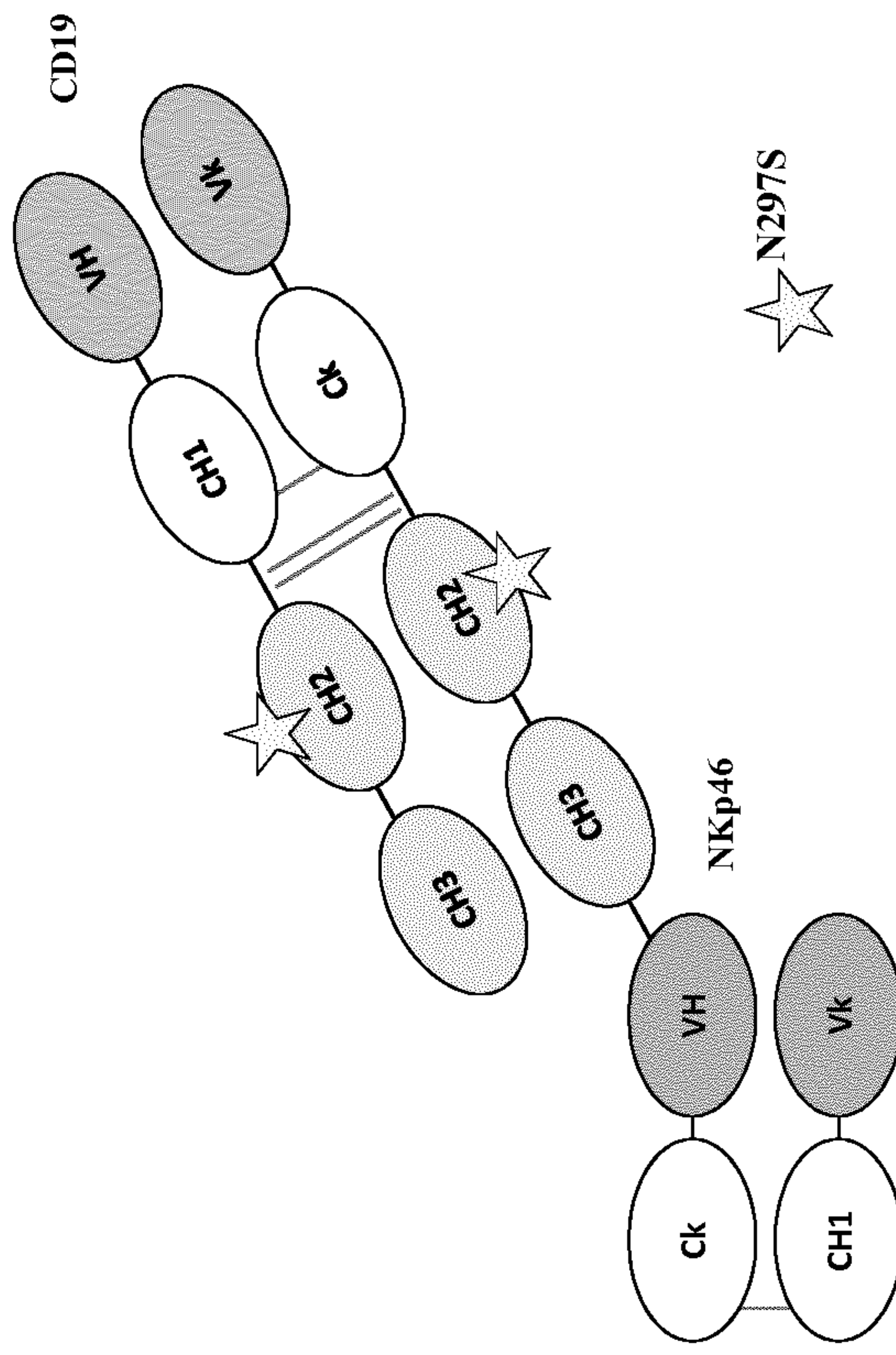


Figure 2F

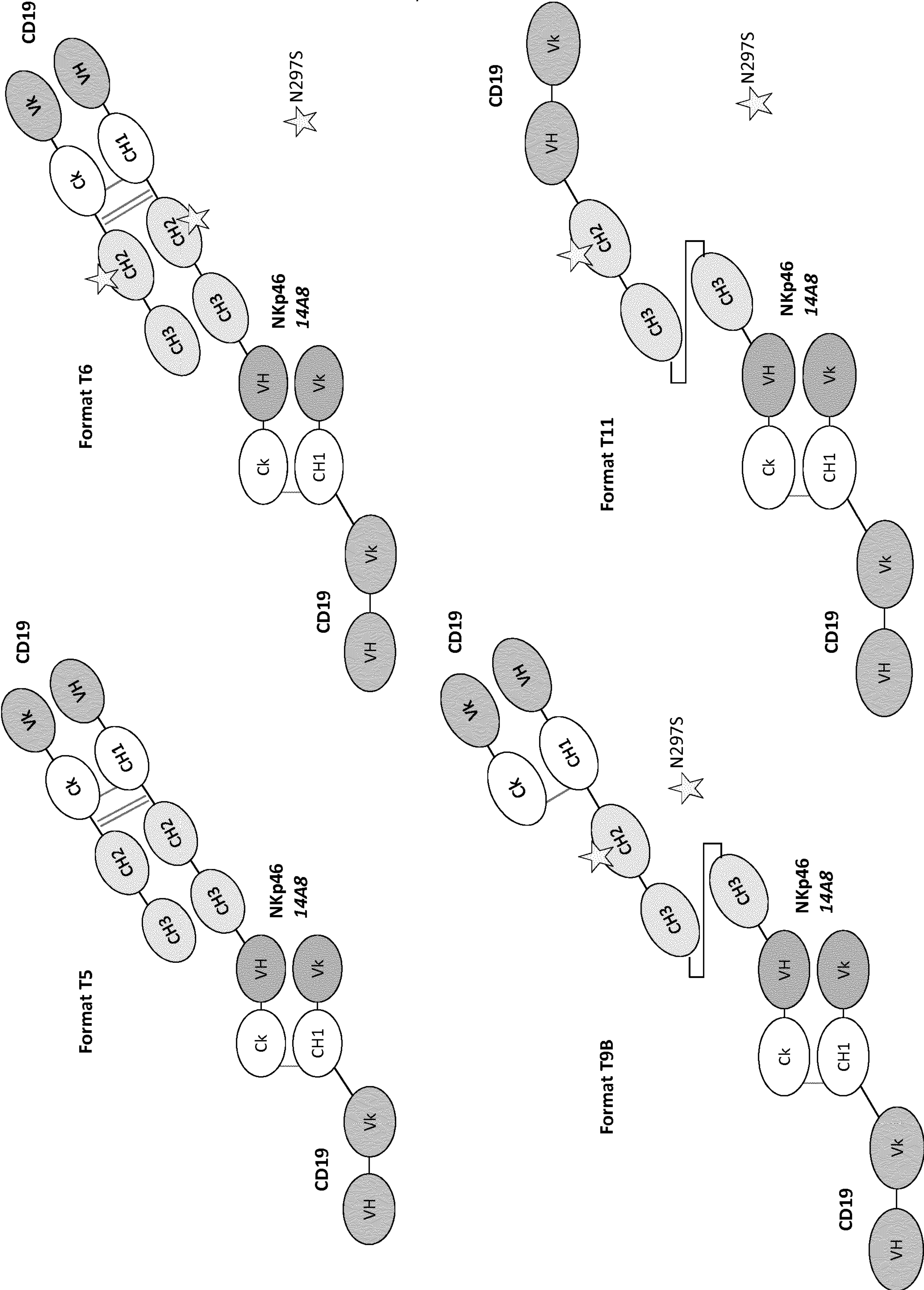


Figure 3A

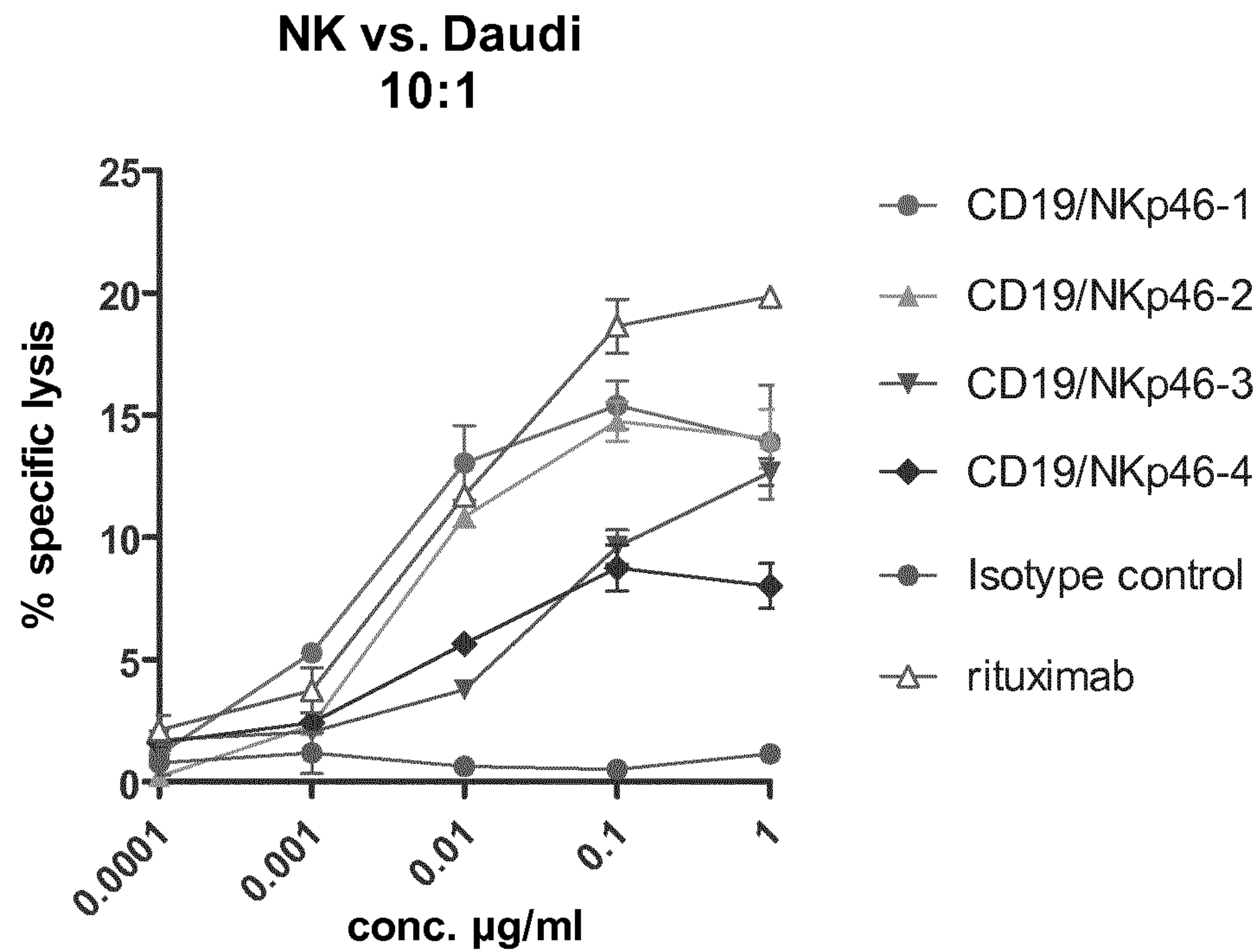


