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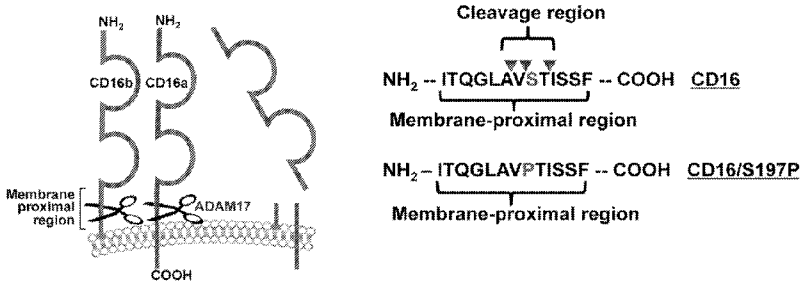

FIG. 2

(57) Abstract: This disclosure describes, generally, a modified form of CD 16, genetically-modified cells that express the modified CD 16, and methods that involve the genetically-modified cells. The modified form of CD 16 can exhibit increased anti-tumor and/or anti- viral activity due, at least in part, to reduced susceptibility to ADAM17-mediated shedding upon NK cell stimulation.

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POLYPEPTIDES, CELLS, AND METHODS INVOLVING ENGINEERED CD16

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application Serial No. 61/971,996, filed March 28, 2014, which is incorporated herein by reference.

SUMMARY

This disclosure describes, generally, a modified form of CD16, genetically-modified cells that express the modified CD16, and methods that involve the genetically-modified cells. The modified form of CD16 can exhibit increased anti-tumor and/or anti-viral activity due, at least in part, to reduced susceptibility to metalloprotease-mediated shedding upon NK cell stimulation.

In one aspect, therefore, this disclosure describes a cell genetically modified to express a CD16 polypeptide that has a membrane proximal region and an amino acid modification in the membrane proximal region.

In another aspect, this disclosure describes a cell that includes a polynucleotide that encodes a CD16 polypeptide that has membrane proximal region and an amino acid modification in the membrane proximal region.

In either aspect, the amino acid medication reflects an addition of one or more amino acids, a deletion of one or more amino acids, or a substitution of one or more amino acids compared to the wild-type amino acid sequence of the CD16 membrane proximal region. In some of these embodiments, the substitution of one or more amino acids includes a substitution of the serine residue at position 197 of SEQ ID NO:1.

In either aspect, the cell can be a Natural Killer (NK) cell, a neutrophil, a monocyte, or a T cell.

In either aspect, the modified CD16 polypeptide exhibits reduced susceptibility to ADAM17-mediated shedding compared to a wild-type CD16 polypeptide.

In either aspect, the modified CD16 polypeptide exhibits reduced susceptibility to cleavage upon NK cell stimulation compared to a wild-type CD1 polypeptide.

In another aspect, this disclosure describes a method that generally involves administering to a patient in need of such treatment a therapy that includes (a) administering to the patient a therapeutic NK effector, and (b) administering to the patient the any embodiment of the genetically-modified cell summarized above.

In some embodiments, the therapeutic NK effector includes a therapeutic agent. In some of these embodiments, the therapeutic agent can include an antibody, or a therapeutic antibody fragment. In some of these embodiments, the antibody, or antibody fragment, specifically binds to a viral antigen. In other embodiments, the antibody, or antibody fragment, specifically binds to a tumor antigen.

In some embodiments, the therapeutic agent can include a bi-specific killer engager (BiKE) or a tri-specific killer cell engager (TriKE).

In yet another aspect, this disclosure describes a method for improving immunotherapy to a patient, in which the immunotherapy involves administering to the patient a therapeutic NK effector. Generally the method includes further administering to the patient any embodiment of the genetically-modified cell summarized above.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Location of ectodomain cleavage sites in human CD16. (A) Tryptic peptides of soluble CD16 immunoprecipitated from the cell supernatant of PMA-activated human NK cells or neutrophils were subjected to mass spectrometry analysis. Four high confidence peptides with non-tryptic C-termini were identified: 1 peptide from soluble CD16 released by NK cells (Peptide #1, upper left) and 3 peptides from soluble CD16 released by neutrophils (Peptide #2, lower left; Peptide #3, upper right; and Peptide #4, lower right). (B) Illustration of Peptides #1-4 (underlined) and putative cleavage sites (arrowheads) in CD16a (SEQ ID NO:1) and CD16b (SEQ ID NO:2). Amino acid 176 distinguishes CD16a (F) from CD16b (V) in the identified

peptides. Amino acids 1-16 indicate a predicted signal sequences of CD16a and CD16b. Amino acids 210-229 indicate the transmembrane region of CD16a. Amino acid numbering begins with methionine in the signal sequence. The amino acid sequences of CD16a and CD16b are from the NCBI reference sequences NM_000569.6 and NM_000570.4, respectively.

FIG. 2. Schematic illustration of CD16 ectodomain shedding, the cleavage region, and the engineered serine-197 to proline mutation. CD16a and CD16b undergo ectodomain shedding by ADAM17 within a membrane proximal region, as indicated. The CD16 cleavage region within the membrane proximal region is based on mass spectrometry analysis that revealed three distinct cleavage sites in close proximity (arrowheads). Site-directed mutagenesis was performed to substitute serine-197 in CD16 (amino acids 190-202 of SEQ ID NO:1) with a proline (CD16/S197P).

FIG. 3. Effects of the engineered S197P mutation on CD16a and CD16b shedding. Transfected HEK293 (human embryonic kidney) cells separately expressed CD16b and CD16b/S197P (A) or CD16a and CD16a/S197P (B) at similar levels, as determined by flow cytometry (left panels). The different transfectants were treated with or without PMA (15 ng/ml for 30 minutes at 37°C) and soluble levels of CD16 in the media supernatant were quantified by ELISA (right panels). Each treatment condition was repeated three times for each experiment and the data are representative of three independent experiments. Bar graphs show mean ± SD. Statistical significance is indicated as ***P<0.001. (C) Transfected HEK293 cells expressed L-selectin (CD62L) or L-selectin and CD16b/S197P. Surface levels of L-selectin and CD16b/S197P on transfected and mock-transfected cells were measured using flow cytometry (histogram plots). Transfectants expressing L-selectin or L-selectin and CD16b/S197P were incubated in the presence or absence of PMA for 30 minutes at 37°C, and the mean fluorescence intensity (MFI) of L-selectin staining determined (bar graph). Each treatment condition was repeated three times for each experiment and the data are representative of two independent experiments. Bar graphs show mean ± SD. Statistical significance is indicated as *P<0.05. For all histogram plots, the x-axis = Log 10 fluorescence and the y-axis = cell number.

FIG. 4. Effects of the engineered S197P mutation on CD16a shedding in NK cells. NK92 cells transduced with empty vector (vector only), CD16a, or CD16a/S197P were treated without (Unstim.) or with PMA (100 ng/ml) for 30 minutes at 37°C (A), with IL-12 and IL-18 (100 ng/ml and 400 ng/ml, respectively) for 24 hours at 37°C (B), or with Raji cells and rituximab for

60 minutes at 37°C (C). Cell surface levels of CD16a were determined by flow cytometry. Isotype-matched negative control antibody staining is indicated by a dotted line. (D) Parent NK92 cells and transduced cells expressing CD16a or CD16a/S197P were treated with Raji cells and rituximab in the presence or absence of the ADAM17 inhibitor BMS566394 (5 μM) for 60 minutes at 37°C. Soluble CD16a levels were determined by ELISA. Each treatment condition was repeated three times and the data are representative of three independent experiments. Bar graphs show mean ± SD. Statistical significance is indicated as ***P<0.001. (E) NK92 cells expressing CD16a or CD16a/S197P were stained with the anti-ADAM17 mAbs M220, 623, 633, or an isotype-matched negative control antibody, as indicated. (F) $CD56^{+}CD45^{+}$ NK cells derived from mock-transduced iPSCs (left panel) or iPSCs expressing recombinant CD16a or CD16a/S197P (right panels) were incubated with or without K562 target cells for four hours at 37°C. For all histogram plots, the x-axis = Log 10 fluorescence, the y-axis = cell number, and the data are representative of at least 3 independent experiments.

FIG. 5. Effects of the engineered S197P mutation on CD16a function. (A) NK92 cells expressing CD16a or CD16a/S197P at equivalent levels (left panel) were treated with monomeric human IgG (0-20 μg/ml). As controls, cells were also treated with monomeric human IgA (20 μg/ml), and NK92 parent cells were treated with IgG (20 μg/ml) (bar). Antibody binding was determined by flow cytometry, as described in Materials and Methods. The bar graph shows mean ± SD of at least three separate experiments. Statistical significance is indicated as *P<0.05 versus IgG (0 μg/ml), IgA, or NK92 parent cells + IgG. (B) Mock transduced NK92 cells or NK92 cells expressing CD16a or CD16a/S197P were incubated in the absence (Unstim.) or presence of Raji cells treated with or without anti-CD20 rituximab for the indicated time points at 37°C. NK92 cell activation was assessed by the up-regulation in CD107a staining by flow cytometry. For the histogram plots, the x-axis = Log 10 fluorescence and the y-axis = cell number. Data are representative of at least 3 independent experiments.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

This disclosure describes, generally, a modified form of CD16a, genetically-modified cells that express the modified CD16a, and methods that involve the genetically-modified cells. The modified form of CD16a can exhibit increased anti-tumor and/or anti-viral activity due, at

least in part, to reduced susceptibility to metaaloprotease-mediated shedding upon NK cell stimulation.

In contrast to many solid cancer types, the survival rate of women with epithelial ovarian cancer has changed little in the last 30 years. Moreover, current standard therapies for recurrent ovarian cancer provide a low (<20%) response rate. Despite ubiquitous HER2 overexpression by ovarian cancer samples, treatment with the anti-HER2 antibody trastuzumab provides only limited responses in patients with advanced ovarian cancer. This resistance to trastuzumab may arise from dysfunctional NK cell-mediated antibody-dependent cell cytotoxicity. Thus, there is an urgent need for innovative therapeutic strategies. We describe a novel approach for providing therapeutic treatment strategy.

One concern with ovarian cancer is that the milieu in which tumor cells develop can be highly pro-inflammatory, and thus likely to promote CD16a cleavage on infiltrating NK cells and consequently diminishing antibody-dependent cell cytotoxicity. Several antibodies have emerged as effective targeted therapies for treating human malignancies. Their efficacy is due in part to antibody interactions with FcγRIIIa/CD16a on Natural Killer (NK) cells and induction of cancer cell killing by antibody-dependent cell cytotoxicity. Human IgG Fc receptor CD16 (FcγRIII) consists of two isoforms: CD16a (FcγRIIIa) and CD16b (FcγRIIIb). CD16a is expressed by Natural Killer (NK) cells and CD16b is expressed by neutrophils. NK Cell activation results in a rapid down-regulation in the surface levels of both isoforms of CD16 by a process referred to as ectodomain shedding—a proteolytic event that involves the metalloprotease ADAM17 and occurs at a single extracellular region proximal to the plasma membrane (FIG. 1A).

As noted above, ovarian cancer patients may be resistant to NK cell-mediated immunotherapies—i.e., the tumors are not sensitive to NK cell-mediated therapies. For example, ovarian cancer cells typically express the epidermal growth factor receptor HER2, yet its targeting with the therapeutic antibody trastuzumab has provided only a limited clinical response. This resistance may result, at least in part, from ectodomain shedding—i.e., NK cell activation by cytokines, target cell interaction, and/or tumor infiltration can result in CD16a cleavage and impaired antibody-dependent cell cytotoxicity. Thus, blocking the process of ectodomain shedding has clinical significance.

We have determined the cleavage sites of CD16a and CD16b using mass spectrometry and cloned the cDNAs of CD16a and CD16b from human blood leukocytes. Each cDNA was

mutated in a directed manner to induce a single amino acid change. Serine at location 197 was changed to a proline. (FIG. 1B). This mutation blocks the cleavage of CD16a and CD16b, and prevents their down-regulation upon cell activation. The expression of cleavage-resistant CD16a in *ex vivo* expanded NK cells maintain high surface levels of this IgG Fc receptor, which enhances NK cell stimulation, the efficacy of therapeutic antibodies, and cancer call killing.

ADAM17 has a number of cell surface substrates, but possesses no consensus sequence for proteolysis that can be used to predict the site of CD16a cleavage. Therefore, we used LC-MS-MS to determine the C-terminus cleavage site in soluble CD16 released from activated human peripheral blood leukocytes. We observed three putative cleavage locations in close proximity in the membrane proximal region of CD16 (FIG. 2, arrowheads), a region that is identical between CD16a and CD16b. Although ADAM17 proteolysis does not require a consensus sequence, the secondary structure of the cleavage region is important. In an attempt to block CD16a cleavage, we substituted serine-197 with a proline ($CD16a^{197P}$) to introduce a conformational change.

We identified the location of CD16 cleavage by immunoprecipitating CD16 from the media supernatant of activated NK cells and, separately, from the media supernatant of neutrophils. The immunoprecipitated CD16 was treated with PNGaseF to remove N-glycans, trypsin digested, and the generated peptides subjected to mass spectrometric analysis. Four different peptide patterns of high confidence were identified containing non-tryptic C-termini (FIG. 1A).

For CD16 enriched from the media supernatant of activated NK cells, we observed only one peptide pattern, which corresponds to amino acids glycine-174 through alanine-195 (Peptide #1, FIG. 1A) of SEQ ID NO:1. The membrane proximal regions of CD16a and CD16b have identical amino acid sequences except for residue 176. A phenylalanine at this location is indicative of CD16a, which was present in Peptide #1 (FIG. 1A and B). This peptide revealed a non-tryptic P1/P1′ cleavage position at alanine-195/valine-196 (FIG. 1B).

For CD16 enriched from the media supernatant of activated neutrophils, we detected three different peptide patterns with non-tryptic C-termini (Peptides #2-4, FIG. 1A and 1B). Peptide #2 corresponds to amino acids glycine-174 through alanine-195 of SEQ ID NO:2, Peptide #3 corresponds to amino acids glycine-174 through valine-196 of SEQ ID NO:2, and Peptide #4 corresponds to amino acids asparagine-180 through threonine-198 of SEQ ID NO:2.

Peptide #2 and Peptide #3 contained a valine at position 176, indicative of CD16b, and revealed P1/P1′ positions at alanine-195/valine-196 and at valine-196/serine-197 (FIG. 1B). Peptide #4 possessed a P1/P1′ position at threonine-198/isoleucine-199 (FIG. 1B). Though this peptide was derived from soluble CD16 from enriched neutrophils, it does not contain an amino acid at position 176 to identify the isoform (FIG. 1B). Regardless, the high confidence peptide revealed a third cleavage site in CD16. Taken together, these findings demonstrate the presence of a cleavage region in CD16 rather than a single specific cleavage site.

We further examined the cleavage region in CD16 by using site-directed mutagenesis to determine whether CD16a and CD16b cleavage could be disrupted in cell-based assays. ADAM17 tends to prefer an α-helical conformation in the substrate region that interacts with its catalytic site. Moreover, proteomic studies of ADAM17 cleavage site specificities revealed a very low preference for proline residues at the P1′, P2′, or P3′ positions. We therefore substituted serine-197 in the cleavage regions of CD16a and CD16b with a proline (S197P, as indicated in FIG. 2).

CD16b and CD16b/S197P were separately expressed in the human kidney cell line HEK293, which does not express endogenous CD16. The HEK293 transfectants expressed CD16b or CD16b/S197P at similar levels on their surface (FIG. 3A). High levels of CD16b were released from the transfected HEK293, which was increased further upon their treatment with PMA, as determined by ELISA (FIG. 3A). However, soluble levels of CD16b/S197P generated by untreated or PMA-treated HEK293 cells were markedly lower than those of CD16b (FIG. 3A).

We also examined the effects of the S197P mutation on CD16a cleavage using the same approach. Surface expression of CD16a requires association with γ chain dimmers. We therefore used HEK293 cells stably expressing human γ chain. Comparing HEK293 transfectants expressing equivalent surface levels of CD16a or CD16a/S197P (FIG. 3B), we determined the soluble levels of each receptor in the media supernatant of untreated and PMA-treated cells. Again, significantly lower levels of soluble CD16a/S197P were observed when compared to CD16a (FIG. 3B).

To evaluate whether the engineered S197P mutation in CD16 might disrupt ADAM17 activity, we also transfected HEK293 cells expressing or lacking CD16b/S197P with L-selectin, a well described ADAM17 substrate normally expressed by leukocytes. Both transfectants

expressed equivalent levels of L-selectin, which was similarly down-regulated following their activation with PMA (FIG. 3C), demonstrating that the S197P mutation affected CD16 shedding and not ADAM17 activity.