Figure 3B

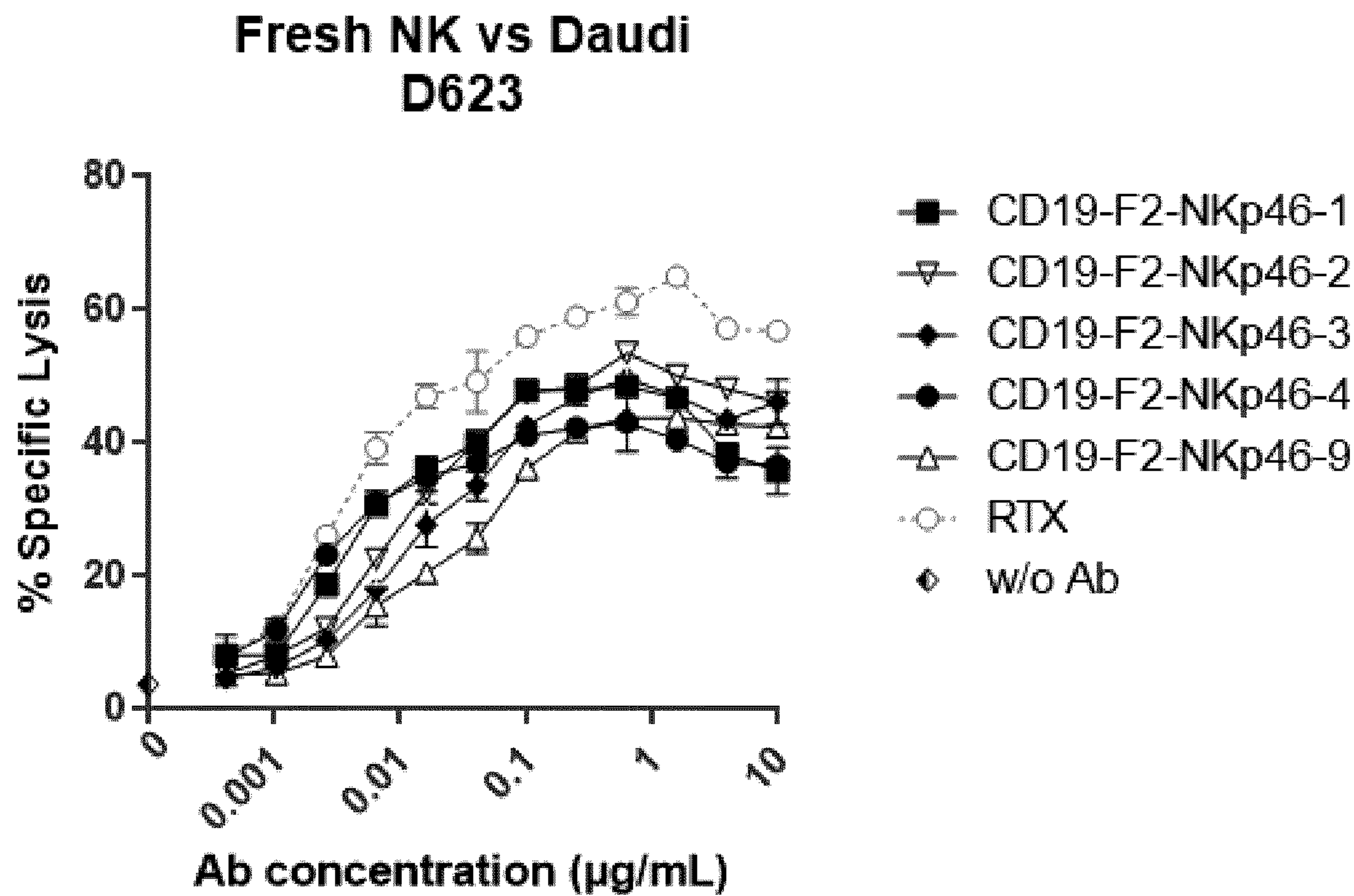


Figure 4A

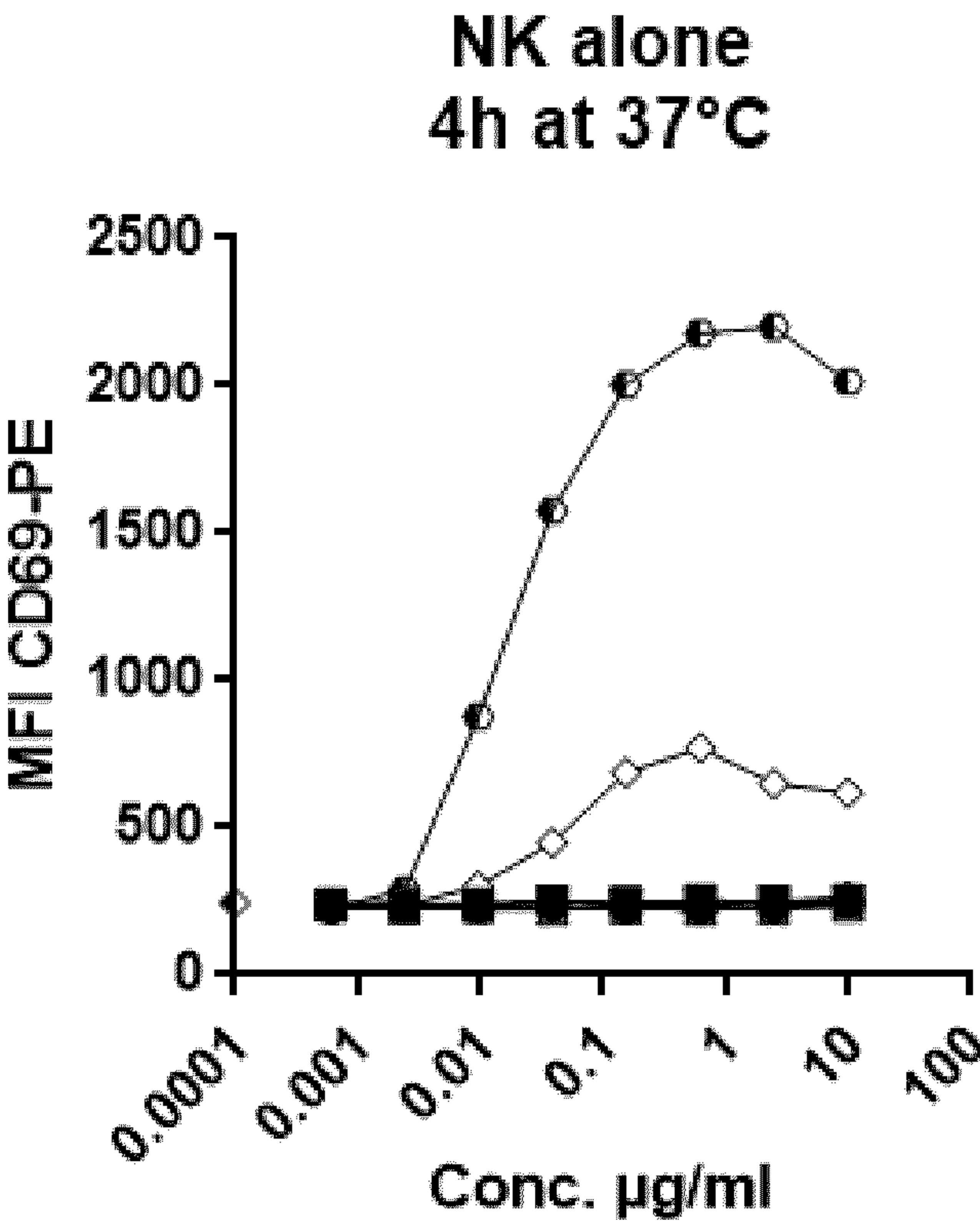
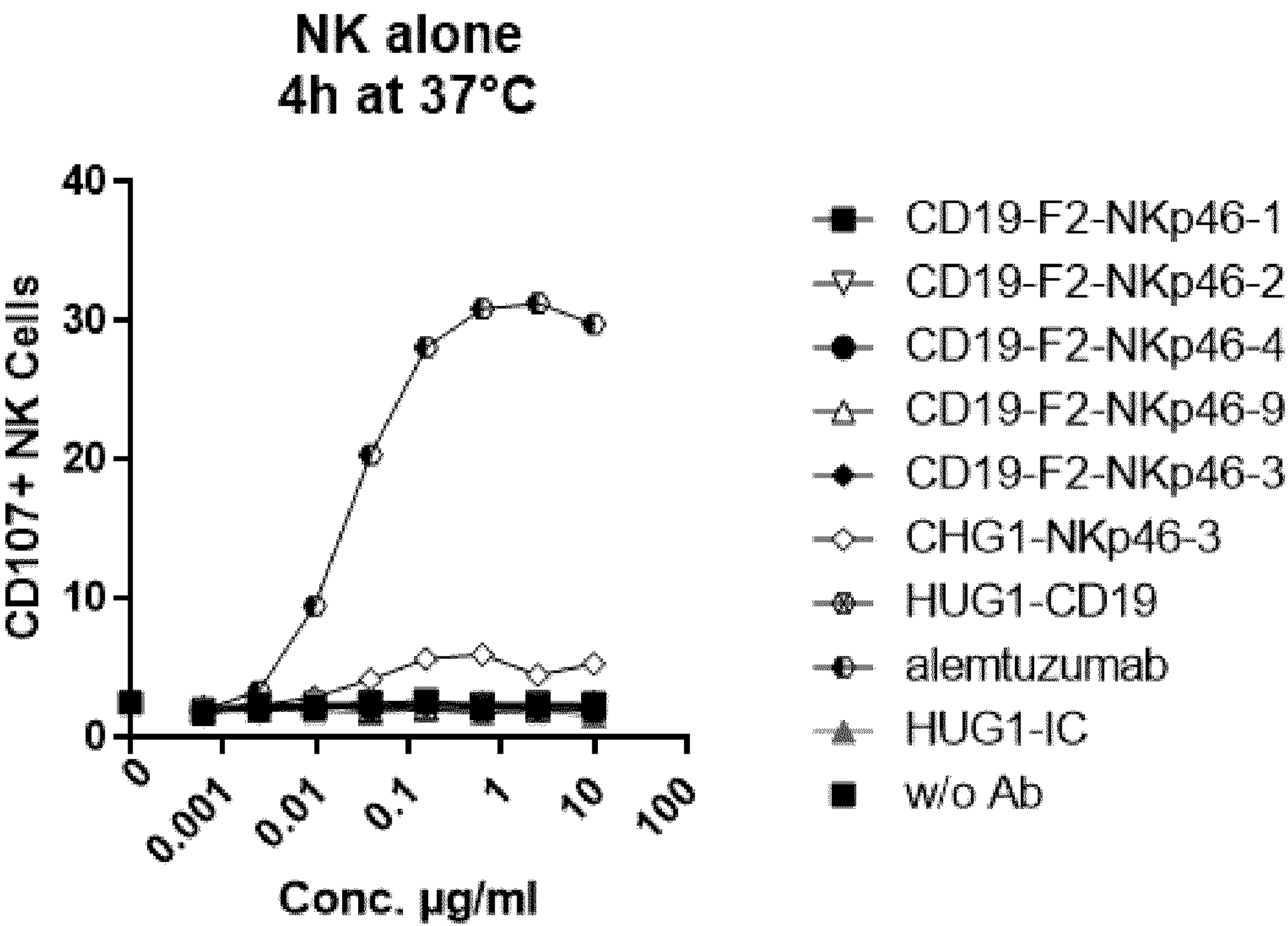


Figure 4B

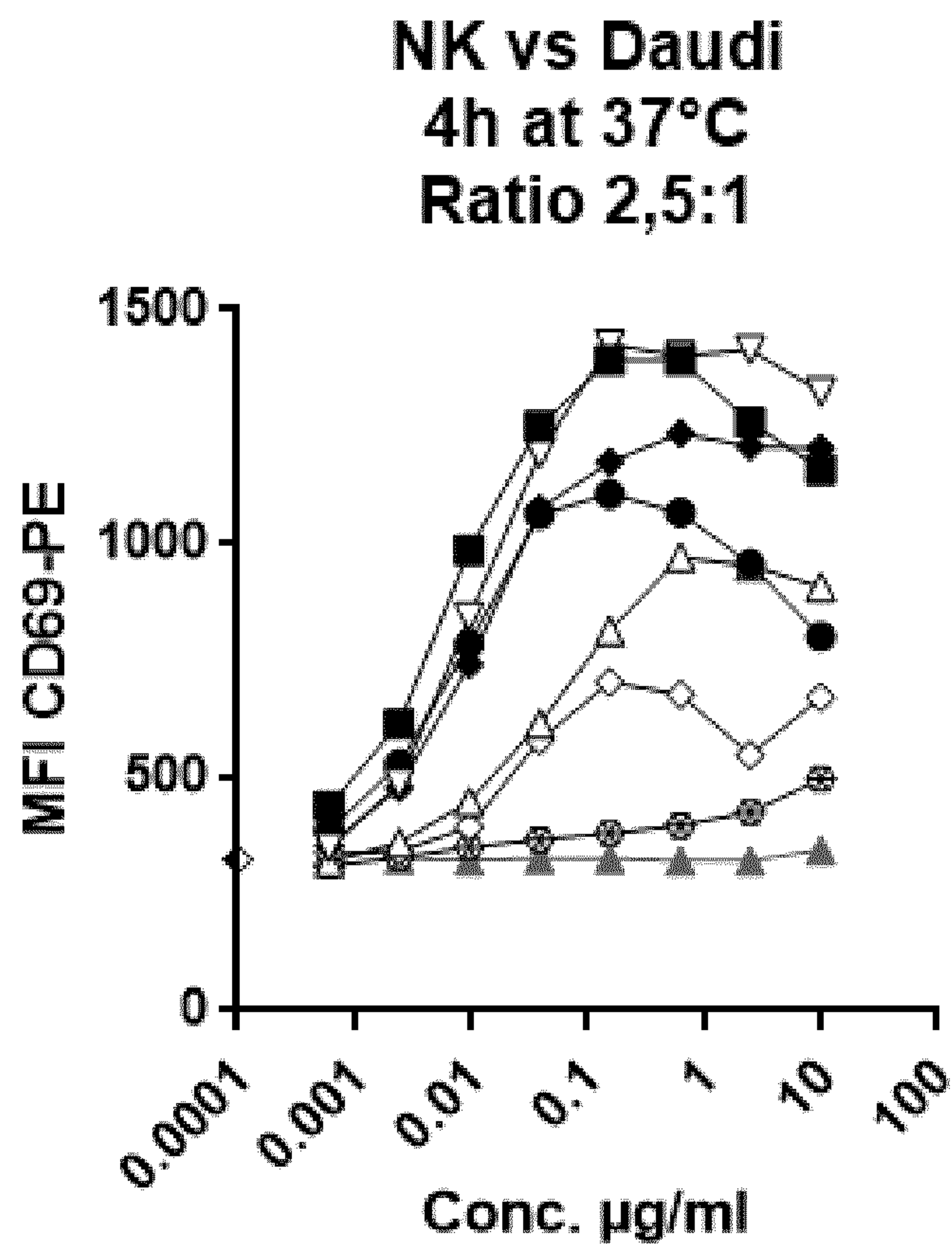
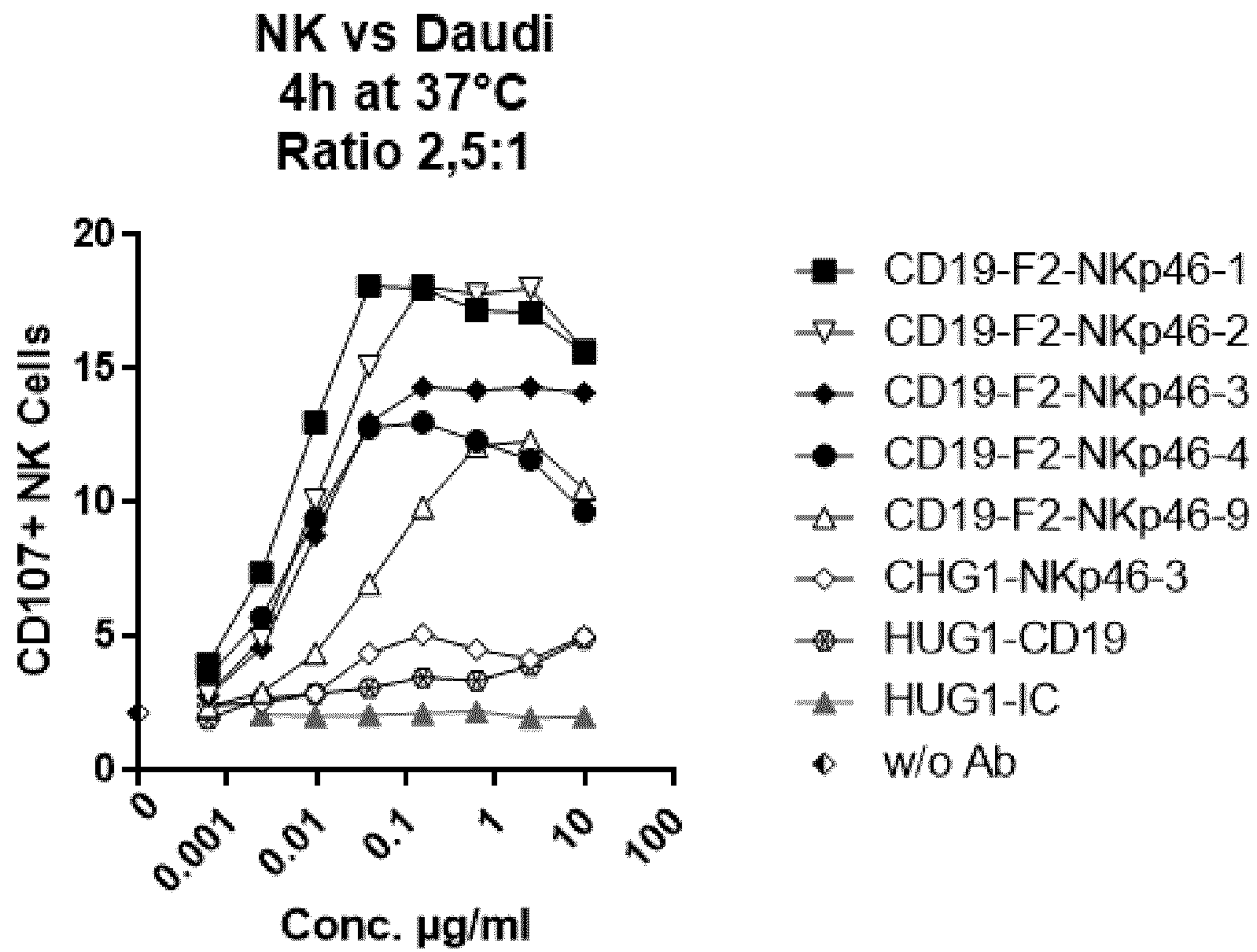