To assess the effects of the S197P mutation on CD16a shedding in NK cells, we used the human NK cell line NK92 (Gong et al., 1994, *Leukemia* 8:652-658). These cells lack expression of endogenous CD16a, but recombinant CD16a can be stably expressed. We transduced NK92 cells to separately express CD16a and CD16a/S197P. Cells expressing equivalent levels of these receptors were activated with PMA and cell surface CD16 levels were examined by flow cytometry. CD16a, but not CD16a/S197P, underwent a marked down-regulation in cell surface expression (FIG. 4A). IL-12 and IL-18 are physiological stimuli of NK cells that individually or in combination can induce CD16a shedding. NK92 cells treated with IL-12 and IL-18 demonstrated an appreciable down-regulation in their cell surface expression of CD16a but not CD16a/S197P (FIG. 4B). Direct engagement of cell bound IgG by CD16a also can induce its shedding, which we examined here by incubating NK92 cells expressing CD16a or CD16a/S197P with the CD20-positive Burkitt's lymphoma cell line Raji in the presence or absence of the anti-CD20 mAb rituximab. Raji cells treated with rituximab induced the down-regulation of CD16a, but not CD16a/S197P (FIG. 4C).

BMS566394 is a highly selective ADAM17 inhibitor with a potency orders of magnitude higher for ADAM17 than for other metalloproteases. BMS566394 blocked CD16a shedding with similar efficiency as the S197P mutation, but had no additional blocking effect on activated NK92 cells expressing CD16a/S197P (FIG. 4D). These findings provide further evidence that ADAM17 is the primary sheddase that cleaves CD16a within its cleavage region. It is possible, however, that ADAM17 expression levels were not equivalent in the NK92 cells expressing CD16a or CD16a/S197P, accounting for their dissimilar shedding. We therefore stained NK92 cells expressing CD16a or CD16a/S197P with multiple anti-ADAM17 mAbs and observed identical cell surface levels (FIG. 4E).

To establish the effect of the S197P mutation on CD16a shedding by primary NK cells, we used human iPSCs to generate engineered NK cells. We have previously reported on deriving functional NK cells from iPSCs and their similarity to peripheral blood NK cells (Knorr et al., 2013 *Stem Cells Transl Med.* 2:274-283; Ni et al., 2014, *Stem Cells* 32:1021-1031). CD16a and CD16a/S197P cDNA were cloned into a *Sleeping Beauty* transposon plasmid for gene insertion

and stable expression in iPSC cells, which were subsequently differentiated into mature NK cells. NK cells derived from mock transduced iPSC cells expressed low levels of endogenous CD16a, whereas transduced CD16a and CD16a/S197P were expressed at higher levels (FIG. 4F). NK cell activation occurs through various receptors upon their interaction with K562 cells, including BY55/CD160, resulting in ADAM17 activation and CD16a shedding. We stimulated the iPSC-derived NK cells with K562 cells and found that CD16a underwent a marked down-regulation in cell surface expression, whereas the expression of CD16a/S197P remained stable (FIG. 4F).

Endogenous and recombinant CD16a have sufficient affinity to bind monomeric IgG. To examine the effects of the S197P mutation on CD16a function, we compared the IgG binding capacities of CD16a and CD16a/S197P. NK92 cells expressing CD16a or CD16a/S197P at equivalent levels bound IgG in a similar dose-dependent manner (FIG. 5A). Controls consisted of IgA binding to NK92 cells expressing CD16a or CD16a/S197P, and IgG binding to NK92 parent cells. Both occurred at essentially background levels (FIG. 5A). These findings demonstrate specific and equivalent IgG binding by CD16a and CD16a/S197P.

CD16a is a potent activating receptor in NK cells, and we examined whether the engineered S197P mutation affected the capacity of CD16a to induce cell activation upon engagement of antibody-treated tumor cells. NK92 cell activation was assessed by measuring the up-regulation of CD107a, which occurs very rapidly upon degranulation and is a sensitive marker of NK cell activation. Mock transduced NK92 cells incubated with Raji cells treated with or without rituximab demonstrated low level and similar up-regulation CD107a (FIG. 5B). NK92 cells expressing CD16a or CD16a/S197P at equivalent levels when incubated with Raji cells alone marginally up-regulated CD107a as well, whereas their incubation with Raji cells treated with rituximab resulted in a considerable up-regulation of CD107a (FIG. 5B). Taken together, the above findings indicate that the engineered S197P mutation in CD16a did not impair its function.

Thus, we show that the engineered S197P mutation in CD16a and CD16b effectively blocked their shedding in cell-based assays that involved native ADAM17. The S197P mutation in CD16a also blocked shedding of the receptor in the human NK cell line NK92, but it did not impair receptor function. NK92 cells expressing equivalent levels of CD16a or CD16a/S197P bound monomeric IgG with similar efficiency over a range of antibody concentrations. In

addition, NK92 cells expressing CD16a or CD16a/S197P up-regulated the activation marker CD107a in a comparable manner upon their engagement of rituximab bound to Raji cells.

Pluripotent stem cells allow genetic manipulation to generate engineered NK cells. This disclosure describes the generation of engineered NK cells from transduced iPSCs expressing wild-type CD16a or CD16a/S197P. As with NK92 cells, CD16a underwent shedding in the iPSCs-derived NK cells, demonstrating normal ADAM17 activity upon cell activation, whereas CD16a/S197P was not shed.

CD16a and NK cell cytotoxic function can undergo a considerable down-regulation in cancer patients. The cDNAs encoding CD16a/S197P can be used to generate stable human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). These stem cells can then be differentiated into primary NK cells that express CD16a/S197P. Other cell populations that express cleavage resistant CD16a/S197P (e.g., monocytes) or CD16b/S197P (e.g., neutrophils) also can be derived from hESCs/iPSCs.

To generate an NK cell immunotherapy to be used in human patients against various forms of cancer or infection, the CD16a/S197P-expressing NK cells can mediate increased antibody-dependent cell cytotoxicity (ADCC) activity or other CD16a-mediated activity (e.g., IFNγ and TNFα production). For example, the CD16a/S197P-expressing NK cells may be combined with therapeutic antibodies (e.g., trastuzumab or rituximab), a bi-specific killer engager (BiKE, e.g., CD16×CD33, CD16×CD19, or CD16×EP-CAM bi-specific killer cell engager) or a tri-specific killer cell engager (TriKE). Other therapeutic cell populations (e.g., neutrophils, monocytes, T cells, etc.) also can be produced with increased CD16-mediated activity.

Expression of CD16a/S197P in human iPSCs or human ESCs can produce an NK cell population with enhanced ADCC activity against neoplastic conditions such as, for example, HER2 ovarian cancer. In some cases, the neoplastic condition may be treated with a therapeutic antibody such as, for example, trastuzumab. Mature NK cells may be derived from human embryonic stem cells and iPSCs.

Wild-type CD16a and/or CD16a/S197P can be cloned to generate a stable iPSC line or a stable ECS line expressing the individual CD16a receptors. Any suitable cloning method may be used. Exemplary cloning methods include, for example, viral-based methods, transposon vectors (e.g., *Sleeping Beauty*), or nucleofection. In one example, iPSCs may be modified using the

Sleeping Beauty transposon vector. The vector can contain a selection system such as, for example, GFP/zeocin resistance fusion protein, which allows a dual selection system (zeocin resistance and flow cytometric sorting). The iPSCs can be differentiated into mature NK cells, as previously described (Ni et al., 2011, *J. Virol.* 85:43–50; Knorr et al. 2013, *Stem Cells Transl Med* 2:274–283; Woll et al., 2009, *Blood* 113:6094–6101). Expression of transgenic receptors in iPSCs can lead to a high level of expression in the derived NK cells. CD16 expression in undifferentiated iPSCs may disrupt NK cell differentiation. In such cases, CD16 expression may be delayed using, for example, a CD56 or a natural CD16a promoter, so that CD16 expression better coincides with normal NK cell differentiation.

One can compare NK cells expressing equivalent levels of wild-type CD16a versus CD16a/S197P. Expression levels of the CD16 constructs can be matched by FACS sorting based on GFP expression, which occurs in a proportional manner to the CD16 constructs. Matched CD16a levels can be verified by FACS. NK cell cytotoxicity against HER2-expressing ovarian cancer cells can be assessed by a standard chromium release assay in the presence or absence of a therapeutic antibody such as, for example, trastuzumab. Antibody-dependent cell cytotoxicity with non-chromium labeled ovarian cancer cells can be evaluated. One can evaluate NK cell production of cytokines (e.g., IFNγ, TNFα) and soluble levels of CD16a by ELISA, and the cell surface levels of CD16a and other activation markers (e.g., CD107a, CD62L) by FACS.

The human tumor xenograft model described in Example 3 can be used to evaluate the anti-cancer activity of NK cells that express non-cleavable CD16a *in vivo*. Unlike human CD16, mouse CD16 does not undergo ectodomain shedding upon cell stimulation, and thus determining the effects of CD16a shedding on NK cell-mediated ADCC cannot be modeled in normal mice. Table 1 provides a representative set of experimental groupings and treatments.

Table 1. Tumor xenograft model

Group	**n**	**Treatment#**
1	5	No treatment
2	5	OVCAR3 cells only
3	5	OVCAR3 + NK cells/WT-CD16a
4	5	OVCAR3 + NK cells/ WT-CD16a + trastuzumab
5	5	OVCAR3 + NK cells/CD16a^{197P}

6	5	OVCAR3 + NK cells/ $CD16a^{197P}$ + trastuzumab
7	5	OVCAR3 + NK cells/vector only
8	5	OVCAR3 + NK cells/vector + trastuzumab

#Treatment performed at least twice and data pooled.

Tumor growth and/or regression can be monitored weekly by conventional methods including, for example, bioluminescent imaging, ultrasound, CT, MRI, another imaging technology, and/or weighing the mice (Woll et al., 2009, *Blood* 113:6094–6101). Mice also can be bled (e.g., weekly) to quantify human NK cell survival. The expression and/or cell surface levels of various effector function markers (e.g., IFNγ, CD16a) can be evaluated using conventional techniques such as, for example, by FACS. Mice can be followed for any suitable period such as, for example, 60 days. At the time of sacrifice, internal organs (e.g., spleen, liver, lungs, kidney, and/or ovaries) can be examined for evidence of metastasis (e.g., by bioluminescence), as previously described (Woll et al., 2009, *Blood* 113:6094–6101).

Our analyses allow one to define and compare the antibody-dependent cell cytotoxicity activity and *in vivo* potency of iPSC-derived NK cells expressing wild-type CD16a versus CD16a/S197P. Thus, we describe herein a modified form of CD16a, genetically-modified cells (e.g., NK cells, neutrophils, monocytes, T cells, etc.) that express the modified CD16a, and methods that involve the genetically-modified cells. For example, NK cells expressing the modified form of CD16a, CD16a/S197P, exhibit increased anti-ovarian cancer activity due, at least in part, to reduced susceptibility to ADAM17-mediated shedding upon NK cell stimulation. This, in turn, increases antibody-dependent cell cytotoxicity activity upon engaging antibody-tagged cancer cells such as, for example, cancer cells tagged with a therapeutic antibody. Moreover, antibody recognition by NK cells increases contact stability with tumor cells and bolsters NK cell activity through other activating receptors, such as NKG2D.

The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements; the terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims; unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one; and the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

In the preceding description, particular embodiments may be described in isolation for clarity. Unless otherwise expressly specified that the features of a particular embodiment are incompatible with the features of another embodiment, certain embodiments can include a combination of compatible features described herein in connection with one or more embodiments.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Mass spectrometry

Peripheral blood collection from healthy individuals was performed in accordance with protocols approved by the University of Minnesota Institutional Review Board according to protocol # 9708M00134. Human neutrophil and NK cell isolation was performed as previously described (Wang et al., 2013, *Biochim Biophys Acta.* 1833:680-685; Long et al., 2010, *J Leukoc Biol.* 87:1097-1101; Long et al., 2012, *J Leukoc Biol.* 92:667-672). Enriched neutrophils or NK cells (1×10^7/ml in PBS; Mediatech, Inc. Manassas, VA) were activated with PMA (15 ng/ml or 50 ng/ml, respectively; Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C. Cell supernatants were filtered (0.45 μm pore size) and CD16 was immunoprecipitated using the mAb 3G8 (BioLegend, Inc., San Diego, CA) and the Pierce direct immunopreciptation kit (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's instructions. Purified CD16 was deglycosylated by chitin binding domain-tagged Remove-iT PNGase F (New England BioLabs, Inc., Ipswich, MA), according to the manufacturer's instructions. Briefly, 10-20μg of purified CD16 was denatured in the presence of 40 mM DTT at 55°C for 10 minutes and then incubated with 3 μl of REMOVE-IT PNGase F (New England BioLabs, inc., Ipswich, MA) at 37°C for one hour. REMOVE-IT PNGase F was then removed from the reaction using chitin magnetic beads.

CD16 was subjected to SDS-PAGE and gel bands corresponding to soluble CD16 were detected by a krypton fluorescent protein stain (Thermo Fisher Scientific, Rockford, IL), verified by CD16 immunoblot analysis of adjacent lanes in the same gel, and were then excised and subjected to standard in-gel digestion with trypsin. Digested peptides extracted from the gel were dried down and reconstituted for liquid chromatography-mass spectrometry analysis in 98:2:0.01, water:acetonitrile:formic acid and ≤1 μg aliquots were analyzed by mass spectrometry (VELOS ORBITRAP, Thermo Fisher Scientific, Rockford, IL) in a data dependent scan mode, as described previously (Lin-Moshier et al., 2013, *J Biol Chem.* 288:355-367). Database searches were performed with Protein Pilot 4.5 (AB Sciex, Framingham, MA), which uses the Paragon scoring algorithm (Shilov et al., 2007, *Mol Cell Proteomics* 6:1638-1655), against the NCBI reference sequence *Homo sapiens* protein FASTA database to which the contaminant database (thegpm.org/cRAP/index,109 proteins) was appended. Search parameters were: cysteine iodoacetamide; trypsin; instrument Orbi MS (1-3ppm) Orbi MS/MS; biological modifications ID focus, which includes asparagine deamidation; a thorough search effort; and False Discovery Rate analysis (with reversed database).

Generation of cDNA expression constructs

CD16b occurs as two allelic variants termed NA1 and NA2, differing by four amino acids in the N-terminal portion of its extracellular region. Both allelic variants of CD16b are cleaved with similar efficiency by ADAM17. For this study, we examined only the NA1 variant. There are also two allelic variants of CD16a that have either a valine or phenylalanine residue at position 176. These two allelic variants of CD16a were cleaved with similar efficiency by ADAM17. For this study, we examined only the valine allelic variant CD16a.

CD16a and CD16b were amplified from human leukocyte cDNA, separately cloned into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA) at the *BamHI* and *EcoRI* restriction enzyme sites as previously described (Wang et al., 2013, *Biochim Biophys Acta*. 1833:680-685; Dong et al., 2014, *Arthritis Rheumatol*. 66:1291-1299). The constructs were then subjected to Quik-Change Site-directed Mutagenesis (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions to convert the serine at position 197 to a proline in CD16a and CD16b. All constructs were sequenced to confirm the presence of the intended mutation and the absence of any spontaneous mutations.

The CD16a cDNA was subsequently cloned into the bi-cistronic retroviral expression vector pBMN-IRES-EGFP, provided by Dr. G. Nolan (Stanford University, Stanford, CA), at the *BamHI* and *EcoRI* restriction enzyme sites. The CD16a constructs were also cloned into a bicistronic *Sleeping Beauty* transposon plasmid (pKT2-IRES-GFP:zeo) as previously described (Wilber et al., 2007, *Stem Cells* 25:2919-2927; Tian et al., 2009, *Stem Cells* 27:2675-2685). Briefly, wild-type CD16a and CD16a/S197P were PCR amplified using the primers: 5'-CCG GAA TTC CAG TGT GGC ATC ATG TGG CAG CTG CTC-3' (sense, SEQ ID NO:XX) and 5'-CCG GAA TTC TCA TTT GTC TTG AGG GTC CTT TCT-3' (antisense, SEQ ID NO:YY). *EcoRI* sites are underlined. The *EcoRI*-digested CD16a and CD16a/S197P PCR fragments were separately cloned into pKT2-IRES-GFP:zeo. Correct CD16a orientation and sequence were confirmed by PCR and sequencing analyses. We have previously cloned full-length human L-selectin (CD62L) cDNA (Feehan et al., 1996, *J Biol Chem.* 271:7019-7024; Matala et al., 2001, *J Immunol.* 167:1617-1623), which was transferred to the pcDNA3.1 vector at the restriction enzyme site *Xba1*. Full-length human FcRγ cDNA was cloned as previously described (Dong et al., 2014, *Arthritis Rheumatol.* 66:1291-1299), with the modification that a pcDNA3.1 vector was used.

Generation of cell lines expressing recombinant L-selectin, CD16a, and CD16b

HEK293 cells (a human embryonic kidney cell line) and NK92 cells (a human NK cell line) (ATCC, Manassas, VA) were cultured according to the depository's instructions. HEK293 cells were transiently transfected with pcDNA3.1 with or without CD16b, CD16b/S197P, and/or L-selectin using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. HEK293 cells stably expressing human FcRγ were transiently transfected with pcDNA3.1 with or without CD16a or CD16a/S197P by the same approach. NK92 cells were stably transduced with pBMN-IRES-EGFP with or without CD16a or CD16a/S197P by retrovirus generation and infection procedures described previously (Matala et al., 2001, *J Immunol.* 167:1617-1623; Walcheck et al., 2003, *J Leukoc Biol.* 74:389-394; Wang et al., 2009, *J Immunol.* 182:2449-2457). Construct expression was assessed by EGFP fluorescence and CD16 staining, as determined by flow cytometry. Human iPSCs (UCBiPS7, derived from umbilical cord blood CD34 cells) were maintained on mouse embryonic fibroblasts (Knorr et al., 2013, *Stem Cells Transl Med.* 2:274-283; Ni et al., 2014, *Stem Cells* 32:1021-1031). Stable

expression of CD16a or CD16a/S197P was performed using a *Sleeping Beauty* transposon system as previously described (Wilber et al., 2007, *Stem Cells* 25:2919-2927; Tian et al., 2009, *Stem Cells* 27:2675-2685). Briefly, iPSCs were nucleofected with pKT2-IRES-GFP:zeo in combination with transposase DNA in nucleofector solution V (Lonza Inc., Gaithersburg, MD) using program setting B16. Nucleofected cells were immediately suspended in iPSC growth medium containing zeocin (50 μg/ml) and seeded onto mouse embryonic fibroblasts.

NK cell derivation from CD16a-hESC and CD16a-iPSC cells

Hematopoietic differentiation of hESCs and iPSCs was performed as previously described (Ng et al., 2005, *Blood* 106: 1601–1603; Ng et al., 2008, *Nat Protoc* 3:768–776; Le Garff-Tavernier et al., 2010, *Aging Cell* 9: 527–535). Briefly, 3000 single cells were seeded per well of 96-well round bottom plates in BPEL media with stem cell factor (SCF, 40 ng/ml), vascular endothelial growth factor (VEGF, 20 ng/ml) and bone morphogenic protein 4 (BMP4, 20 ng/ml). BPEL media contained Iscove's Modified Dulbecco's Medium (IMDM, 86 ml, Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), F12 Nutrient Mixture with Glutmax I (86 mL, Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), 10% deionized Bovine Serum Albumin (BSA, 5 ml, Sigma-Aldrich, St. Louis, MO), 5% Polyvinyl alcohol (10 ml, Sigma-Aldrich, St. Louis, MO), linolenic acid (20 μl of 1 gm/ml solution, Sigma-Aldrich, St. Louis, MO), linoleic acid (20 μl of 1 gm/ml solution, Sigma), SYNTHECOL 500x solution (Sigma-Aldrich, St. Louis, MO), a-monothioglyceral (3.9 μl/100 ml, Sigma-Aldrich, St. Louis, MO), Protein-free hybridoma mix II (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), ascorbic acid (5 mg/ml, Sigma), GLUTAMAX I (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), Insulin-transferrin-selenium 100x solution (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), Penicillin/streptomycin (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA).

At day 11 of hematopoietic differentiation, spin embryoid bodies were directly transferred into 24-well plates with or without EL08-1D2 stromal cells in NK media supplied with cytokines (Le Garff-Tavernier et al., 2010, *Aging Cell* 9:527–535). After 4-5 weeks of culture, single cell suspensions were stained with APC-, PE-, FITC- and PerCP-cy5.5-coupled IgG or specific antibodies against human blood surface antigens: CD45-PE, CD56-APC, CD56-PE, CD16-PerCP-cy5.5, NKG2D-PE, NKp44-PE, NKp46-PE, CD158b-FITC, CD158e1/2-FITC

(BD Pharmingen, San Jose, CA), CD158a/h-PE and CD158i-PE (Beckman Coulter, Inc., Pasadena, CA). Antibody stains were assessed by flow cytometry.

Cell stimulation

HEK293 and NK92 cells in RPMI 1640 media (Mediatech, Inc., Manassas, VA) were activated with 15 ng/ml and 100 ng/ml, respectively, PMA for 30 minutes at 37°C. NK92 cells were activated with IL-12 (PeproTech Inc, Rocky Hill, NJ) and IL-18 (R&D Systems, Inc., Minneapolis, MN) at 100 ng/ml and 400 ng/ml, respectively, for the indicated time points. NK92 cell activation through CD16a was mediated by their incubation with the CD20-positive Burkitt's lymphoma cell line Raji (ATCC, grown according to the depository's instructions) (1:1 ratio) treated with the anti-CD20 mAb rituximab (1 μg/ml) (Genentech, Inc., South San Francisco, CA), as described previously (Romee et al., 2013, *Blood* 121:3599-3608). Excess rituximab was removed by washing the Raji cells. In some experiments, NK92 cells were pre-incubated for 30 minutes with the selective ADAM17 inhibitor BMS566394 (5 μM) (Bristol-Myers Squibb Company, Princeton, NJ). NK cells derived from iPSCs were stimulated with the human erythroleukemic cell line K562 (ATCC, grown according to the depository's instructions), as previously described (Romee et al., 2013, *Blood* 121:3599-3608). Briefly, iPSC-derived NK cells were incubated with K562 target cells (2:1 ratio) for four hours at 37°C.

Antibody binding assay

Cell binding to monomeric human IgG and IgA (Sigma-Aldrich, St. Louis, MO) was performed as previously described with some modifications (Dong et al., 2014, *Arthritis Rheumatol.* 66:1291-1299). NK92 parent cells or transduced cells expressing CD16a or CD16a/S197P at 5×10^6/ml in PBS were incubated with IgG or IgA at the indicated concentrations in triplicate for one hour at 4°C. The cells were extensively washed and incubated with APC-conjugated donkey anti-human Fc (heavy and light chain) antibody (Jackson Immunoresearch, West Grove, PA) according to the manufacturer's instructions. The cells were washed and then immediately analyzed by flow cytometry.

Flow cytometry and ELISA

For cell staining, nonspecific antibody binding sites were blocked and cells were stained with the indicated antibodies and examined by flow cytometry, as previously described (Wang et al., 2013, *Biochim Biophys Acta.* 1833:680-685; Romee et al., 2013, *Blood* 121:3599-3608). Flow cytometric analysis was performed on FACSCanto and LS RII instruments (BD Biosciences, San Jose, CA). Human CD16 was detected by the mAbs 3G8 (BioLegend, Inc., San Diego, CA) and DJ130c (Santa Cruz Biotech, Santa Cruz, CA). CD107a was detected by the mAb H4A3 (Biolegend, Inc., San Diego, CA). ADAM17 was detected by the mAbs M220 (Doedens et al., 2000, *J Biol Chem.* 275:14598-14607), 111633, and 111623 (R&D Systems, Inc., Minneapolis, MN). Human L-selectin was detected by the mAb LAM1-116 (Ancell Corp., Stillwater, MN). Isotype-matched negative control mAbs were used to evaluate levels of nonspecific staining. The CD16 ELISA was performed by a custom cytometric bead assay, as previously described (Wang et al., 2013, *Biochim Biophys Acta.* 1833:680-685).

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad, San Diego, CA) using ANOVA and student's t test where appropriate. A p value of < 0.05 was considered significant.

Example 2

Comparison of NK cells expressing equivalent levels of WT CD16a and CD16a^{197P} (CD16a/S197P)

Expression levels of the CD16 constructs are matched by FACS sorting based on GFP expression (as done for NK92 cells described above, FIG. 2), which occurs in a proportional manner to the CD16 constructs. Matched CD16a levels are verified by FACS for all assays. As a control, iPSC-derived NK cells modified with empty *Sleeping Beauty* transposon vector (expressing only GFP) are evaluated. iPSC-derived NK cells express low levels of endogenous CD16a (data not shown). NK cell cytotoxicity against HER2-expressing ovarian cancer cells is assessed by a standard chromium release assay in the presence or absence of trastuzumab. Antibody-dependent cell cytotoxicity with non-chromium labeled ovarian cancer cells is also performed. NK cell production of cytokines (e.g., IFNγ, TNFα) and soluble levels of CD16a are

evaluated by ELISA. Cell surface levels of CD16a and other activation markers (e.g., CD107a, CD62L) are evaluated by FACS.

Example 3

Human tumor xenograft model for testing whether iPSC-derived NK cells expressing $CD16a^{197P}$ (CD16a/S197P) have increased *in vivo* anti-ovarian cancer activity in the presence of trastuzumab.

A xenograft model using NOD/SCID/$\gamma c^{-/-}$ (NSG) mice and human ovarian cancer cell lines stably engineered to express firefly luciferase for bioluminescent imaging (Geller et al., 2013, *Cytotherapy* 15:1297–1306) is used to test intraperitoneal (ip) delivery of NK cell activity against ovarian cancer cells. The OVCAR3 ovarian cancer cell line, which over-expresses HER2, is used as the *in vivo* target (Hellstrom et al., 2001, *Cancer Res* 61:2420–2423). Sublethally-irradiated (225 cGY) NSG female mice are injected intraperitoneally with OVCAR3 (2×10^5 cells) generated to express luciferase for bioluminescent imaging to quantify tumor growth or regression (Geller et al., 2013, *Cytotherapy* 15:1297–1306). Tumors are allowed to grow for seven days before the mice get a single intraperitoneal injection of 20×10^6 NK cells. Mice are then given IL-2 (5 μg/mouse) every other day for four weeks as previously described (Woll et al., 2009, *Blood* 113: 6094–6101) to promote *in vivo* survival of NK cells. Trastuzumab is administered at a dose of 50 μg intraperitoneally once weekly for four weeks, a previously used dose in this model (Warburton et al., 2004, *Clinical cancer research* 10:2512–2524). The *in vivo* potency of iPSC-derived NK cells expressing equivalent levels of WT CD16 or $CD16a^{197P}$ (CDa6a/S197P) are compared. Controls include iPSC-derived NK cells expressing GFP alone (vector only), and a cohort of mice receiving ovarian cancer cells only. All mice get the same IL-2 treatment.

Tumor growth/regression are monitored weekly by bioluminescent imaging and weighing the mice, as previously described (Woll et al., 2009, *Blood* 113: 6094–6101). Mice are also bled weekly to quantify human NK cell survival. The expression/cell surface levels of various effector function markers (e.g., IFNγ, CD16a) are evaluated by FACS. Mice are followed for ~60 days. At the time of sacrifice, internal organs (spleen, liver, lungs, kidney, and ovaries) are examined by bioluminescence for evidence of metastasis, as previously described (Woll et al., 2009, *Blood* 113: 6094–6101).

EXEMPLARY EMBODIMENTS

Embodiment 1. A cell genetically modified to express a CD16 polypeptide that comprises a membrane proximal region and an amino acid modification in the membrane proximal region.

Embodiment 2. A cell comprising:

a polynucleotide that encodes a CD16 polypeptide that comprises a membrane proximal region and an amino acid modification in the membrane proximal region.

Embodiment 3. The cell of Embodiment 1 or Embodiment 2 wherein the amino acid medication reflects an addition of one or more amino acids, a deletion of one or more amino acids, or a substitution of one or more amino acids compared to the wild-type amino acid sequence of the CD16 membrane proximal region.

Embodiment 4. The cell of Embodiment 3 wherein the substitution of one or more amino acids comprises a substitution of the serine residue at position 197 of SEQ ID NO:1.

Embodiment 5. The cell of any preceding Embodiment wherein the cell is a Natural Killer (NK) cell.

Embodiment 6. The cell of any preceding Embodiment wherein the cell is a neutrophil.

Embodiment 7. The cell of any preceding Embodiment wherein the cell is a monocyte.

Embodiment 8. The cell of any preceding Embodiment wherein the modified CD16 polypeptide exhibits reduced susceptibility to ADAM17-mediated shedding compared to a wild-type CD16 polypeptide.

Embodiment 9. The cell of any preceding Embodiment wherein the modified CD16 polypeptide exhibits reduced susceptibility to cleavage upon NK cell stimulation compared to a wild-type CD16 polypeptide.

Embodiment 10. A method comprising administering to a patient in need of such treatment a therapy that comprises:

administering to the patient a therapeutic NK effector; and

administering to the patient the cell of any one of claims 1-9.

Embodiment 11. The method of Embodiment 10 wherein the therapeutic NK effector comprises a therapeutic agent.

Embodiment 12. The method of Embodiment 11 wherein the therapeutic agent specifically recognizes a tumor antigen.

Embodiment 13. The method of Embodiment 12 wherein the therapeutic agent comprises an antibody or an antibody fragment that specifically recognizes the tumor antigen.

Embodiment 14. The method of Embodiment 13 wherein the tumor antigen comprises HER2.

Embodiment 15. The method of Embodiment 13 or Embodiment 14 wherein the antibody comprises trastuzumab or rituximab.

Embodiment 16. The method of Embodiment 10 wherein the therapeutic NK effector comprises a bi-specific killer engager (BiKE)

Embodiment 17. The method of Embodiment 16 wherein the BiKE comprises a CD16×CD33 BiKE, a CD16×CD19 BiKE, or a CD16×EP-CAM BiKE.

Embodiment 18. The method of Embodiment 10 wherein the therapeutic NK effector comprises a tri-specific killer cell engager (TriKE).

Embodiment 19. The method of any one of Embodiments 11 or 16-18 wherein the therapeutic agent specifically recognizes a viral target.

Embodiment 20. A method for improving therapy to a patient that includes administering to the patient a therapeutic NK effector, the method comprising:

administering to the patient the cell of any one of claims 1-9.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as

being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A cell genetically modified to express a CD16 polypeptide that comprises a membrane proximal region and an amino acid modification in the membrane proximal region.

2. A cell comprising:

a polynucleotide that encodes a CD16 polypeptide that comprises a membrane proximal region and an amino acid modification in the membrane proximal region.

3. The cell of claim 1 or claim 2 wherein the amino acid medication reflects an addition of one or more amino acids, a deletion of one or more amino acids, or a substitution of one or more amino acids compared to the wild-type amino acid sequence of the CD16 membrane proximal region.

4. The cell of claim 3 wherein the substitution of one or more amino acids comprises a substitution of the serine residue at position 197 of SEQ ID NO:1.

5. The cell of claim 1 or claim 2 wherein the cell is a Natural Killer (NK) cell.

6. The cell of claim 1 or claim 2 wherein the cell is a neutrophil.

7. The cell of claim 1 or claim 2 wherein the cell is a monocyte.

8. The cell of claim 1 or claim 2 wherein the modified CD16 polypeptide exhibits reduced susceptibility to ADAM17-mediated shedding compared to a wild-type CD16 polypeptide.

9. The cell of claim 1 or claim 2 wherein the modified CD16 polypeptide exhibits reduced susceptibility to cleavage upon NK cell stimulation compared to a wild-type CD16 polypeptide.

10. A method comprising:

administering to a patient in need of such treatment a therapy that comprises:

administering to the patient a therapeutic NK effector; and
administering to the patient the cell of claim 1 or claim 2.

11. The method of claim 10 wherein the therapeutic NK effector comprises a therapeutic agent.

12. The method of claim 11 wherein the therapeutic agent specifically recognizes a tumor antigen.

13. The method of claim 12 wherein the therapeutic agent comprises an antibody or an antibody fragment that specifically recognizes the tumor antigen.

14. The method of claim 13 wherein the tumor antigen comprises HER2.

15. The method of claim 13 or claim 14 wherein the antibody comprises trastuzumab or rituximab.

16. The method of claim 10 wherein the therapeutic NK effector comprises a bi-specific killer engager (BiKE)

17. The method of claim 16 wherein the BiKE comprises a CD16×CD33 BiKE, a CD16×CD19 BiKE, or a CD16×EP-CAM BiKE.

18. The method of claim 10 wherein the therapeutic NK effector comprises a tri-specific killer cell engager (TriKE).

19. The method of claim 11 wherein the therapeutic agent specifically recognizes a viral target.

20. A method for improving therapy to a patient that includes administering to the patient a therapeutic NK effector, the method comprising:

administering to the patient the cell of claim 1 or claim 2.