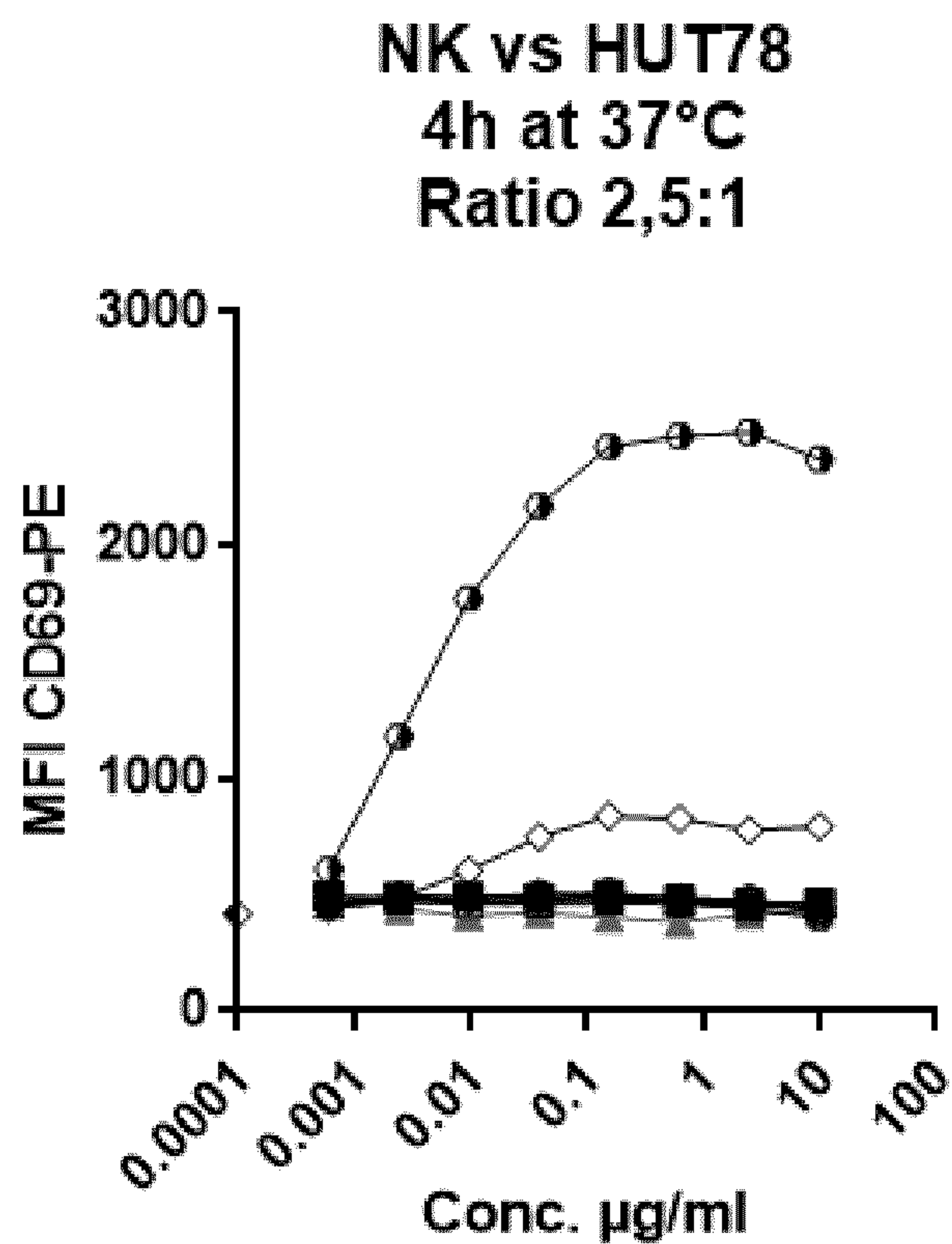
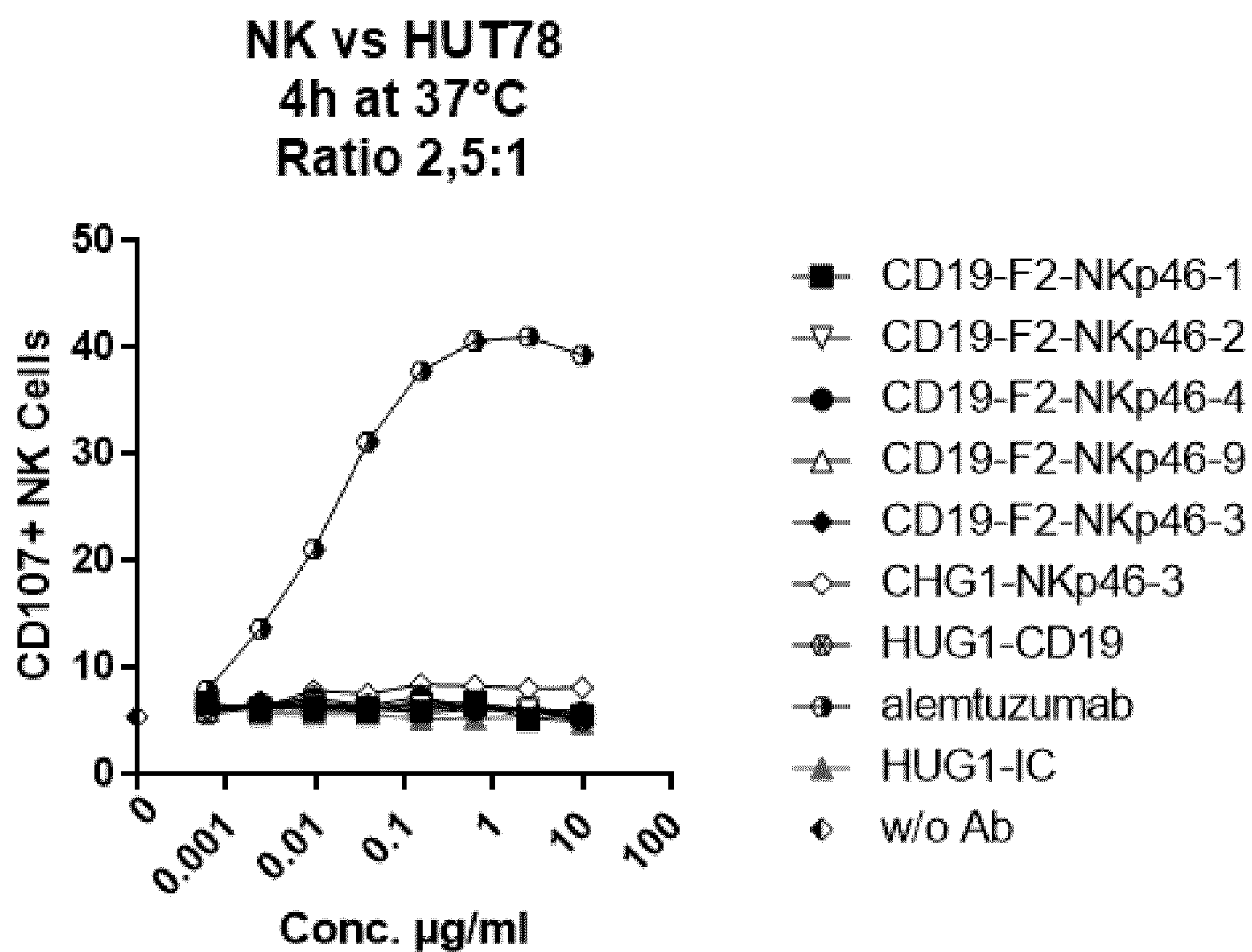