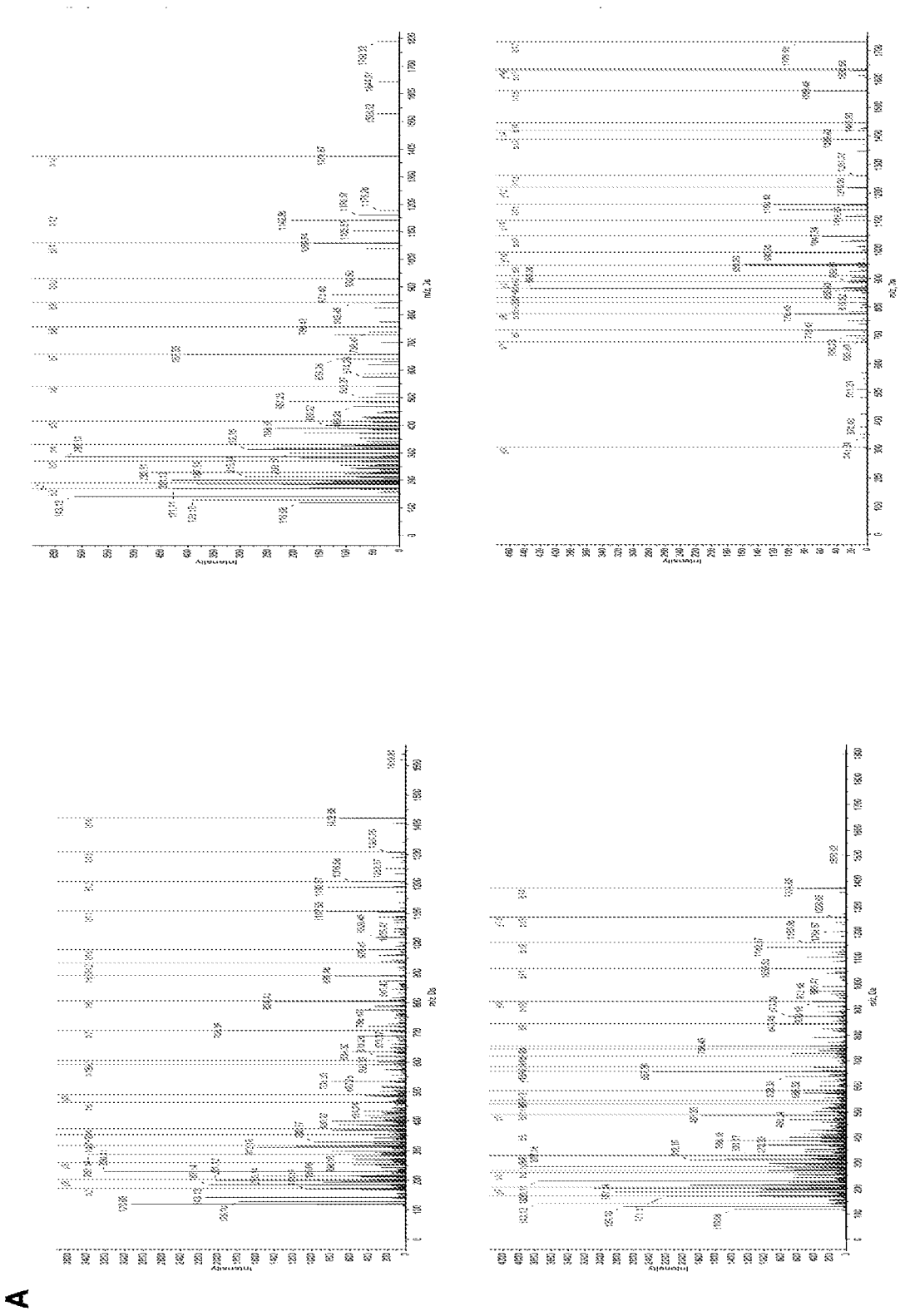


FIG. 1

WO 2015/148926

PCT/US2015/022998

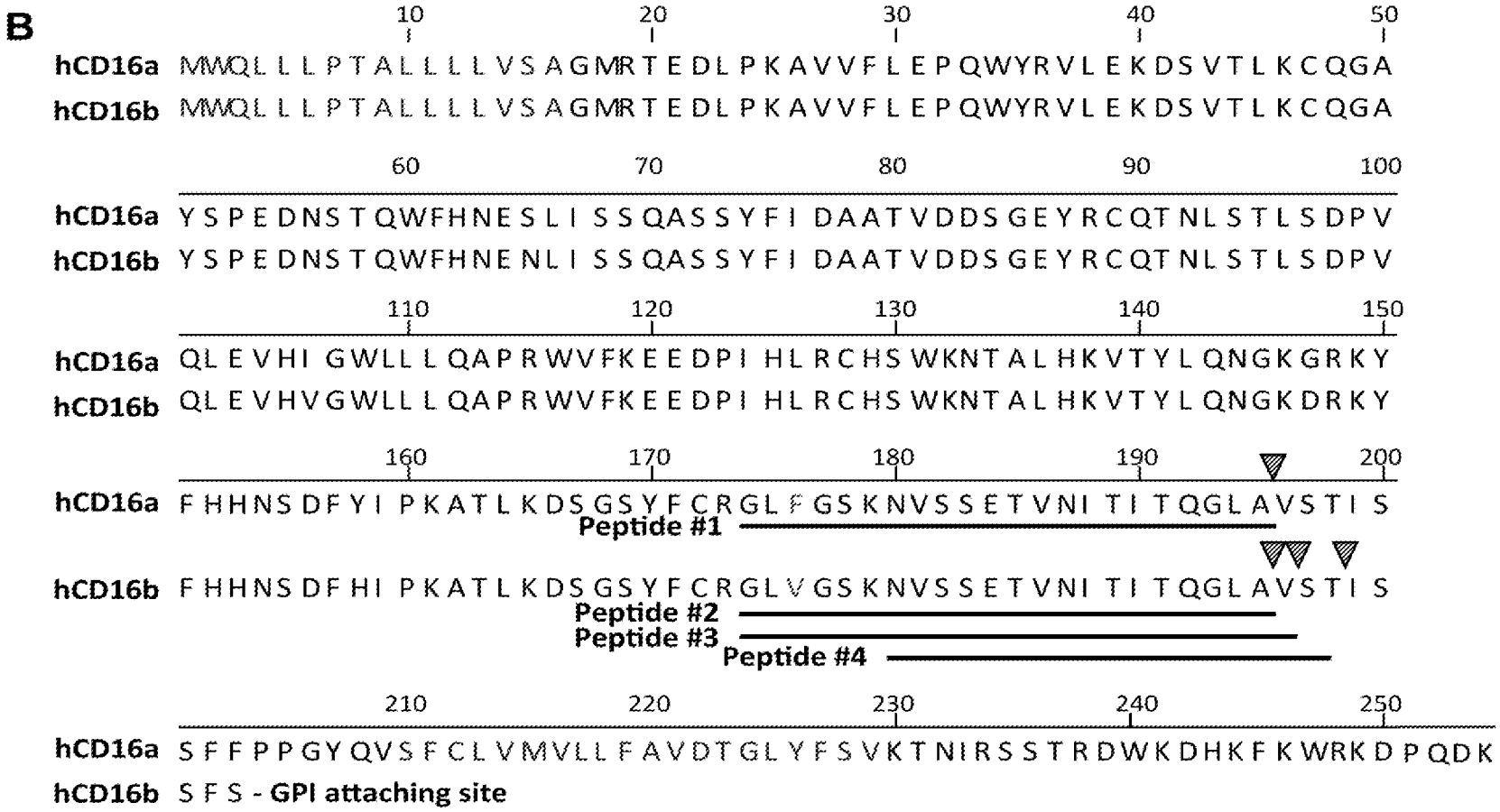
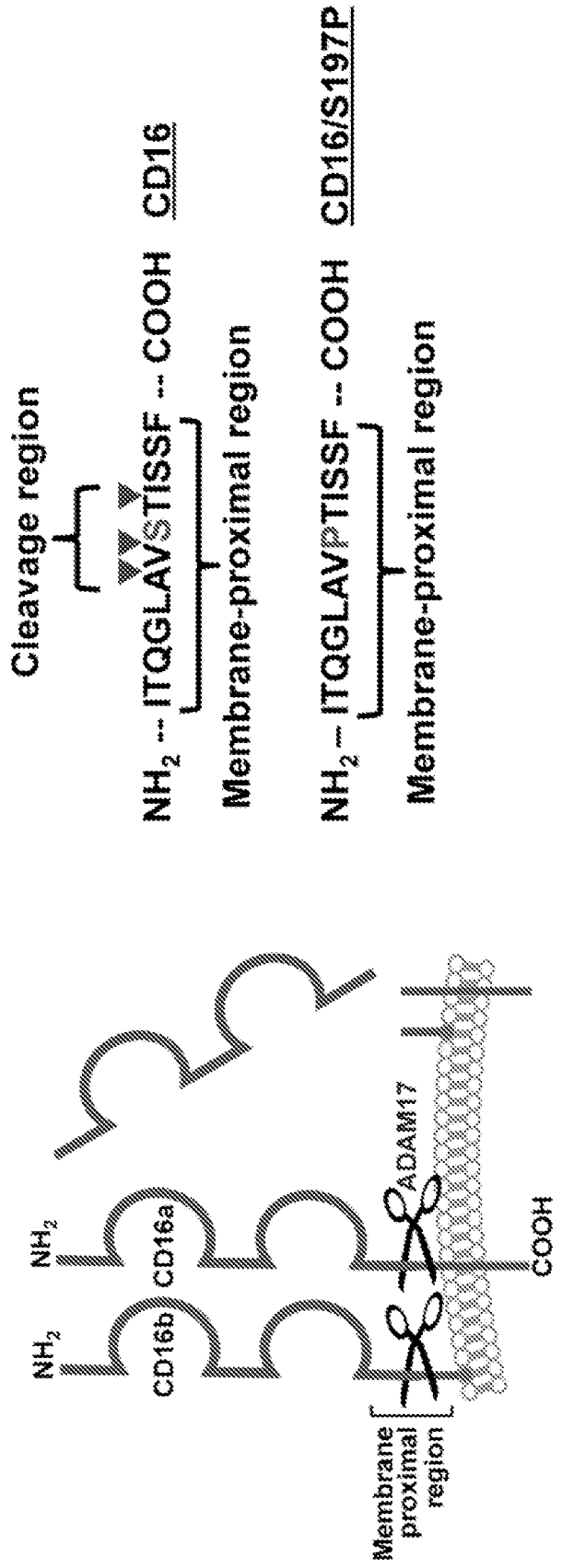


FIG. 1

3/6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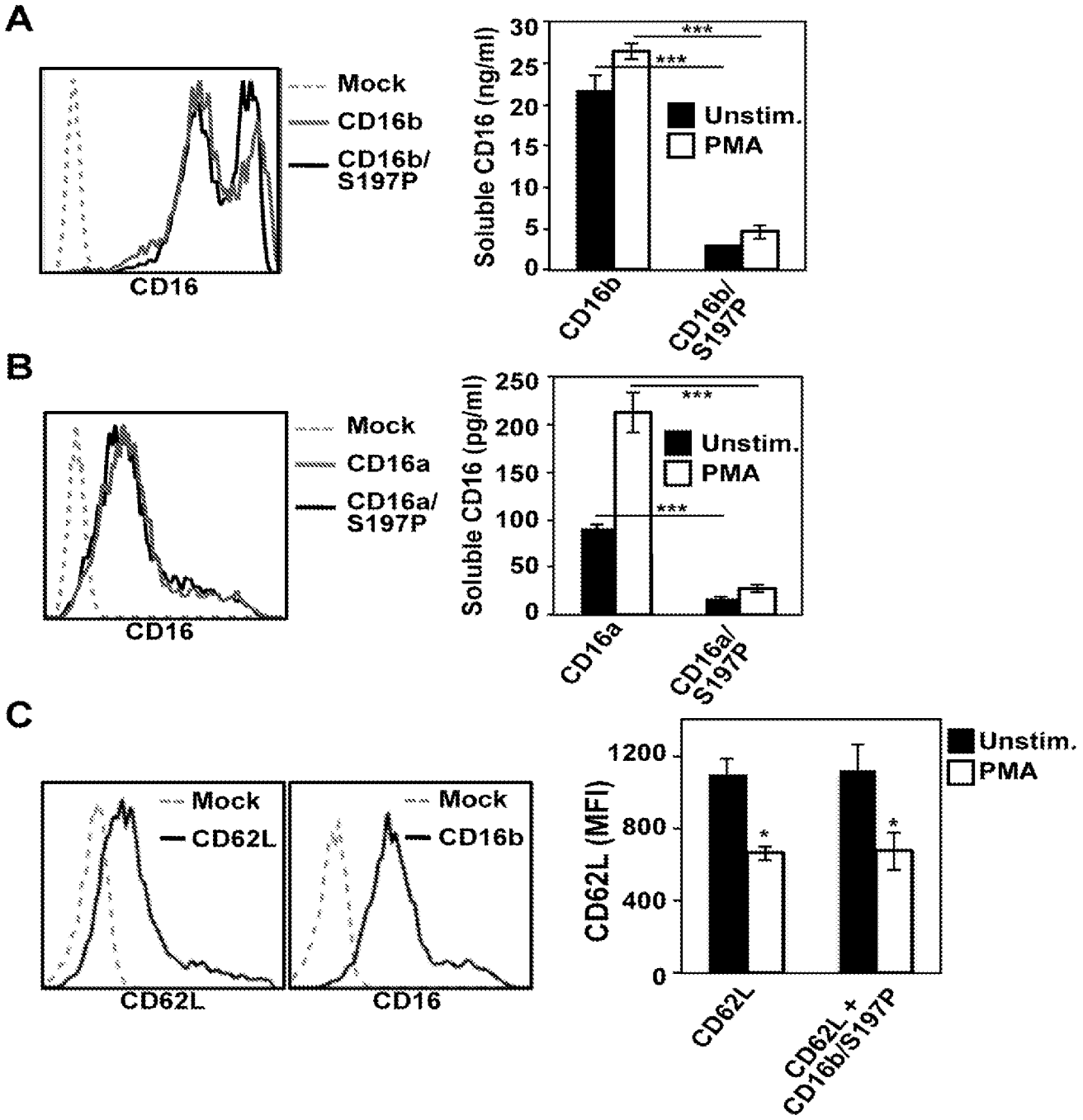
NH2 -- ITQGLAVSTISSF -- COOH CD16
Membrane-proximal region
NH2 -- ITQGLAVPTISSF -- COOH CD16/S197P
Membrane-proximal region
NH2
CD16b
NH2
CD16a
ADAM17
COOH
Membrane proximal region

FIG. 2


A
Mock
CD16b
CD16b/
S197P
CD16
Soluble CD16 (ng/ml)
30
25
20
15
10
5
0
***
***
Unstim.
PMA
CD16b
CD16b/
S197P
B
Mock
CD16a
CD16a/
S197P
CD16
Soluble CD16 (pg/ml)
250
200
150
100
50
0
***
***
Unstim.
PMA
CD16a
CD16a/
S197P
C
Mock
CD62L
CD62L
Mock
CD16b
CD16
CD62L (MFI)
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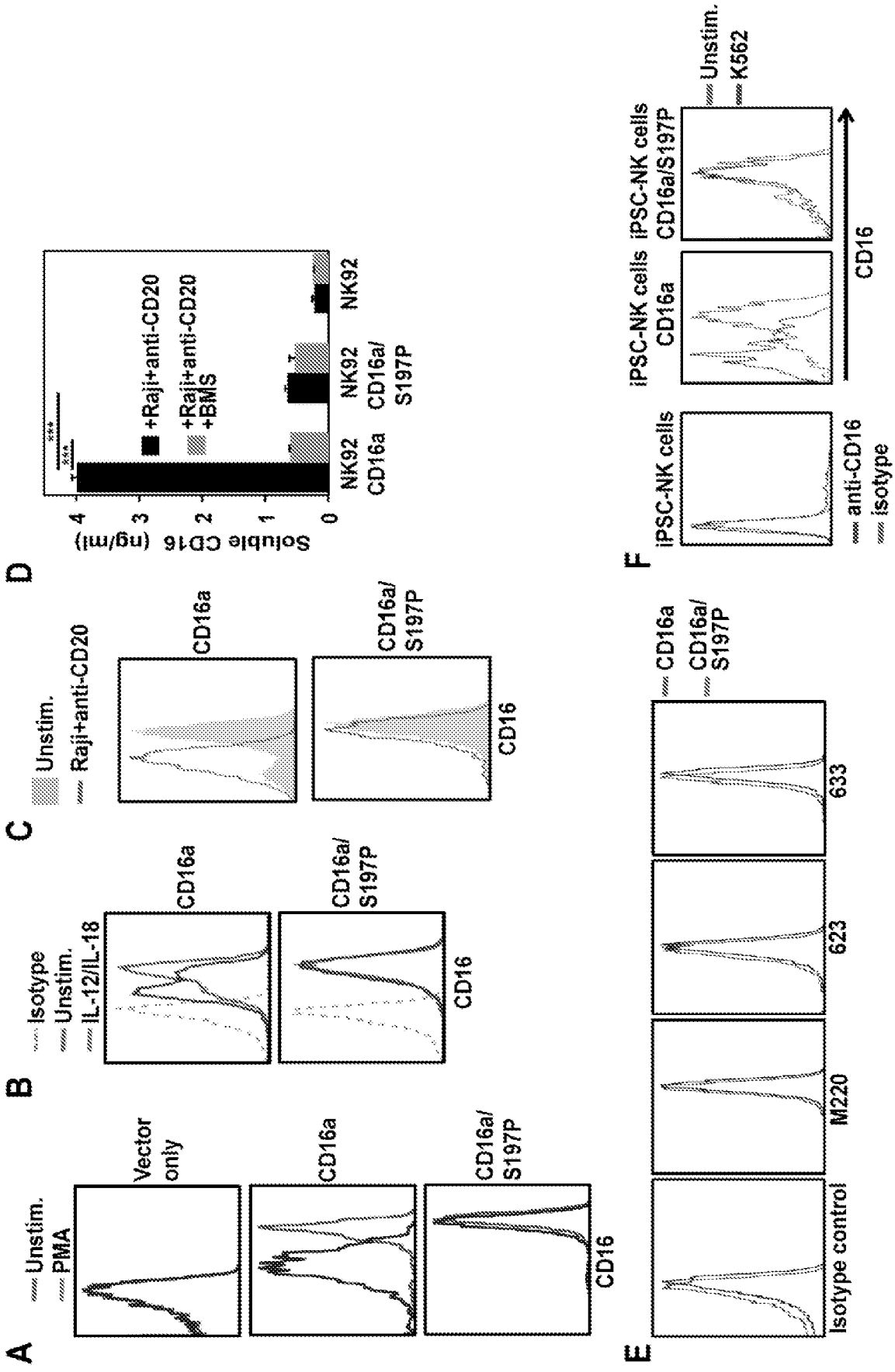
800
400
0
*
*
Unstim.
PMA
CD62L
CD62L +
CD16b/S197P

FIG. 3

5/6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PMA
Vector only
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CD16
B
Isotype
Unstim.
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CD16a
CD16a/ S197P
CD16
C
Unstim.
Raji+anti-CD20
CD16a
CD16a/ S197P
CD16
D
Soluble CD16 (ng/ml)
0
1
2
3
4
***
***
+Raji+anti-CD20
+Raji+anti-CD20 +BMS
NK92 CD16a
NK92 CD16a/ S197P
NK92
E
CD16a
CD16a/ S197P
Isotype control
M220
623
633
F
iPSC-NK cells
anti-CD16
isotype
iPSC-NK cells CD16a
iPSC-NK cells CD16a/S197P
CD16
Unstim.
K562

FIG. 4

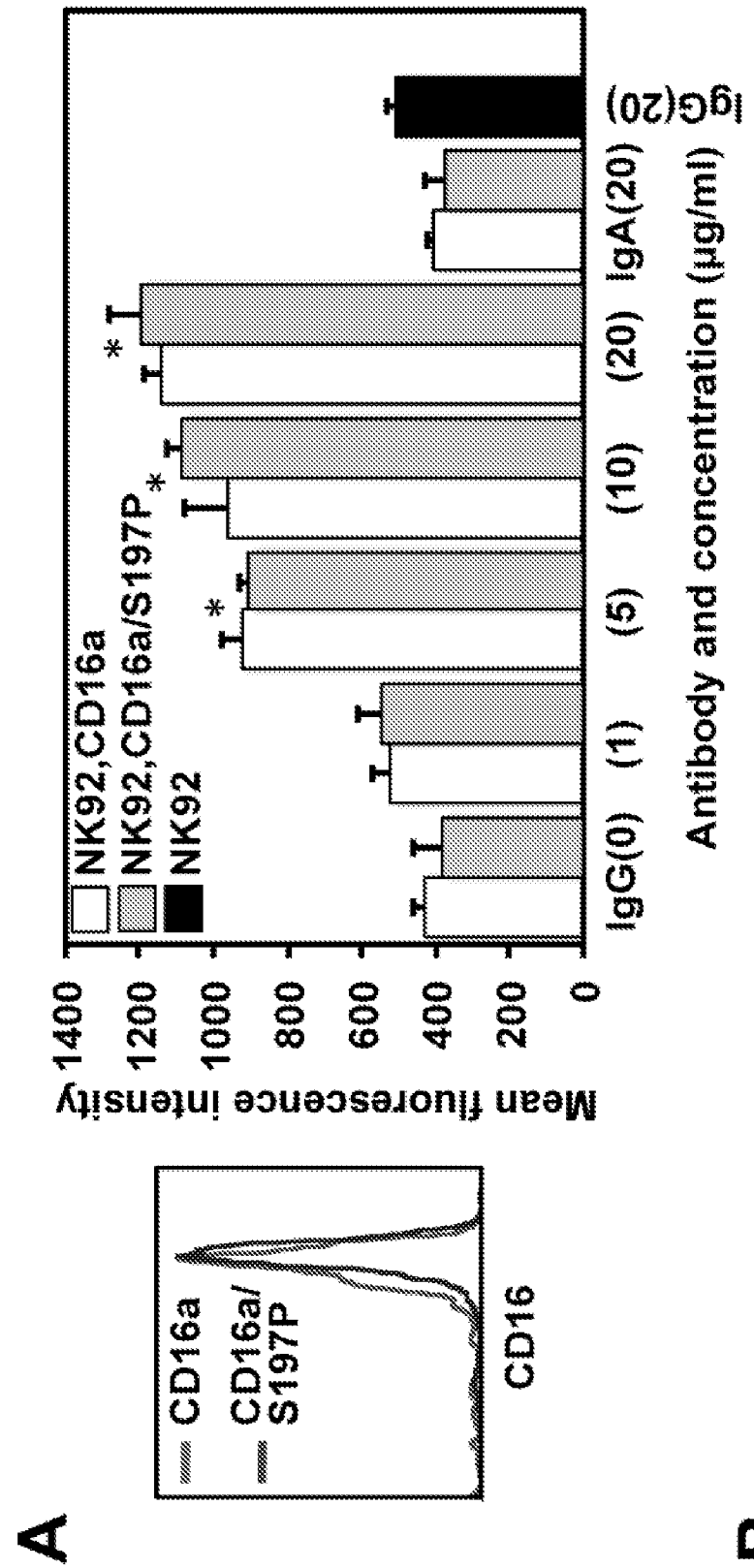
A
CD16a
CD16a/
S197P
CD16
Mean fluorescence intensity
1400
1200
1000
800
600
400
200
0
NK92,CD16a
NK92,CD16a/S197P
NK92
*
*
*
IgG(0) (1) (5) (10) (20) IgA(20) IgG(20)
Antibody and concentration (μg/ml)
B
NK92
mock
NK92
CD16a
NK92
CD16a/S197P
Unstim.
+Raji, 20min
+Raji+anti-CD20,
20 min
+Raji+anti-CD20,
40 min
CD107a

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/022998

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/735
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/196870 A1 (LEDBETTER JEFFREY A [US] ET AL) 6 August 2009 (2009-08-06)	1,2
Y	whole document esp. paragraphs [337,338,381] and seq id no 426	3-20
Y	WO 2010/040091 A1 (UNIV ARIZONA [US]; CHANG YUNG [US]; YAN HAO [US]) 8 April 2010 (2010-04-08) whole document esp. [5,165], seq id no 6, claims 43,50	3-20
X	EP 1 734 119 A2 (APPLIED RESEARCH SYSTEMS [AN] MERCK SERONO SA [CH]) 20 December 2006 (2006-12-20)	1,2
Y	the whole document esp. paragraphs [17,21]	3-20

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 June 2015	02/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brück, Marianne

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/022998

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [X] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/022998

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2009196870 A1	06-08-2009	AT 525399 T	15-10-2011
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Published:

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(54) Title: POLYPEPTIDES, CELLS, AND METHODS INVOLVING ENGINEERED CD16

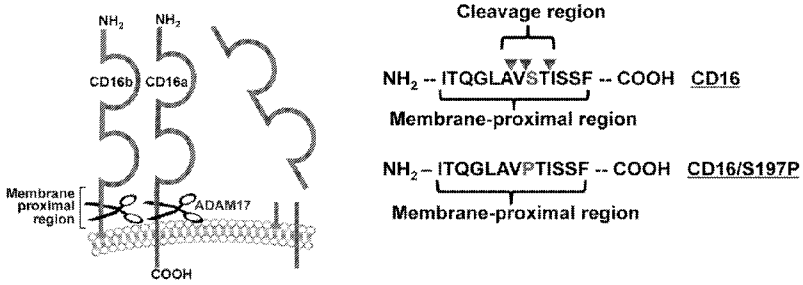

FIG. 2

(57) Abstract: This disclosure describes, generally, a modified form of CD 16, genetically-modified cells that express the modified CD 16, and methods that involve the genetically-modified cells. The modified form of CD 16 can exhibit increased anti-tumor and/or anti- viral activity due, at least in part, to reduced susceptibility to ADAM17-mediated shedding upon NK cell stimulation.

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POLYPEPTIDES, CELLS, AND METHODS INVOLVING ENGINEERED CD16

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application Serial No. 61/971,996, filed March 28, 2014, which is incorporated herein by reference.

SUMMARY

This disclosure describes, generally, a modified form of CD16, genetically-modified cells that express the modified CD16, and methods that involve the genetically-modified cells. The modified form of CD16 can exhibit increased anti-tumor and/or anti-viral activity due, at least in part, to reduced susceptibility to metalloprotease-mediated shedding upon NK cell stimulation.

In one aspect, therefore, this disclosure describes a cell genetically modified to express a CD16 polypeptide that has a membrane proximal region and an amino acid modification in the membrane proximal region.

In another aspect, this disclosure describes a cell that includes a polynucleotide that encodes a CD16 polypeptide that has membrane proximal region and an amino acid modification in the membrane proximal region.

In either aspect, the amino acid medication reflects an addition of one or more amino acids, a deletion of one or more amino acids, or a substitution of one or more amino acids compared to the wild-type amino acid sequence of the CD16 membrane proximal region. In some of these embodiments, the substitution of one or more amino acids includes a substitution of the serine residue at position 197 of SEQ ID NO:1.

In either aspect, the cell can be a Natural Killer (NK) cell, a neutrophil, a monocyte, or a T cell.

In either aspect, the modified CD16 polypeptide exhibits reduced susceptibility to ADAM17-mediated shedding compared to a wild-type CD16 polypeptide.

In either aspect, the modified CD16 polypeptide exhibits reduced susceptibility to cleavage upon NK cell stimulation compared to a wild-type CD1 polypeptide.

In another aspect, this disclosure describes a method that generally involves administering to a patient in need of such treatment a therapy that includes (a) administering to the patient a therapeutic NK effector, and (b) administering to the patient the any embodiment of the genetically-modified cell summarized above.

In some embodiments, the therapeutic NK effector includes a therapeutic agent. In some of these embodiments, the therapeutic agent can include an antibody, or a therapeutic antibody fragment. In some of these embodiments, the antibody, or antibody fragment, specifically binds to a viral antigen. In other embodiments, the antibody, or antibody fragment, specifically binds to a tumor antigen.

In some embodiments, the therapeutic agent can include a bi-specific killer engager (BiKE) or a tri-specific killer cell engager (TriKE).

In yet another aspect, this disclosure describes a method for improving immunotherapy to a patient, in which the immunotherapy involves administering to the patient a therapeutic NK effector. Generally the method includes further administering to the patient any embodiment of the genetically-modified cell summarized above.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Location of ectodomain cleavage sites in human CD16. (A) Tryptic peptides of soluble CD16 immunoprecipitated from the cell supernatant of PMA-activated human NK cells or neutrophils were subjected to mass spectrometry analysis. Four high confidence peptides with non-tryptic C-termini were identified: 1 peptide from soluble CD16 released by NK cells (Peptide #1, upper left) and 3 peptides from soluble CD16 released by neutrophils (Peptide #2, lower left; Peptide #3, upper right; and Peptide #4, lower right). (B) Illustration of Peptides #1-4 (underlined) and putative cleavage sites (arrowheads) in CD16a (SEQ ID NO:1) and CD16b (SEQ ID NO:2). Amino acid 176 distinguishes CD16a (F) from CD16b (V) in the identified

peptides. Amino acids 1-16 indicate a predicted signal sequences of CD16a and CD16b. Amino acids 210-229 indicate the transmembrane region of CD16a. Amino acid numbering begins with methionine in the signal sequence. The amino acid sequences of CD16a and CD16b are from the NCBI reference sequences NM_000569.6 and NM_000570.4, respectively.

FIG. 2. Schematic illustration of CD16 ectodomain shedding, the cleavage region, and the engineered serine-197 to proline mutation. CD16a and CD16b undergo ectodomain shedding by ADAM17 within a membrane proximal region, as indicated. The CD16 cleavage region within the membrane proximal region is based on mass spectrometry analysis that revealed three distinct cleavage sites in close proximity (arrowheads). Site-directed mutagenesis was performed to substitute serine-197 in CD16 (amino acids 190-202 of SEQ ID NO:1) with a proline (CD16/S197P).

FIG. 3. Effects of the engineered S197P mutation on CD16a and CD16b shedding. Transfected HEK293 (human embryonic kidney) cells separately expressed CD16b and CD16b/S197P (A) or CD16a and CD16a/S197P (B) at similar levels, as determined by flow cytometry (left panels). The different transfectants were treated with or without PMA (15 ng/ml for 30 minutes at 37°C) and soluble levels of CD16 in the media supernatant were quantified by ELISA (right panels). Each treatment condition was repeated three times for each experiment and the data are representative of three independent experiments. Bar graphs show mean ± SD. Statistical significance is indicated as ***P<0.001. (C) Transfected HEK293 cells expressed L-selectin (CD62L) or L-selectin and CD16b/S197P. Surface levels of L-selectin and CD16b/S197P on transfected and mock-transfected cells were measured using flow cytometry (histogram plots). Transfectants expressing L-selectin or L-selectin and CD16b/S197P were incubated in the presence or absence of PMA for 30 minutes at 37°C, and the mean fluorescence intensity (MFI) of L-selectin staining determined (bar graph). Each treatment condition was repeated three times for each experiment and the data are representative of two independent experiments. Bar graphs show mean ± SD. Statistical significance is indicated as *P<0.05. For all histogram plots, the x-axis = Log 10 fluorescence and the y-axis = cell number.

FIG. 4. Effects of the engineered S197P mutation on CD16a shedding in NK cells. NK92 cells transduced with empty vector (vector only), CD16a, or CD16a/S197P were treated without (Unstim.) or with PMA (100 ng/ml) for 30 minutes at 37°C (A), with IL-12 and IL-18 (100 ng/ml and 400 ng/ml, respectively) for 24 hours at 37°C (B), or with Raji cells and rituximab for

60 minutes at 37°C (C). Cell surface levels of CD16a were determined by flow cytometry. Isotype-matched negative control antibody staining is indicated by a dotted line. (D) Parent NK92 cells and transduced cells expressing CD16a or CD16a/S197P were treated with Raji cells and rituximab in the presence or absence of the ADAM17 inhibitor BMS566394 (5 μM) for 60 minutes at 37°C. Soluble CD16a levels were determined by ELISA. Each treatment condition was repeated three times and the data are representative of three independent experiments. Bar graphs show mean ± SD. Statistical significance is indicated as ***P<0.001. (E) NK92 cells expressing CD16a or CD16a/S197P were stained with the anti-ADAM17 mAbs M220, 623, 633, or an isotype-matched negative control antibody, as indicated. (F) $CD56^{+}CD45^{+}$ NK cells derived from mock-transduced iPSCs (left panel) or iPSCs expressing recombinant CD16a or CD16a/S197P (right panels) were incubated with or without K562 target cells for four hours at 37°C. For all histogram plots, the x-axis = Log 10 fluorescence, the y-axis = cell number, and the data are representative of at least 3 independent experiments.

FIG. 5. Effects of the engineered S197P mutation on CD16a function. (A) NK92 cells expressing CD16a or CD16a/S197P at equivalent levels (left panel) were treated with monomeric human IgG (0-20 μg/ml). As controls, cells were also treated with monomeric human IgA (20 μg/ml), and NK92 parent cells were treated with IgG (20 μg/ml) (bar). Antibody binding was determined by flow cytometry, as described in Materials and Methods. The bar graph shows mean ± SD of at least three separate experiments. Statistical significance is indicated as *P<0.05 versus IgG (0 μg/ml), IgA, or NK92 parent cells + IgG. (B) Mock transduced NK92 cells or NK92 cells expressing CD16a or CD16a/S197P were incubated in the absence (Unstim.) or presence of Raji cells treated with or without anti-CD20 rituximab for the indicated time points at 37°C. NK92 cell activation was assessed by the up-regulation in CD107a staining by flow cytometry. For the histogram plots, the x-axis = Log 10 fluorescence and the y-axis = cell number. Data are representative of at least 3 independent experiments.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

This disclosure describes, generally, a modified form of CD16a, genetically-modified cells that express the modified CD16a, and methods that involve the genetically-modified cells. The modified form of CD16a can exhibit increased anti-tumor and/or anti-viral activity due, at

least in part, to reduced susceptibility to metaaloprotease-mediated shedding upon NK cell stimulation.

In contrast to many solid cancer types, the survival rate of women with epithelial ovarian cancer has changed little in the last 30 years. Moreover, current standard therapies for recurrent ovarian cancer provide a low (<20%) response rate. Despite ubiquitous HER2 overexpression by ovarian cancer samples, treatment with the anti-HER2 antibody trastuzumab provides only limited responses in patients with advanced ovarian cancer. This resistance to trastuzumab may arise from dysfunctional NK cell-mediated antibody-dependent cell cytotoxicity. Thus, there is an urgent need for innovative therapeutic strategies. We describe a novel approach for providing therapeutic treatment strategy.