Figure 4C



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Figure 5A

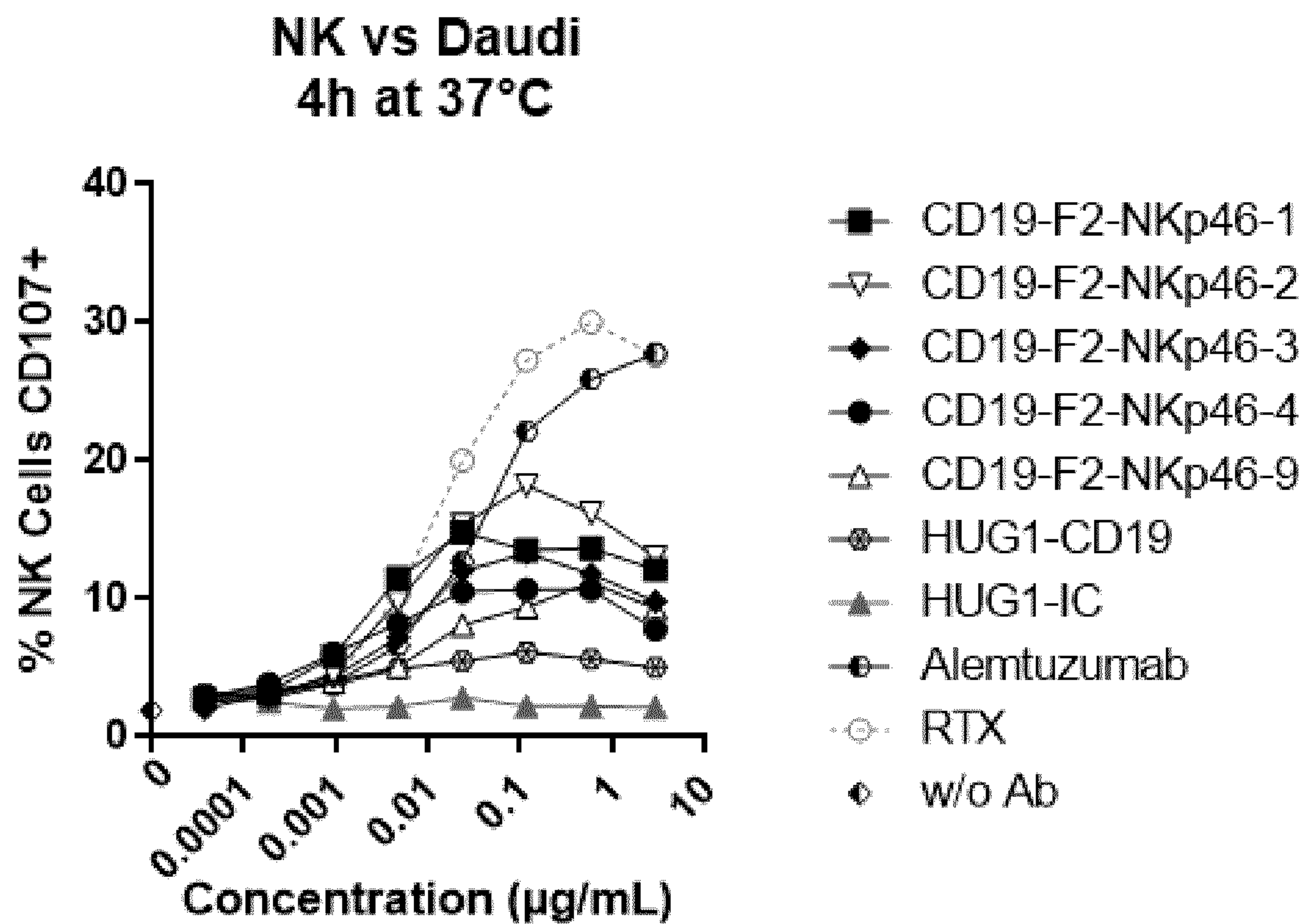


Figure 5B

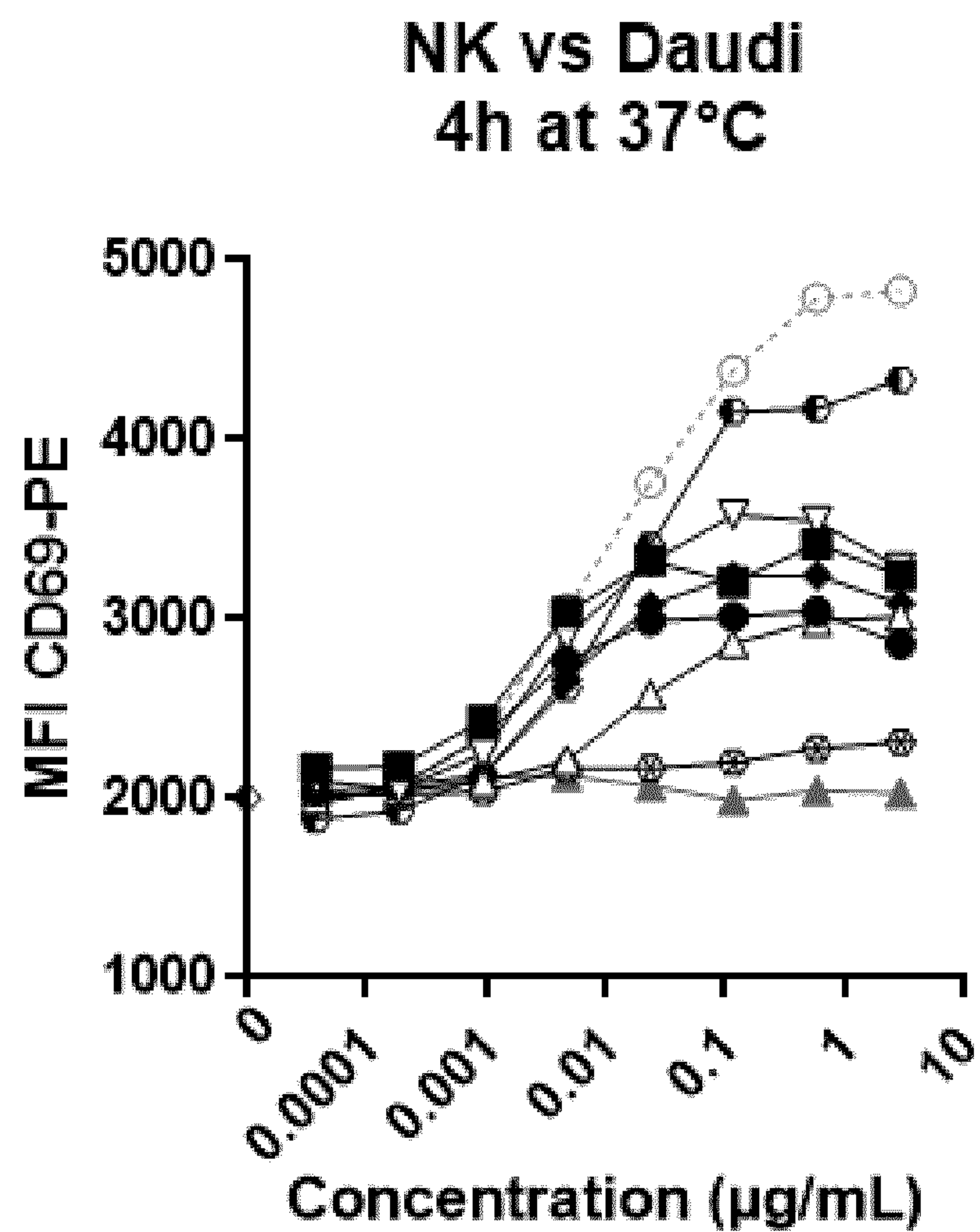