One concern with ovarian cancer is that the milieu in which tumor cells develop can be highly pro-inflammatory, and thus likely to promote CD16a cleavage on infiltrating NK cells and consequently diminishing antibody-dependent cell cytotoxicity. Several antibodies have emerged as effective targeted therapies for treating human malignancies. Their efficacy is due in part to antibody interactions with FcγRIIIa/CD16a on Natural Killer (NK) cells and induction of cancer cell killing by antibody-dependent cell cytotoxicity. Human IgG Fc receptor CD16 (FcγRIII) consists of two isoforms: CD16a (FcγRIIIa) and CD16b (FcγRIIIb). CD16a is expressed by Natural Killer (NK) cells and CD16b is expressed by neutrophils. NK Cell activation results in a rapid down-regulation in the surface levels of both isoforms of CD16 by a process referred to as ectodomain shedding—a proteolytic event that involves the metalloprotease ADAM17 and occurs at a single extracellular region proximal to the plasma membrane (FIG. 1A).

As noted above, ovarian cancer patients may be resistant to NK cell-mediated immunotherapies—i.e., the tumors are not sensitive to NK cell-mediated therapies. For example, ovarian cancer cells typically express the epidermal growth factor receptor HER2, yet its targeting with the therapeutic antibody trastuzumab has provided only a limited clinical response. This resistance may result, at least in part, from ectodomain shedding—i.e., NK cell activation by cytokines, target cell interaction, and/or tumor infiltration can result in CD16a cleavage and impaired antibody-dependent cell cytotoxicity. Thus, blocking the process of ectodomain shedding has clinical significance.

We have determined the cleavage sites of CD16a and CD16b using mass spectrometry and cloned the cDNAs of CD16a and CD16b from human blood leukocytes. Each cDNA was

mutated in a directed manner to induce a single amino acid change. Serine at location 197 was changed to a proline. (FIG. 1B). This mutation blocks the cleavage of CD16a and CD16b, and prevents their down-regulation upon cell activation. The expression of cleavage-resistant CD16a in *ex vivo* expanded NK cells maintain high surface levels of this IgG Fc receptor, which enhances NK cell stimulation, the efficacy of therapeutic antibodies, and cancer call killing.

ADAM17 has a number of cell surface substrates, but possesses no consensus sequence for proteolysis that can be used to predict the site of CD16a cleavage. Therefore, we used LC-MS-MS to determine the C-terminus cleavage site in soluble CD16 released from activated human peripheral blood leukocytes. We observed three putative cleavage locations in close proximity in the membrane proximal region of CD16 (FIG. 2, arrowheads), a region that is identical between CD16a and CD16b. Although ADAM17 proteolysis does not require a consensus sequence, the secondary structure of the cleavage region is important. In an attempt to block CD16a cleavage, we substituted serine-197 with a proline ($CD16a^{197P}$) to introduce a conformational change.

We identified the location of CD16 cleavage by immunoprecipitating CD16 from the media supernatant of activated NK cells and, separately, from the media supernatant of neutrophils. The immunoprecipitated CD16 was treated with PNGaseF to remove N-glycans, trypsin digested, and the generated peptides subjected to mass spectrometric analysis. Four different peptide patterns of high confidence were identified containing non-tryptic C-termini (FIG. 1A).

For CD16 enriched from the media supernatant of activated NK cells, we observed only one peptide pattern, which corresponds to amino acids glycine-174 through alanine-195 (Peptide #1, FIG. 1A) of SEQ ID NO:1. The membrane proximal regions of CD16a and CD16b have identical amino acid sequences except for residue 176. A phenylalanine at this location is indicative of CD16a, which was present in Peptide #1 (FIG. 1A and B). This peptide revealed a non-tryptic P1/P1′ cleavage position at alanine-195/valine-196 (FIG. 1B).

For CD16 enriched from the media supernatant of activated neutrophils, we detected three different peptide patterns with non-tryptic C-termini (Peptides #2-4, FIG. 1A and 1B). Peptide #2 corresponds to amino acids glycine-174 through alanine-195 of SEQ ID NO:2, Peptide #3 corresponds to amino acids glycine-174 through valine-196 of SEQ ID NO:2, and Peptide #4 corresponds to amino acids asparagine-180 through threonine-198 of SEQ ID NO:2.

Peptide #2 and Peptide #3 contained a valine at position 176, indicative of CD16b, and revealed P1/P1′ positions at alanine-195/valine-196 and at valine-196/serine-197 (FIG. 1B). Peptide #4 possessed a P1/P1′ position at threonine-198/isoleucine-199 (FIG. 1B). Though this peptide was derived from soluble CD16 from enriched neutrophils, it does not contain an amino acid at position 176 to identify the isoform (FIG. 1B). Regardless, the high confidence peptide revealed a third cleavage site in CD16. Taken together, these findings demonstrate the presence of a cleavage region in CD16 rather than a single specific cleavage site.

We further examined the cleavage region in CD16 by using site-directed mutagenesis to determine whether CD16a and CD16b cleavage could be disrupted in cell-based assays. ADAM17 tends to prefer an α-helical conformation in the substrate region that interacts with its catalytic site. Moreover, proteomic studies of ADAM17 cleavage site specificities revealed a very low preference for proline residues at the P1′, P2′, or P3′ positions. We therefore substituted serine-197 in the cleavage regions of CD16a and CD16b with a proline (S197P, as indicated in FIG. 2).

CD16b and CD16b/S197P were separately expressed in the human kidney cell line HEK293, which does not express endogenous CD16. The HEK293 transfectants expressed CD16b or CD16b/S197P at similar levels on their surface (FIG. 3A). High levels of CD16b were released from the transfected HEK293, which was increased further upon their treatment with PMA, as determined by ELISA (FIG. 3A). However, soluble levels of CD16b/S197P generated by untreated or PMA-treated HEK293 cells were markedly lower than those of CD16b (FIG. 3A).

We also examined the effects of the S197P mutation on CD16a cleavage using the same approach. Surface expression of CD16a requires association with γ chain dimmers. We therefore used HEK293 cells stably expressing human γ chain. Comparing HEK293 transfectants expressing equivalent surface levels of CD16a or CD16a/S197P (FIG. 3B), we determined the soluble levels of each receptor in the media supernatant of untreated and PMA-treated cells. Again, significantly lower levels of soluble CD16a/S197P were observed when compared to CD16a (FIG. 3B).

To evaluate whether the engineered S197P mutation in CD16 might disrupt ADAM17 activity, we also transfected HEK293 cells expressing or lacking CD16b/S197P with L-selectin, a well described ADAM17 substrate normally expressed by leukocytes. Both transfectants

expressed equivalent levels of L-selectin, which was similarly down-regulated following their activation with PMA (FIG. 3C), demonstrating that the S197P mutation affected CD16 shedding and not ADAM17 activity.

To assess the effects of the S197P mutation on CD16a shedding in NK cells, we used the human NK cell line NK92 (Gong et al., 1994, *Leukemia* 8:652-658). These cells lack expression of endogenous CD16a, but recombinant CD16a can be stably expressed. We transduced NK92 cells to separately express CD16a and CD16a/S197P. Cells expressing equivalent levels of these receptors were activated with PMA and cell surface CD16 levels were examined by flow cytometry. CD16a, but not CD16a/S197P, underwent a marked down-regulation in cell surface expression (FIG. 4A). IL-12 and IL-18 are physiological stimuli of NK cells that individually or in combination can induce CD16a shedding. NK92 cells treated with IL-12 and IL-18 demonstrated an appreciable down-regulation in their cell surface expression of CD16a but not CD16a/S197P (FIG. 4B). Direct engagement of cell bound IgG by CD16a also can induce its shedding, which we examined here by incubating NK92 cells expressing CD16a or CD16a/S197P with the CD20-positive Burkitt's lymphoma cell line Raji in the presence or absence of the anti-CD20 mAb rituximab. Raji cells treated with rituximab induced the down-regulation of CD16a, but not CD16a/S197P (FIG. 4C).

BMS566394 is a highly selective ADAM17 inhibitor with a potency orders of magnitude higher for ADAM17 than for other metalloproteases. BMS566394 blocked CD16a shedding with similar efficiency as the S197P mutation, but had no additional blocking effect on activated NK92 cells expressing CD16a/S197P (FIG. 4D). These findings provide further evidence that ADAM17 is the primary sheddase that cleaves CD16a within its cleavage region. It is possible, however, that ADAM17 expression levels were not equivalent in the NK92 cells expressing CD16a or CD16a/S197P, accounting for their dissimilar shedding. We therefore stained NK92 cells expressing CD16a or CD16a/S197P with multiple anti-ADAM17 mAbs and observed identical cell surface levels (FIG. 4E).

To establish the effect of the S197P mutation on CD16a shedding by primary NK cells, we used human iPSCs to generate engineered NK cells. We have previously reported on deriving functional NK cells from iPSCs and their similarity to peripheral blood NK cells (Knorr et al., 2013 *Stem Cells Transl Med.* 2:274-283; Ni et al., 2014, *Stem Cells* 32:1021-1031). CD16a and CD16a/S197P cDNA were cloned into a *Sleeping Beauty* transposon plasmid for gene insertion

and stable expression in iPSC cells, which were subsequently differentiated into mature NK cells. NK cells derived from mock transduced iPSC cells expressed low levels of endogenous CD16a, whereas transduced CD16a and CD16a/S197P were expressed at higher levels (FIG. 4F). NK cell activation occurs through various receptors upon their interaction with K562 cells, including BY55/CD160, resulting in ADAM17 activation and CD16a shedding. We stimulated the iPSC-derived NK cells with K562 cells and found that CD16a underwent a marked down-regulation in cell surface expression, whereas the expression of CD16a/S197P remained stable (FIG. 4F).

Endogenous and recombinant CD16a have sufficient affinity to bind monomeric IgG. To examine the effects of the S197P mutation on CD16a function, we compared the IgG binding capacities of CD16a and CD16a/S197P. NK92 cells expressing CD16a or CD16a/S197P at equivalent levels bound IgG in a similar dose-dependent manner (FIG. 5A). Controls consisted of IgA binding to NK92 cells expressing CD16a or CD16a/S197P, and IgG binding to NK92 parent cells. Both occurred at essentially background levels (FIG. 5A). These findings demonstrate specific and equivalent IgG binding by CD16a and CD16a/S197P.

CD16a is a potent activating receptor in NK cells, and we examined whether the engineered S197P mutation affected the capacity of CD16a to induce cell activation upon engagement of antibody-treated tumor cells. NK92 cell activation was assessed by measuring the up-regulation of CD107a, which occurs very rapidly upon degranulation and is a sensitive marker of NK cell activation. Mock transduced NK92 cells incubated with Raji cells treated with or without rituximab demonstrated low level and similar up-regulation CD107a (FIG. 5B). NK92 cells expressing CD16a or CD16a/S197P at equivalent levels when incubated with Raji cells alone marginally up-regulated CD107a as well, whereas their incubation with Raji cells treated with rituximab resulted in a considerable up-regulation of CD107a (FIG. 5B). Taken together, the above findings indicate that the engineered S197P mutation in CD16a did not impair its function.

Thus, we show that the engineered S197P mutation in CD16a and CD16b effectively blocked their shedding in cell-based assays that involved native ADAM17. The S197P mutation in CD16a also blocked shedding of the receptor in the human NK cell line NK92, but it did not impair receptor function. NK92 cells expressing equivalent levels of CD16a or CD16a/S197P bound monomeric IgG with similar efficiency over a range of antibody concentrations. In

addition, NK92 cells expressing CD16a or CD16a/S197P up-regulated the activation marker CD107a in a comparable manner upon their engagement of rituximab bound to Raji cells.

Pluripotent stem cells allow genetic manipulation to generate engineered NK cells. This disclosure describes the generation of engineered NK cells from transduced iPSCs expressing wild-type CD16a or CD16a/S197P. As with NK92 cells, CD16a underwent shedding in the iPSCs-derived NK cells, demonstrating normal ADAM17 activity upon cell activation, whereas CD16a/S197P was not shed.

CD16a and NK cell cytotoxic function can undergo a considerable down-regulation in cancer patients. The cDNAs encoding CD16a/S197P can be used to generate stable human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). These stem cells can then be differentiated into primary NK cells that express CD16a/S197P. Other cell populations that express cleavage resistant CD16a/S197P (e.g., monocytes) or CD16b/S197P (e.g., neutrophils) also can be derived from hESCs/iPSCs.

To generate an NK cell immunotherapy to be used in human patients against various forms of cancer or infection, the CD16a/S197P-expressing NK cells can mediate increased antibody-dependent cell cytotoxicity (ADCC) activity or other CD16a-mediated activity (e.g., IFNγ and TNFα production). For example, the CD16a/S197P-expressing NK cells may be combined with therapeutic antibodies (e.g., trastuzumab or rituximab), a bi-specific killer engager (BiKE, e.g., CD16×CD33, CD16×CD19, or CD16×EP-CAM bi-specific killer cell engager) or a tri-specific killer cell engager (TriKE). Other therapeutic cell populations (e.g., neutrophils, monocytes, T cells, etc.) also can be produced with increased CD16-mediated activity.

Expression of CD16a/S197P in human iPSCs or human ESCs can produce an NK cell population with enhanced ADCC activity against neoplastic conditions such as, for example, HER2 ovarian cancer. In some cases, the neoplastic condition may be treated with a therapeutic antibody such as, for example, trastuzumab. Mature NK cells may be derived from human embryonic stem cells and iPSCs.

Wild-type CD16a and/or CD16a/S197P can be cloned to generate a stable iPSC line or a stable ECS line expressing the individual CD16a receptors. Any suitable cloning method may be used. Exemplary cloning methods include, for example, viral-based methods, transposon vectors (e.g., *Sleeping Beauty*), or nucleofection. In one example, iPSCs may be modified using the

Sleeping Beauty transposon vector. The vector can contain a selection system such as, for example, GFP/zeocin resistance fusion protein, which allows a dual selection system (zeocin resistance and flow cytometric sorting). The iPSCs can be differentiated into mature NK cells, as previously described (Ni et al., 2011, *J. Virol.* 85:43–50; Knorr et al. 2013, *Stem Cells Transl Med* 2:274–283; Woll et al., 2009, *Blood* 113:6094–6101). Expression of transgenic receptors in iPSCs can lead to a high level of expression in the derived NK cells. CD16 expression in undifferentiated iPSCs may disrupt NK cell differentiation. In such cases, CD16 expression may be delayed using, for example, a CD56 or a natural CD16a promoter, so that CD16 expression better coincides with normal NK cell differentiation.

One can compare NK cells expressing equivalent levels of wild-type CD16a versus CD16a/S197P. Expression levels of the CD16 constructs can be matched by FACS sorting based on GFP expression, which occurs in a proportional manner to the CD16 constructs. Matched CD16a levels can be verified by FACS. NK cell cytotoxicity against HER2-expressing ovarian cancer cells can be assessed by a standard chromium release assay in the presence or absence of a therapeutic antibody such as, for example, trastuzumab. Antibody-dependent cell cytotoxicity with non-chromium labeled ovarian cancer cells can be evaluated. One can evaluate NK cell production of cytokines (e.g., IFNγ, TNFα) and soluble levels of CD16a by ELISA, and the cell surface levels of CD16a and other activation markers (e.g., CD107a, CD62L) by FACS.

The human tumor xenograft model described in Example 3 can be used to evaluate the anti-cancer activity of NK cells that express non-cleavable CD16a *in vivo*. Unlike human CD16, mouse CD16 does not undergo ectodomain shedding upon cell stimulation, and thus determining the effects of CD16a shedding on NK cell-mediated ADCC cannot be modeled in normal mice. Table 1 provides a representative set of experimental groupings and treatments.

Table 1. Tumor xenograft model

Group	**n**	**Treatment#**
1	5	No treatment
2	5	OVCAR3 cells only
3	5	OVCAR3 + NK cells/WT-CD16a
4	5	OVCAR3 + NK cells/ WT-CD16a + trastuzumab
5	5	OVCAR3 + NK cells/CD16a^{197P}

6	5	OVCAR3 + NK cells/ $CD16a^{197P}$ + trastuzumab
7	5	OVCAR3 + NK cells/vector only
8	5	OVCAR3 + NK cells/vector + trastuzumab

#Treatment performed at least twice and data pooled.

Tumor growth and/or regression can be monitored weekly by conventional methods including, for example, bioluminescent imaging, ultrasound, CT, MRI, another imaging technology, and/or weighing the mice (Woll et al., 2009, *Blood* 113:6094–6101). Mice also can be bled (e.g., weekly) to quantify human NK cell survival. The expression and/or cell surface levels of various effector function markers (e.g., IFNγ, CD16a) can be evaluated using conventional techniques such as, for example, by FACS. Mice can be followed for any suitable period such as, for example, 60 days. At the time of sacrifice, internal organs (e.g., spleen, liver, lungs, kidney, and/or ovaries) can be examined for evidence of metastasis (e.g., by bioluminescence), as previously described (Woll et al., 2009, *Blood* 113:6094–6101).

Our analyses allow one to define and compare the antibody-dependent cell cytotoxicity activity and *in vivo* potency of iPSC-derived NK cells expressing wild-type CD16a versus CD16a/S197P. Thus, we describe herein a modified form of CD16a, genetically-modified cells (e.g., NK cells, neutrophils, monocytes, T cells, etc.) that express the modified CD16a, and methods that involve the genetically-modified cells. For example, NK cells expressing the modified form of CD16a, CD16a/S197P, exhibit increased anti-ovarian cancer activity due, at least in part, to reduced susceptibility to ADAM17-mediated shedding upon NK cell stimulation. This, in turn, increases antibody-dependent cell cytotoxicity activity upon engaging antibody-tagged cancer cells such as, for example, cancer cells tagged with a therapeutic antibody. Moreover, antibody recognition by NK cells increases contact stability with tumor cells and bolsters NK cell activity through other activating receptors, such as NKG2D.

The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements; the terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims; unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one; and the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

In the preceding description, particular embodiments may be described in isolation for clarity. Unless otherwise expressly specified that the features of a particular embodiment are incompatible with the features of another embodiment, certain embodiments can include a combination of compatible features described herein in connection with one or more embodiments.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Mass spectrometry

Peripheral blood collection from healthy individuals was performed in accordance with protocols approved by the University of Minnesota Institutional Review Board according to protocol # 9708M00134. Human neutrophil and NK cell isolation was performed as previously described (Wang et al., 2013, *Biochim Biophys Acta.* 1833:680-685; Long et al., 2010, *J Leukoc Biol.* 87:1097-1101; Long et al., 2012, *J Leukoc Biol.* 92:667-672). Enriched neutrophils or NK cells (1×10^7/ml in PBS; Mediatech, Inc. Manassas, VA) were activated with PMA (15 ng/ml or 50 ng/ml, respectively; Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C. Cell supernatants were filtered (0.45 μm pore size) and CD16 was immunoprecipitated using the mAb 3G8 (BioLegend, Inc., San Diego, CA) and the Pierce direct immunopreciptation kit (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's instructions. Purified CD16 was deglycosylated by chitin binding domain-tagged Remove-iT PNGase F (New England BioLabs, Inc., Ipswich, MA), according to the manufacturer's instructions. Briefly, 10-20μg of purified CD16 was denatured in the presence of 40 mM DTT at 55°C for 10 minutes and then incubated with 3 μl of REMOVE-IT PNGase F (New England BioLabs, inc., Ipswich, MA) at 37°C for one hour. REMOVE-IT PNGase F was then removed from the reaction using chitin magnetic beads.

CD16 was subjected to SDS-PAGE and gel bands corresponding to soluble CD16 were detected by a krypton fluorescent protein stain (Thermo Fisher Scientific, Rockford, IL), verified by CD16 immunoblot analysis of adjacent lanes in the same gel, and were then excised and subjected to standard in-gel digestion with trypsin. Digested peptides extracted from the gel were dried down and reconstituted for liquid chromatography-mass spectrometry analysis in 98:2:0.01, water:acetonitrile:formic acid and ≤1 μg aliquots were analyzed by mass spectrometry (VELOS ORBITRAP, Thermo Fisher Scientific, Rockford, IL) in a data dependent scan mode, as described previously (Lin-Moshier et al., 2013, *J Biol Chem.* 288:355-367). Database searches were performed with Protein Pilot 4.5 (AB Sciex, Framingham, MA), which uses the Paragon scoring algorithm (Shilov et al., 2007, *Mol Cell Proteomics* 6:1638-1655), against the NCBI reference sequence *Homo sapiens* protein FASTA database to which the contaminant database (thegpm.org/cRAP/index,109 proteins) was appended. Search parameters were: cysteine iodoacetamide; trypsin; instrument Orbi MS (1-3ppm) Orbi MS/MS; biological modifications ID focus, which includes asparagine deamidation; a thorough search effort; and False Discovery Rate analysis (with reversed database).

Generation of cDNA expression constructs

CD16b occurs as two allelic variants termed NA1 and NA2, differing by four amino acids in the N-terminal portion of its extracellular region. Both allelic variants of CD16b are cleaved with similar efficiency by ADAM17. For this study, we examined only the NA1 variant. There are also two allelic variants of CD16a that have either a valine or phenylalanine residue at position 176. These two allelic variants of CD16a were cleaved with similar efficiency by ADAM17. For this study, we examined only the valine allelic variant CD16a.

CD16a and CD16b were amplified from human leukocyte cDNA, separately cloned into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA) at the *BamHI* and *EcoRI* restriction enzyme sites as previously described (Wang et al., 2013, *Biochim Biophys Acta.* 1833:680-685; Dong et al., 2014, *Arthritis Rheumatol.* 66:1291-1299). The constructs were then subjected to Quik-Change Site-directed Mutagenesis (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions to convert the serine at position 197 to a proline in CD16a and CD16b. All constructs were sequenced to confirm the presence of the intended mutation and the absence of any spontaneous mutations.

The CD16a cDNA was subsequently cloned into the bi-cistronic retroviral expression vector pBMN-IRES-EGFP, provided by Dr. G. Nolan (Stanford University, Stanford, CA), at the *BamHI* and *EcoRI* restriction enzyme sites. The CD16a constructs were also cloned into a bicistronic *Sleeping Beauty* transposon plasmid (pKT2-IRES-GFP:zeo) as previously described (Wilber et al., 2007, *Stem Cells* 25:2919-2927; Tian et al., 2009, *Stem Cells* 27:2675-2685). Briefly, wild-type CD16a and CD16a/S197P were PCR amplified using the primers: 5'-CCG GAA TTC CAG TGT GGC ATC ATG TGG CAG CTG CTC-3' (sense, SEQ ID NO:XX) and 5'-CCG GAA TTC TCA TTT GTC TTG AGG GTC CTT TCT-3' (antisense, SEQ ID NO:YY). *EcoRI* sites are underlined. The *EcoRI*-digested CD16a and CD16a/S197P PCR fragments were separately cloned into pKT2-IRES-GFP:zeo. Correct CD16a orientation and sequence were confirmed by PCR and sequencing analyses. We have previously cloned full-length human L-selectin (CD62L) cDNA (Feehan et al., 1996, *J Biol Chem.* 271:7019-7024; Matala et al., 2001, *J Immunol.* 167:1617-1623), which was transferred to the pcDNA3.1 vector at the restriction enzyme site *Xba1*. Full-length human FcRγ cDNA was cloned as previously described (Dong et al., 2014, *Arthritis Rheumatol.* 66:1291-1299), with the modification that a pcDNA3.1 vector was used.

Generation of cell lines expressing recombinant L-selectin, CD16a, and CD16b

HEK293 cells (a human embryonic kidney cell line) and NK92 cells (a human NK cell line) (ATCC, Manassas, VA) were cultured according to the depository's instructions. HEK293 cells were transiently transfected with pcDNA3.1 with or without CD16b, CD16b/S197P, and/or L-selectin using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. HEK293 cells stably expressing human FcRγ were transiently transfected with pcDNA3.1 with or without CD16a or CD16a/S197P by the same approach. NK92 cells were stably transduced with pBMN-IRES-EGFP with or without CD16a or CD16a/S197P by retrovirus generation and infection procedures described previously (Matala et al., 2001, *J Immunol.* 167:1617-1623; Walcheck et al., 2003, *J Leukoc Biol.* 74:389-394; Wang et al., 2009, *J Immunol.* 182:2449-2457). Construct expression was assessed by EGFP fluorescence and CD16 staining, as determined by flow cytometry. Human iPSCs (UCBiPS7, derived from umbilical cord blood CD34 cells) were maintained on mouse embryonic fibroblasts (Knorr et al., 2013, *Stem Cells Transl Med.* 2:274-283; Ni et al., 2014, *Stem Cells* 32:1021-1031). Stable

expression of CD16a or CD16a/S197P was performed using a *Sleeping Beauty* transposon system as previously described (Wilber et al., 2007, *Stem Cells* 25:2919-2927; Tian et al., 2009, *Stem Cells* 27:2675-2685). Briefly, iPSCs were nucleofected with pKT2-IRES-GFP:zeo in combination with transposase DNA in nucleofector solution V (Lonza Inc., Gaithersburg, MD) using program setting B16. Nucleofected cells were immediately suspended in iPSC growth medium containing zeocin (50 μg/ml) and seeded onto mouse embryonic fibroblasts.

NK cell derivation from CD16a-hESC and CD16a-iPSC cells

Hematopoietic differentiation of hESCs and iPSCs was performed as previously described (Ng et al., 2005, *Blood* 106: 1601–1603; Ng et al., 2008, *Nat Protoc* 3:768–776; Le Garff-Tavernier et al., 2010, *Aging Cell* 9: 527–535). Briefly, 3000 single cells were seeded per well of 96-well round bottom plates in BPEL media with stem cell factor (SCF, 40 ng/ml), vascular endothelial growth factor (VEGF, 20 ng/ml) and bone morphogenic protein 4 (BMP4, 20 ng/ml). BPEL media contained Iscove's Modified Dulbecco's Medium (IMDM, 86 ml, Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), F12 Nutrient Mixture with Glutmax I (86 mL, Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), 10% deionized Bovine Serum Albumin (BSA, 5 ml, Sigma-Aldrich, St. Louis, MO), 5% Polyvinyl alcohol (10 ml, Sigma-Aldrich, St. Louis, MO), linolenic acid (20 μl of 1 gm/ml solution, Sigma-Aldrich, St. Louis, MO), linoleic acid (20 μl of 1 gm/ml solution, Sigma), SYNTHECOL 500x solution (Sigma-Aldrich, St. Louis, MO), a-monothioglyceral (3.9 μl/100 ml, Sigma-Aldrich, St. Louis, MO), Protein-free hybridoma mix II (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), ascorbic acid (5 mg/ml, Sigma), GLUTAMAX I (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), Insulin-transferrin-selenium 100x solution (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA), Penicillin/streptomycin (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA).

At day 11 of hematopoietic differentiation, spin embryoid bodies were directly transferred into 24-well plates with or without EL08-1D2 stromal cells in NK media supplied with cytokines (Le Garff-Tavernier et al., 2010, *Aging Cell* 9:527–535). After 4-5 weeks of culture, single cell suspensions were stained with APC-, PE-, FITC- and PerCP-cy5.5-coupled IgG or specific antibodies against human blood surface antigens: CD45-PE, CD56-APC, CD56-PE, CD16-PerCP-cy5.5, NKG2D-PE, NKp44-PE, NKp46-PE, CD158b-FITC, CD158e1/2-FITC

(BD Pharmingen, San Jose, CA), CD158a/h-PE and CD158i-PE (Beckman Coulter, Inc., Pasadena, CA). Antibody stains were assessed by flow cytometry.

Cell stimulation

HEK293 and NK92 cells in RPMI 1640 media (Mediatech, Inc., Manassas, VA) were activated with 15 ng/ml and 100 ng/ml, respectively, PMA for 30 minutes at 37°C. NK92 cells were activated with IL-12 (PeproTech Inc, Rocky Hill, NJ) and IL-18 (R&D Systems, Inc., Minneapolis, MN) at 100 ng/ml and 400 ng/ml, respectively, for the indicated time points. NK92 cell activation through CD16a was mediated by their incubation with the CD20-positive Burkitt's lymphoma cell line Raji (ATCC, grown according to the depository's instructions) (1:1 ratio) treated with the anti-CD20 mAb rituximab (1 μg/ml) (Genentech, Inc., South San Francisco, CA), as described previously (Romee et al., 2013, *Blood* 121:3599-3608). Excess rituximab was removed by washing the Raji cells. In some experiments, NK92 cells were pre-incubated for 30 minutes with the selective ADAM17 inhibitor BMS566394 (5 μM) (Bristol-Myers Squibb Company, Princeton, NJ). NK cells derived from iPSCs were stimulated with the human erythroleukemic cell line K562 (ATCC, grown according to the depository's instructions), as previously described (Romee et al., 2013, *Blood* 121:3599-3608). Briefly, iPSC-derived NK cells were incubated with K562 target cells (2:1 ratio) for four hours at 37°C.

Antibody binding assay

Cell binding to monomeric human IgG and IgA (Sigma-Aldrich, St. Louis, MO) was performed as previously described with some modifications (Dong et al., 2014, *Arthritis Rheumatol.* 66:1291-1299). NK92 parent cells or transduced cells expressing CD16a or CD16a/S197P at 5×10^6/ml in PBS were incubated with IgG or IgA at the indicated concentrations in triplicate for one hour at 4°C. The cells were extensively washed and incubated with APC-conjugated donkey anti-human Fc (heavy and light chain) antibody (Jackson Immunoresearch, West Grove, PA) according to the manufacturer's instructions. The cells were washed and then immediately analyzed by flow cytometry.

Flow cytometry and ELISA

For cell staining, nonspecific antibody binding sites were blocked and cells were stained with the indicated antibodies and examined by flow cytometry, as previously described (Wang et al., 2013, *Biochim Biophys Acta.* 1833:680-685; Romee et al., 2013, *Blood* 121:3599-3608). Flow cytometric analysis was performed on FACSCanto and LS RII instruments (BD Biosciences, San Jose, CA). Human CD16 was detected by the mAbs 3G8 (BioLegend, Inc., San Diego, CA) and DJ130c (Santa Cruz Biotech, Santa Cruz, CA). CD107a was detected by the mAb H4A3 (Biolegend, Inc., San Diego, CA). ADAM17 was detected by the mAbs M220 (Doedens et al., 2000, *J Biol Chem.* 275:14598-14607), 111633, and 111623 (R&D Systems, Inc., Minneapolis, MN). Human L-selectin was detected by the mAb LAM1-116 (Ancell Corp., Stillwater, MN). Isotype-matched negative control mAbs were used to evaluate levels of nonspecific staining. The CD16 ELISA was performed by a custom cytometric bead assay, as previously described (Wang et al., 2013, *Biochim Biophys Acta.* 1833:680-685).

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad, San Diego, CA) using ANOVA and student's t test where appropriate. A p value of < 0.05 was considered significant.

Example 2

Comparison of NK cells expressing equivalent levels of WT CD16a and CD16a^{197P} (CD16a/S197P)

Expression levels of the CD16 constructs are matched by FACS sorting based on GFP expression (as done for NK92 cells described above, FIG. 2), which occurs in a proportional manner to the CD16 constructs. Matched CD16a levels are verified by FACS for all assays. As a control, iPSC-derived NK cells modified with empty *Sleeping Beauty* transposon vector (expressing only GFP) are evaluated. iPSC-derived NK cells express low levels of endogenous CD16a (data not shown). NK cell cytotoxicity against HER2-expressing ovarian cancer cells is assessed by a standard chromium release assay in the presence or absence of trastuzumab. Antibody-dependent cell cytotoxicity with non-chromium labeled ovarian cancer cells is also performed. NK cell production of cytokines (e.g., IFNγ, TNFα) and soluble levels of CD16a are

evaluated by ELISA. Cell surface levels of CD16a and other activation markers (e.g., CD107a, CD62L) are evaluated by FACS.

Example 3

Human tumor xenograft model for testing whether iPSC-derived NK cells expressing CD16a^{197P} (CD16a/S197P) have increased *in vivo* anti-ovarian cancer activity in the presence of trastuzumab.

A xenograft model using NOD/SCID/$\gamma c^{-/-}$ (NSG) mice and human ovarian cancer cell lines stably engineered to express firefly luciferase for bioluminescent imaging (Geller et al., 2013, *Cytotherapy* 15:1297–1306) is used to test intraperitoneal (ip) delivery of NK cell activity against ovarian cancer cells. The OVCAR3 ovarian cancer cell line, which over-expresses HER2, is used as the *in vivo* target (Hellstrom et al., 2001, *Cancer Res* 61:2420–2423). Sublethally-irradiated (225 cGY) NSG female mice are injected intraperitoneally with OVCAR3 (2×10^5 cells) generated to express luciferase for bioluminescent imaging to quantify tumor growth or regression (Geller et al., 2013, *Cytotherapy* 15:1297–1306). Tumors are allowed to grow for seven days before the mice get a single intraperitoneal injection of 20×10^6 NK cells. Mice are then given IL-2 (5 μg/mouse) every other day for four weeks as previously described (Woll et al., 2009, *Blood* 113: 6094–6101) to promote *in vivo* survival of NK cells. Trastuzumab is administered at a dose of 50 μg intraperitoneally once weekly for four weeks, a previously used dose in this model (Warburton et al., 2004, *Clinical cancer research* 10:2512–2524). The *in vivo* potency of iPSC-derived NK cells expressing equivalent levels of WT CD16 or CD16a^{197P} (CDa6a/S197P) are compared. Controls include iPSC-derived NK cells expressing GFP alone (vector only), and a cohort of mice receiving ovarian cancer cells only. All mice get the same IL-2 treatment.

Tumor growth/regression are monitored weekly by bioluminescent imaging and weighing the mice, as previously described (Woll et al., 2009, *Blood* 113: 6094–6101). Mice are also bled weekly to quantify human NK cell survival. The expression/cell surface levels of various effector function markers (e.g., IFNγ, CD16a) are evaluated by FACS. Mice are followed for ~60 days. At the time of sacrifice, internal organs (spleen, liver, lungs, kidney, and ovaries) are examined by bioluminescence for evidence of metastasis, as previously described (Woll et al., 2009, *Blood* 113: 6094–6101).