Figure 6A

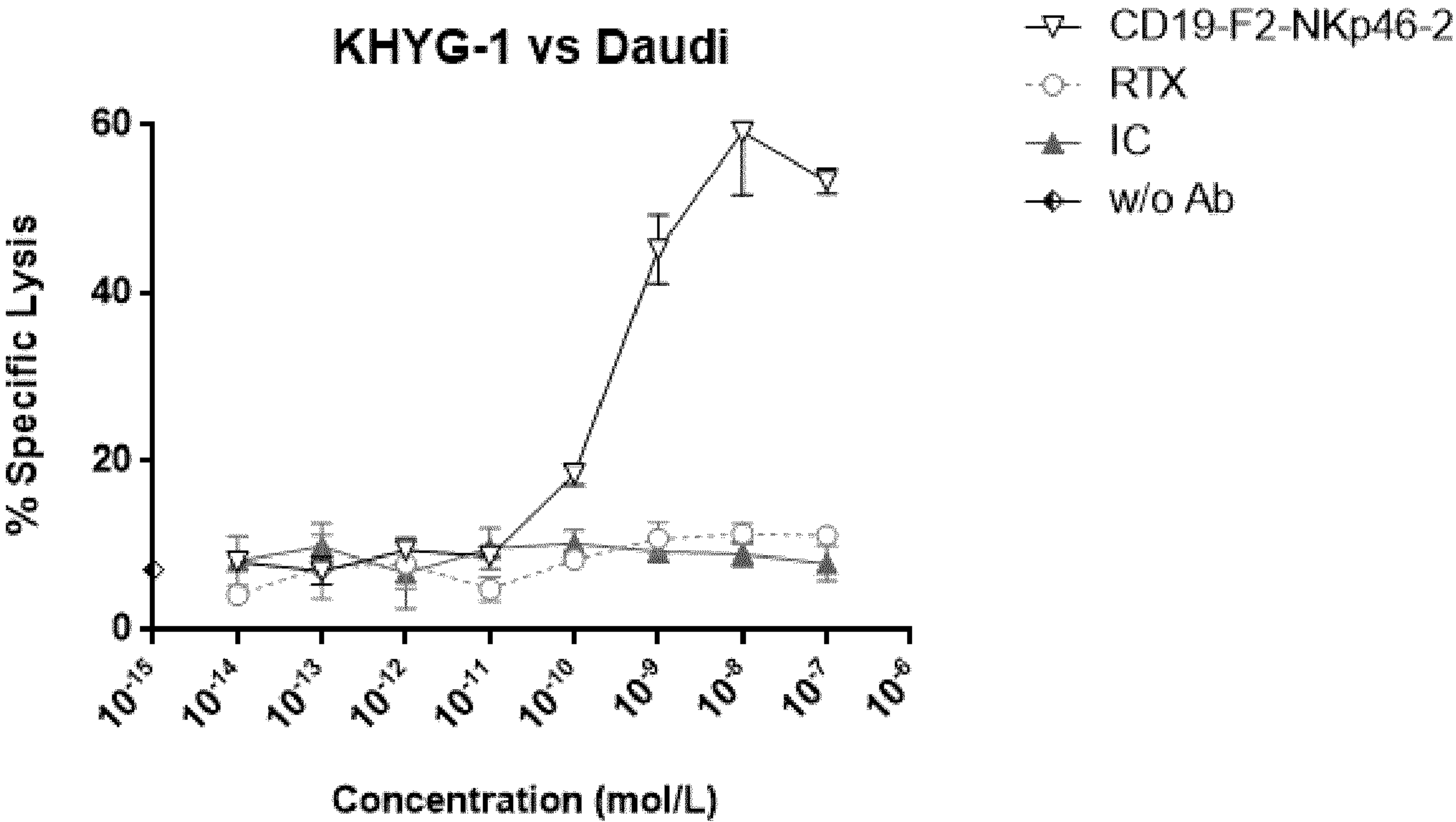


Figure 6B

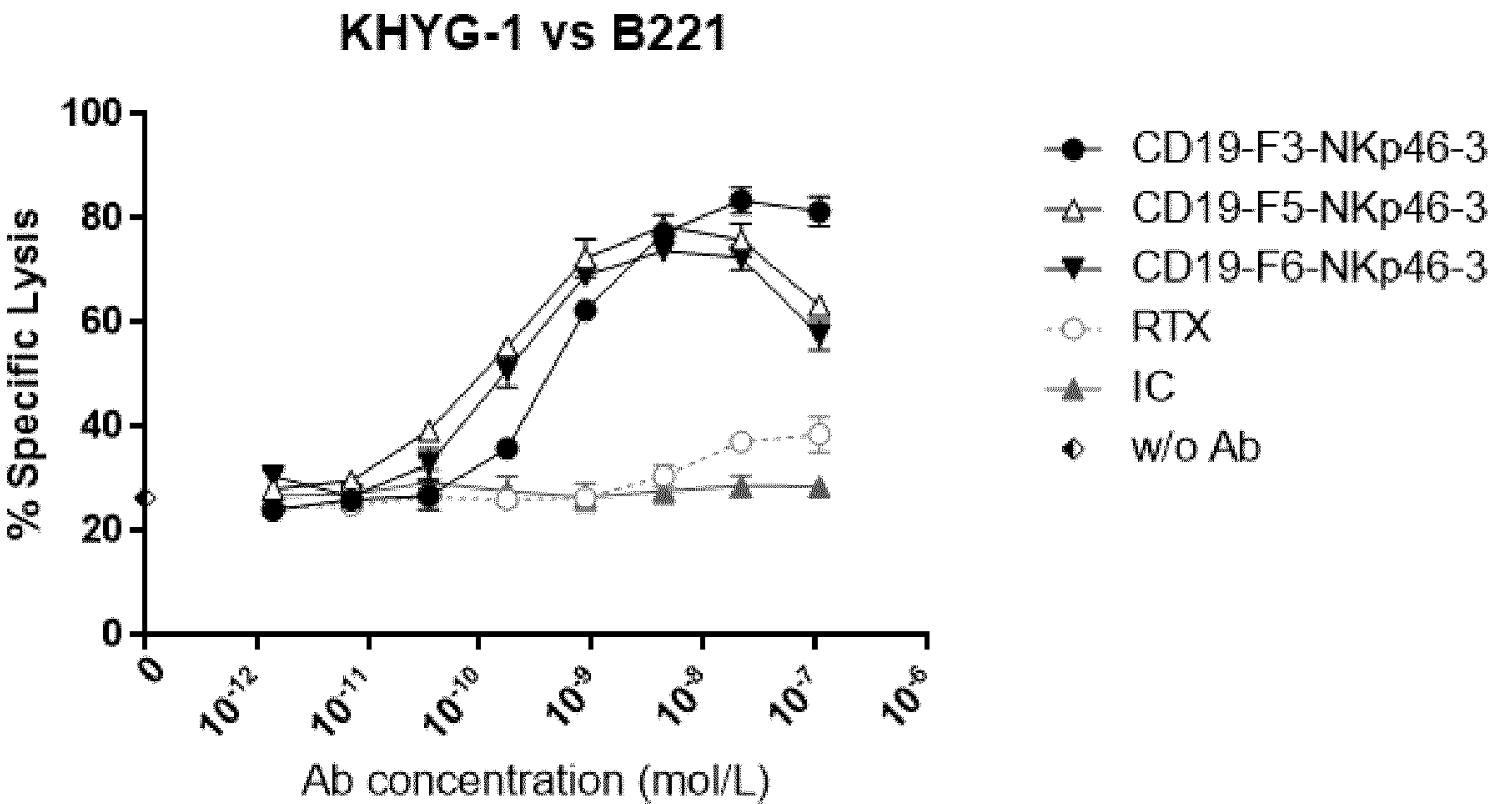
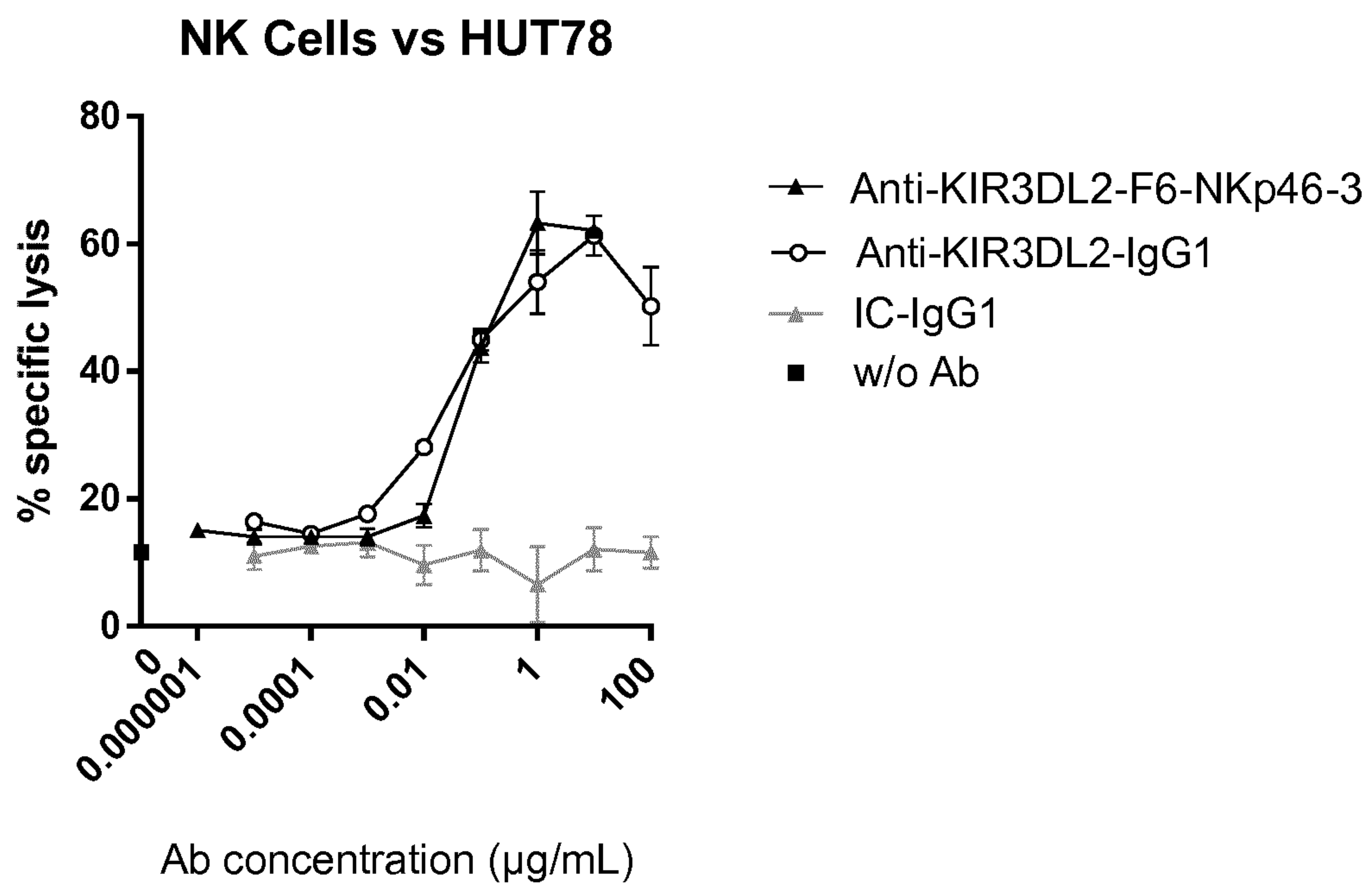


Figure 6C



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Figure 7

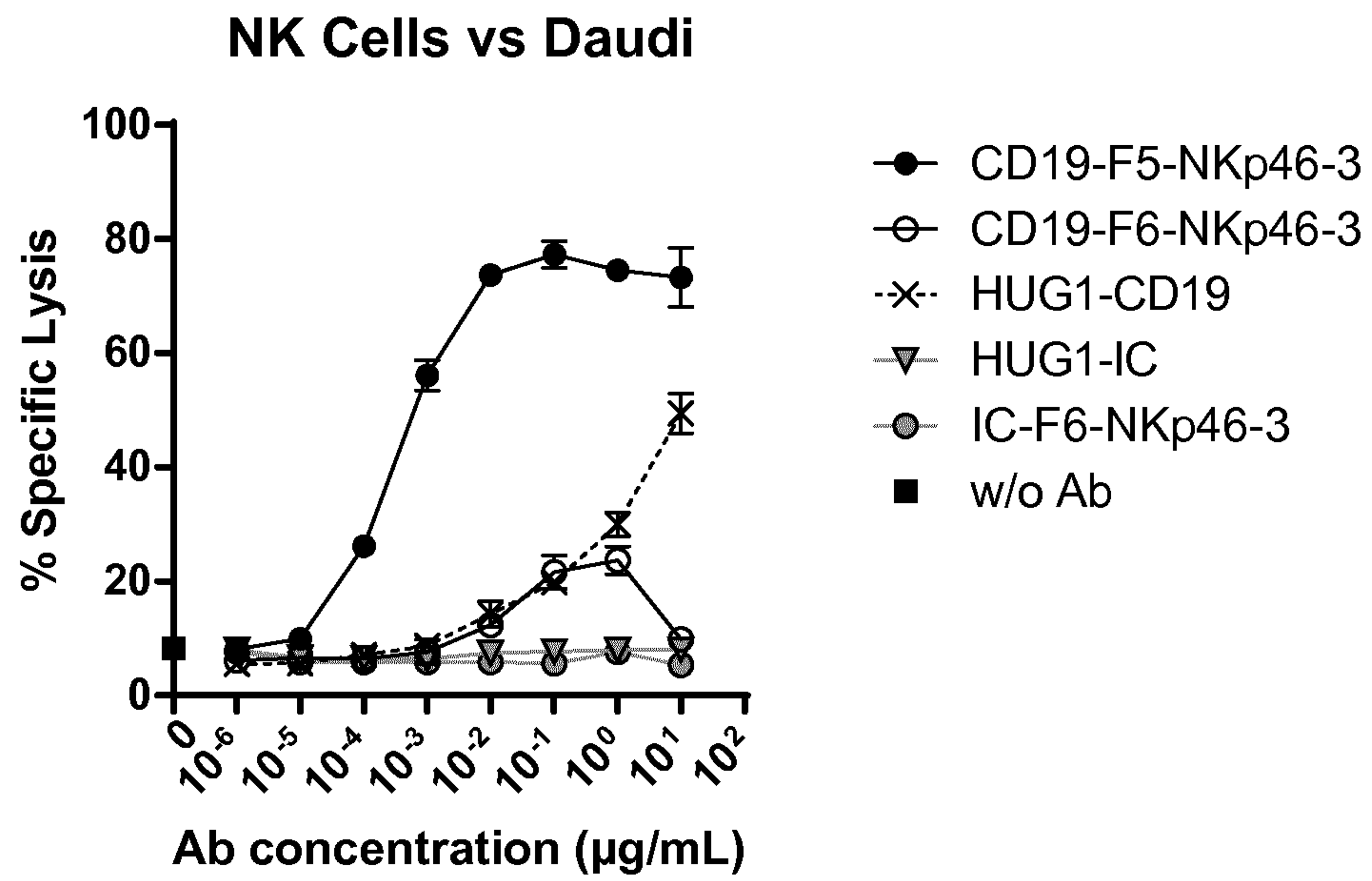


Figure 8

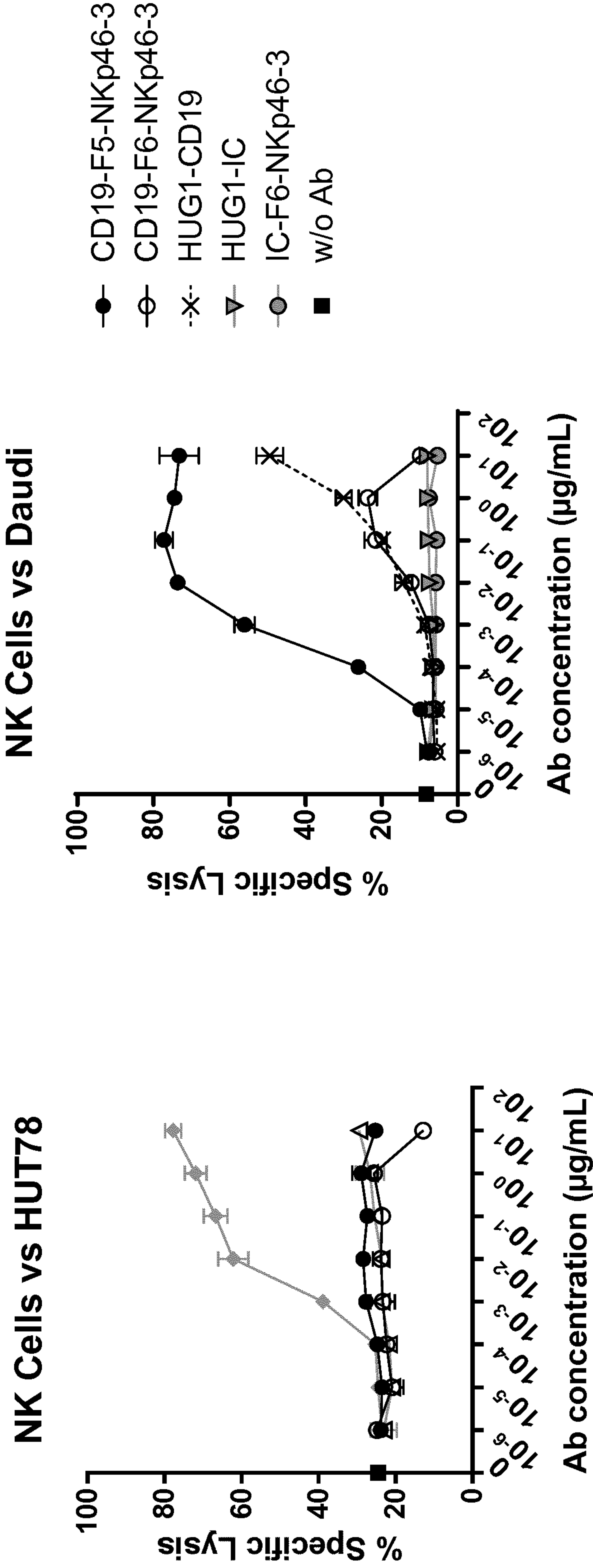
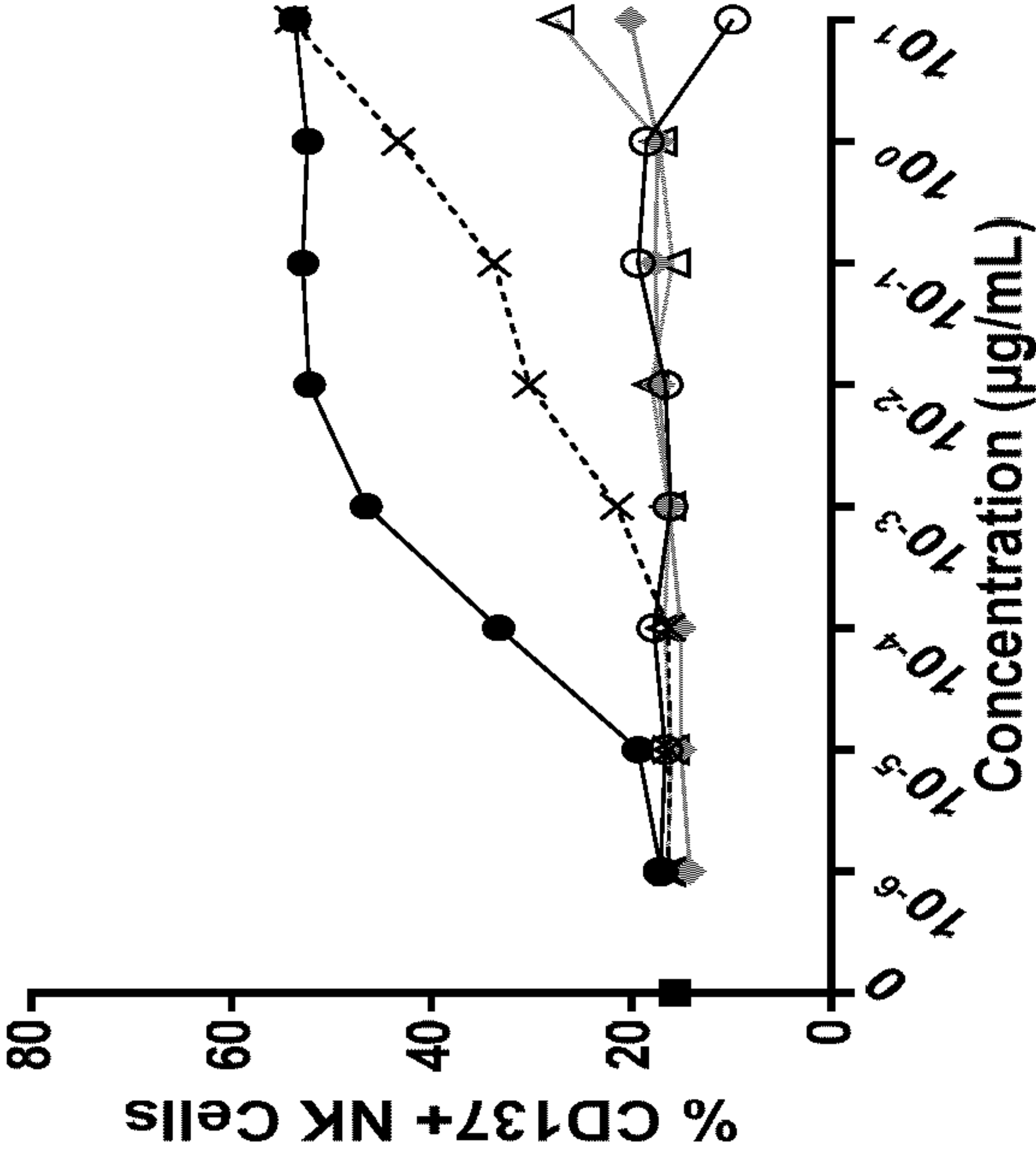


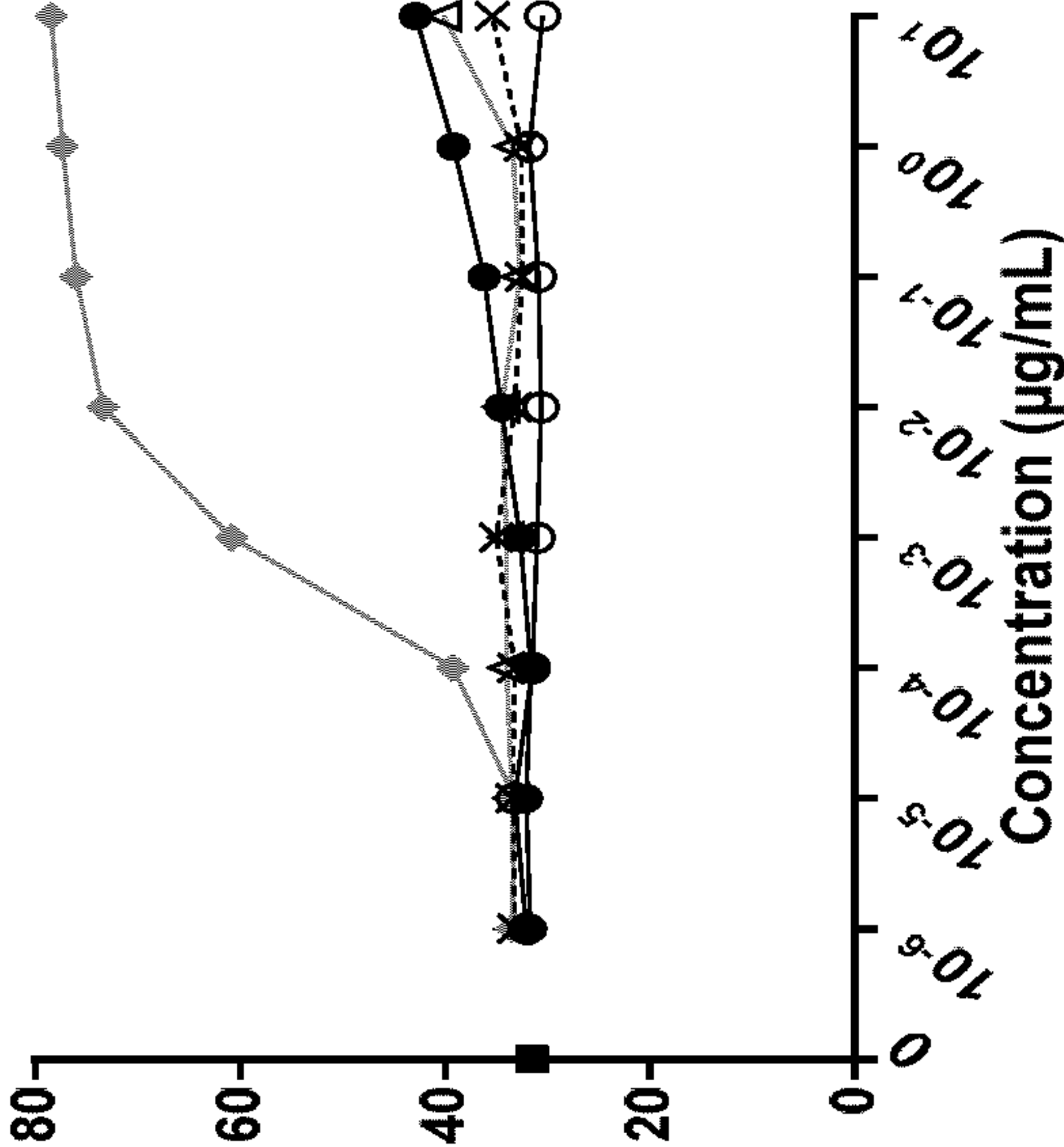
Figure 9

- CD19-F6-NKp46-3
- CD19-F5-NKp46-3
- x- Anti-CD19
- ◆ Anti-KIR3DL2
- △ IC
- w/o Ab

NK vs Daudi



NK vs HUT78



NK alone

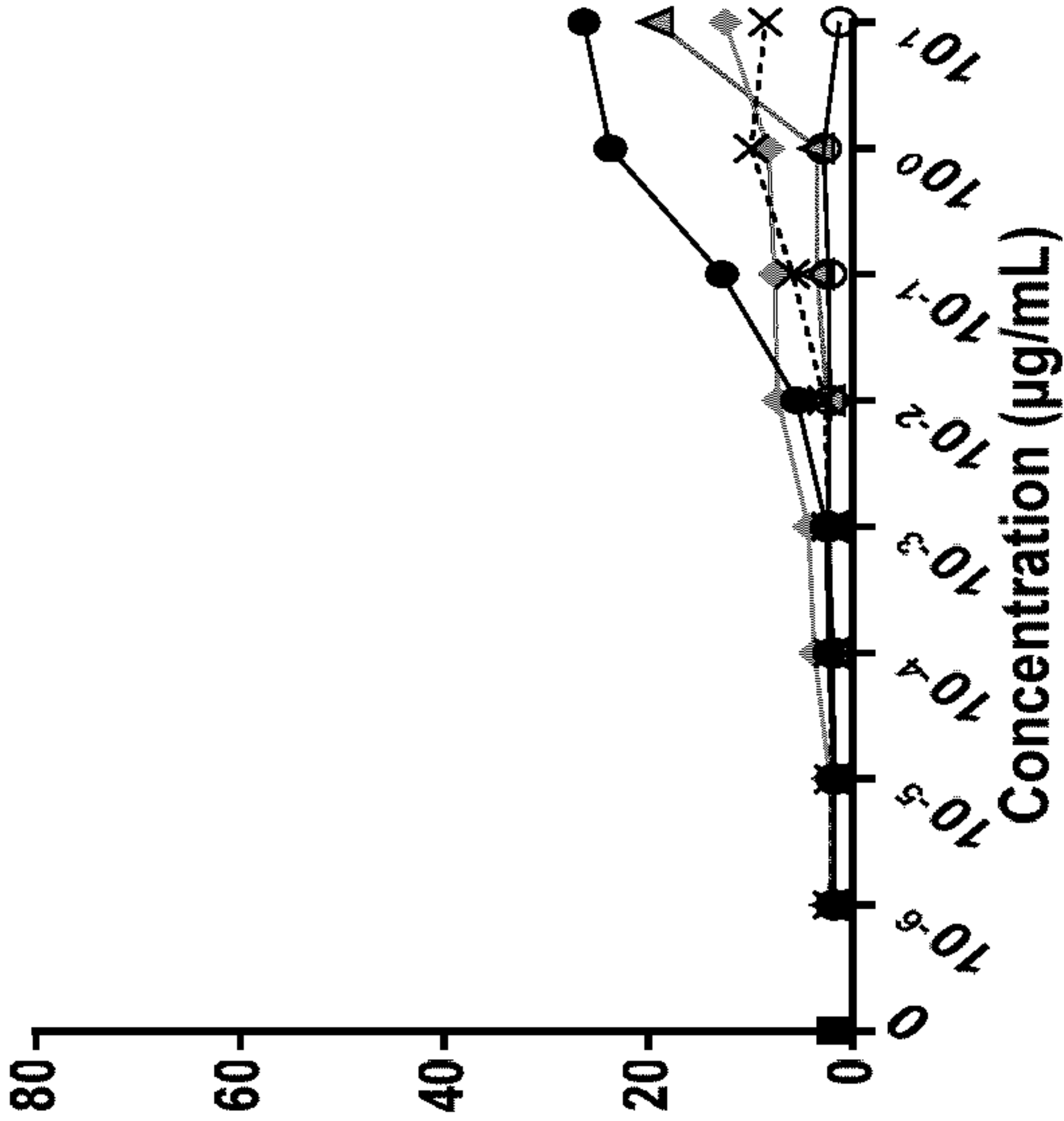


Figure 10

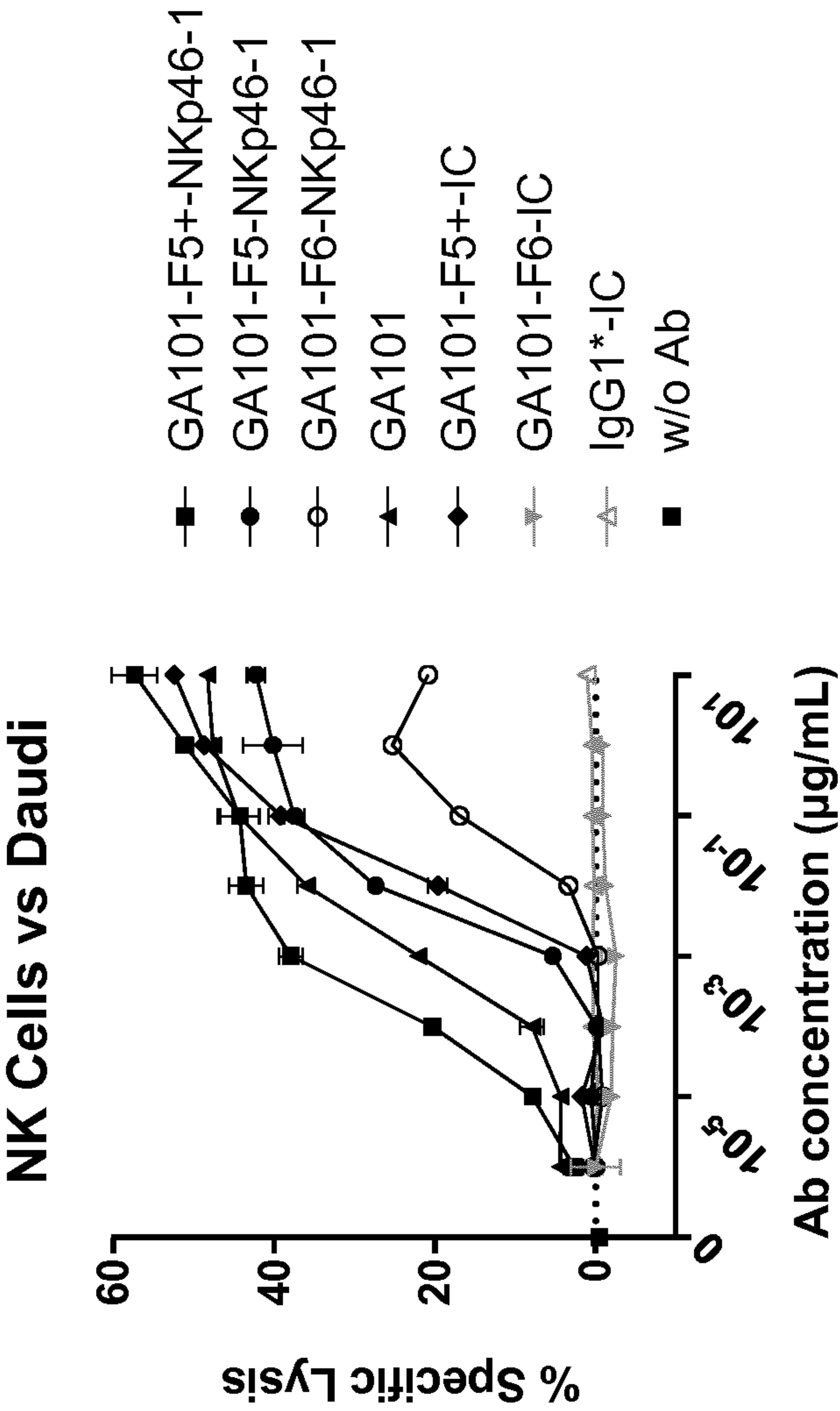


Figure 7

NK Cells vs Daudi