EXEMPLARY EMBODIMENTS

Embodiment 1. A cell genetically modified to express a CD16 polypeptide that comprises a membrane proximal region and an amino acid modification in the membrane proximal region.

Embodiment 2. A cell comprising:

a polynucleotide that encodes a CD16 polypeptide that comprises a membrane proximal region and an amino acid modification in the membrane proximal region.

Embodiment 3. The cell of Embodiment 1 or Embodiment 2 wherein the amino acid medication reflects an addition of one or more amino acids, a deletion of one or more amino acids, or a substitution of one or more amino acids compared to the wild-type amino acid sequence of the CD16 membrane proximal region.

Embodiment 4. The cell of Embodiment 3 wherein the substitution of one or more amino acids comprises a substitution of the serine residue at position 197 of SEQ ID NO:1.

Embodiment 5. The cell of any preceding Embodiment wherein the cell is a Natural Killer (NK) cell.

Embodiment 6. The cell of any preceding Embodiment wherein the cell is a neutrophil.

Embodiment 7. The cell of any preceding Embodiment wherein the cell is a monocyte.

Embodiment 8. The cell of any preceding Embodiment wherein the modified CD16 polypeptide exhibits reduced susceptibility to ADAM17-mediated shedding compared to a wild-type CD16 polypeptide.

Embodiment 9. The cell of any preceding Embodiment wherein the modified CD16 polypeptide exhibits reduced susceptibility to cleavage upon NK cell stimulation compared to a wild-type CD16 polypeptide.

Embodiment 10. A method comprising administering to a patient in need of such treatment a therapy that comprises:

administering to the patient a therapeutic NK effector; and

administering to the patient the cell of any one of claims 1-9.

Embodiment 11. The method of Embodiment 10 wherein the therapeutic NK effector comprises a therapeutic agent.

Embodiment 12. The method of Embodiment 11 wherein the therapeutic agent specifically recognizes a tumor antigen.

Embodiment 13. The method of Embodiment 12 wherein the therapeutic agent comprises an antibody or an antibody fragment that specifically recognizes the tumor antigen.

Embodiment 14. The method of Embodiment 13 wherein the tumor antigen comprises HER2.

Embodiment 15. The method of Embodiment 13 or Embodiment 14 wherein the antibody comprises trastuzumab or rituximab.

Embodiment 16. The method of Embodiment 10 wherein the therapeutic NK effector comprises a bi-specific killer engager (BiKE)

Embodiment 17. The method of Embodiment 16 wherein the BiKE comprises a CD16×CD33 BiKE, a CD16×CD19 BiKE, or a CD16×EP-CAM BiKE.

Embodiment 18. The method of Embodiment 10 wherein the therapeutic NK effector comprises a tri-specific killer cell engager (TriKE).

Embodiment 19. The method of any one of Embodiments 11 or 16-18 wherein the therapeutic agent specifically recognizes a viral target.

Embodiment 20. A method for improving therapy to a patient that includes administering to the patient a therapeutic NK effector, the method comprising:

administering to the patient the cell of any one of claims 1-9.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as

being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A cell genetically modified to express a CD16 polypeptide that comprises a membrane proximal region and an amino acid modification in the membrane proximal region.

2. A cell comprising:

a polynucleotide that encodes a CD16 polypeptide that comprises a membrane proximal region and an amino acid modification in the membrane proximal region.

3. The cell of claim 1 or claim 2 wherein the amino acid medication reflects an addition of one or more amino acids, a deletion of one or more amino acids, or a substitution of one or more amino acids compared to the wild-type amino acid sequence of the CD16 membrane proximal region.

4. The cell of claim 3 wherein the substitution of one or more amino acids comprises a substitution of the serine residue at position 197 of SEQ ID NO:1.

5. The cell of claim 1 or claim 2 wherein the cell is a Natural Killer (NK) cell.

6. The cell of claim 1 or claim 2 wherein the cell is a neutrophil.

7. The cell of claim 1 or claim 2 wherein the cell is a monocyte.

8. The cell of claim 1 or claim 2 wherein the modified CD16 polypeptide exhibits reduced susceptibility to ADAM17-mediated shedding compared to a wild-type CD16 polypeptide.

9. The cell of claim 1 or claim 2 wherein the modified CD16 polypeptide exhibits reduced susceptibility to cleavage upon NK cell stimulation compared to a wild-type CD16 polypeptide.

10. A method comprising:

administering to a patient in need of such treatment a therapy that comprises:

administering to the patient a therapeutic NK effector; and
administering to the patient the cell of claim 1 or claim 2.

11. The method of claim 10 wherein the therapeutic NK effector comprises a therapeutic agent.

12. The method of claim 11 wherein the therapeutic agent specifically recognizes a tumor antigen.

13. The method of claim 12 wherein the therapeutic agent comprises an antibody or an antibody fragment that specifically recognizes the tumor antigen.

14. The method of claim 13 wherein the tumor antigen comprises HER2.

15. The method of claim 13 or claim 14 wherein the antibody comprises trastuzumab or rituximab.

16. The method of claim 10 wherein the therapeutic NK effector comprises a bi-specific killer engager (BiKE)

17. The method of claim 16 wherein the BiKE comprises a CD16×CD33 BiKE, a CD16×CD19 BiKE, or a CD16×EP-CAM BiKE.

18. The method of claim 10 wherein the therapeutic NK effector comprises a tri-specific killer cell engager (TriKE).

19. The method of claim 11 wherein the therapeutic agent specifically recognizes a viral target.

20. A method for improving therapy to a patient that includes administering to the patient a therapeutic NK effector, the method comprising:

administering to the patient the cell of claim 1 or claim 2.

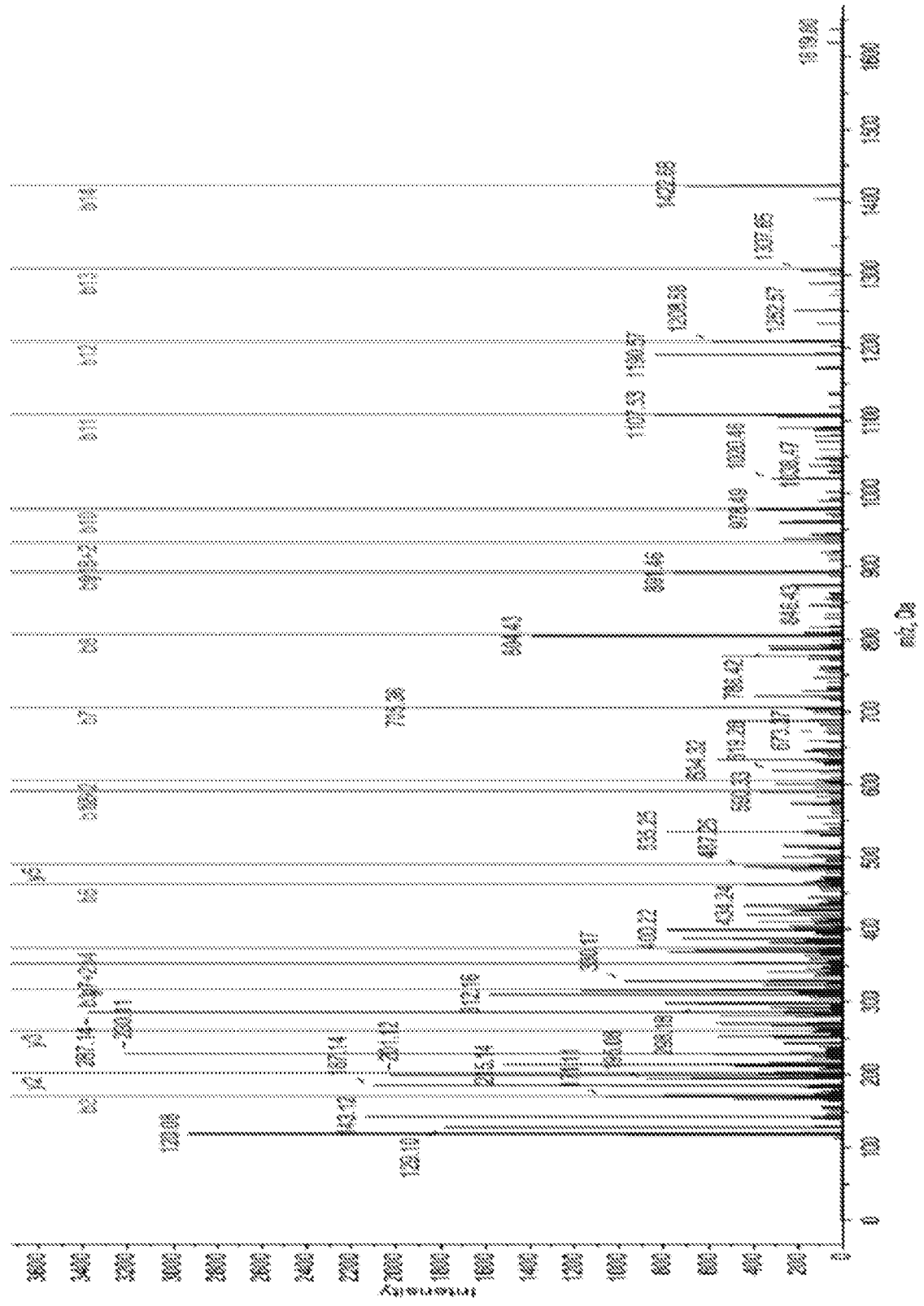


Fig. 1A-1

Fig. 1A-2

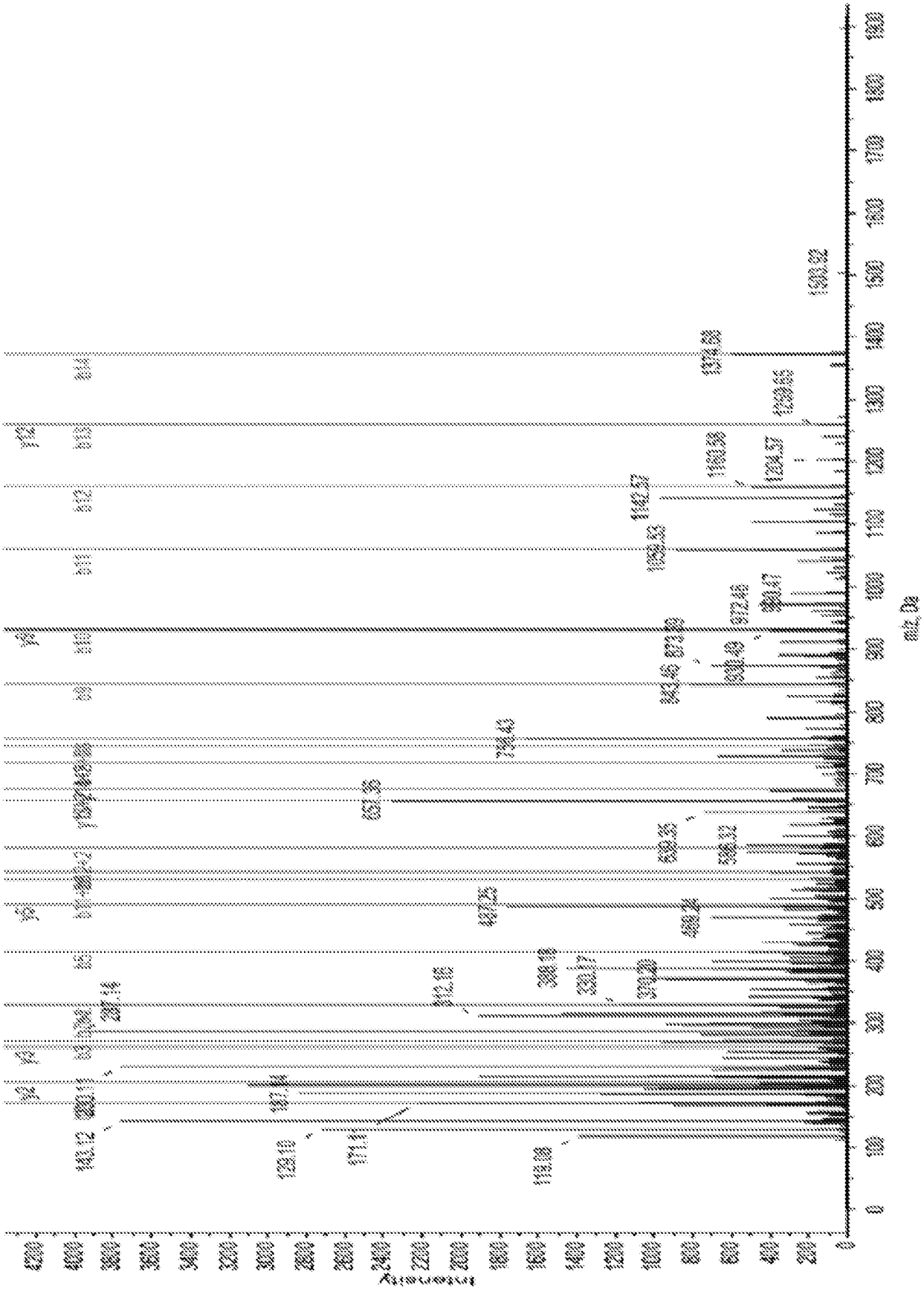


Fig. 1A-3

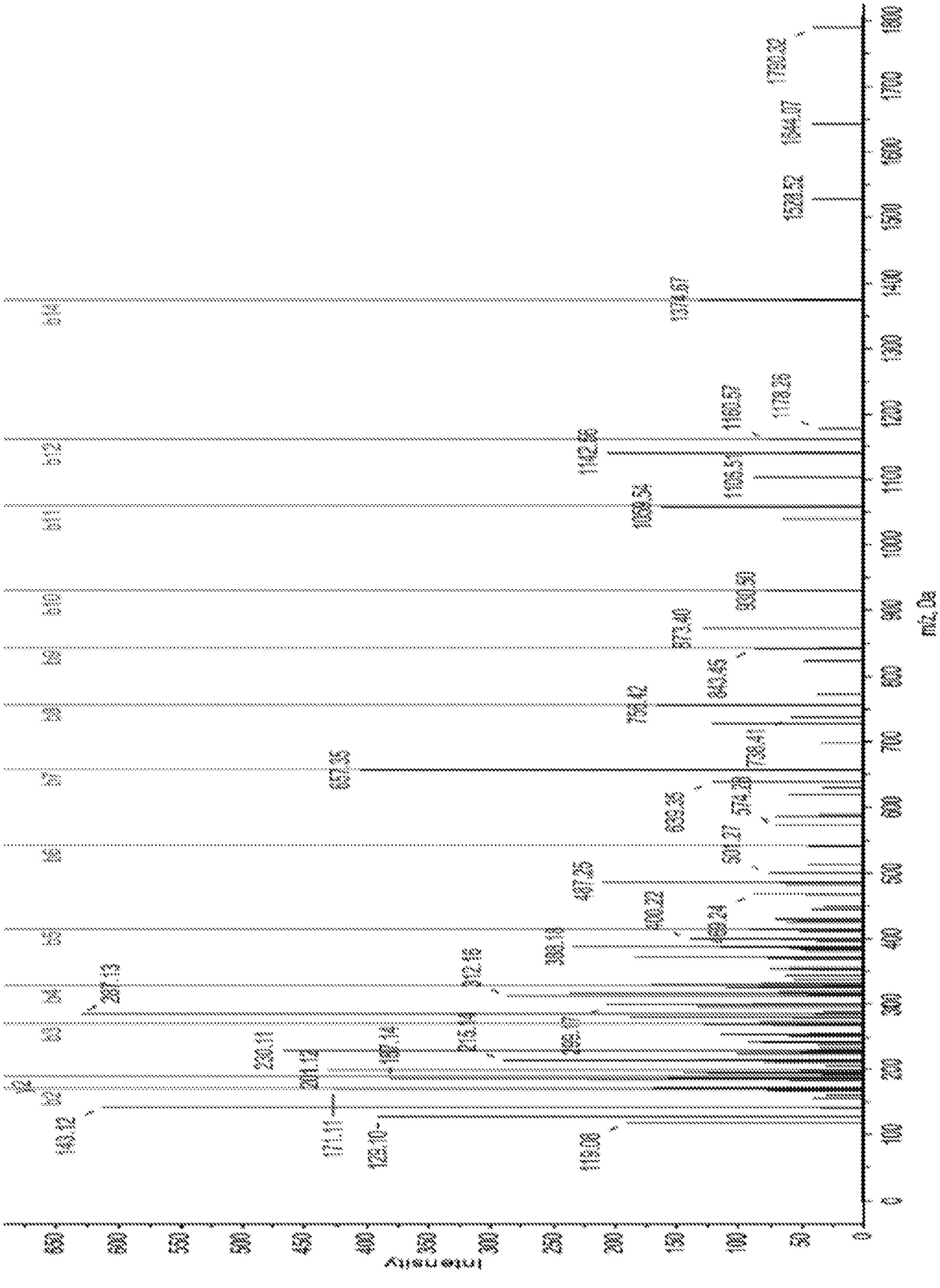
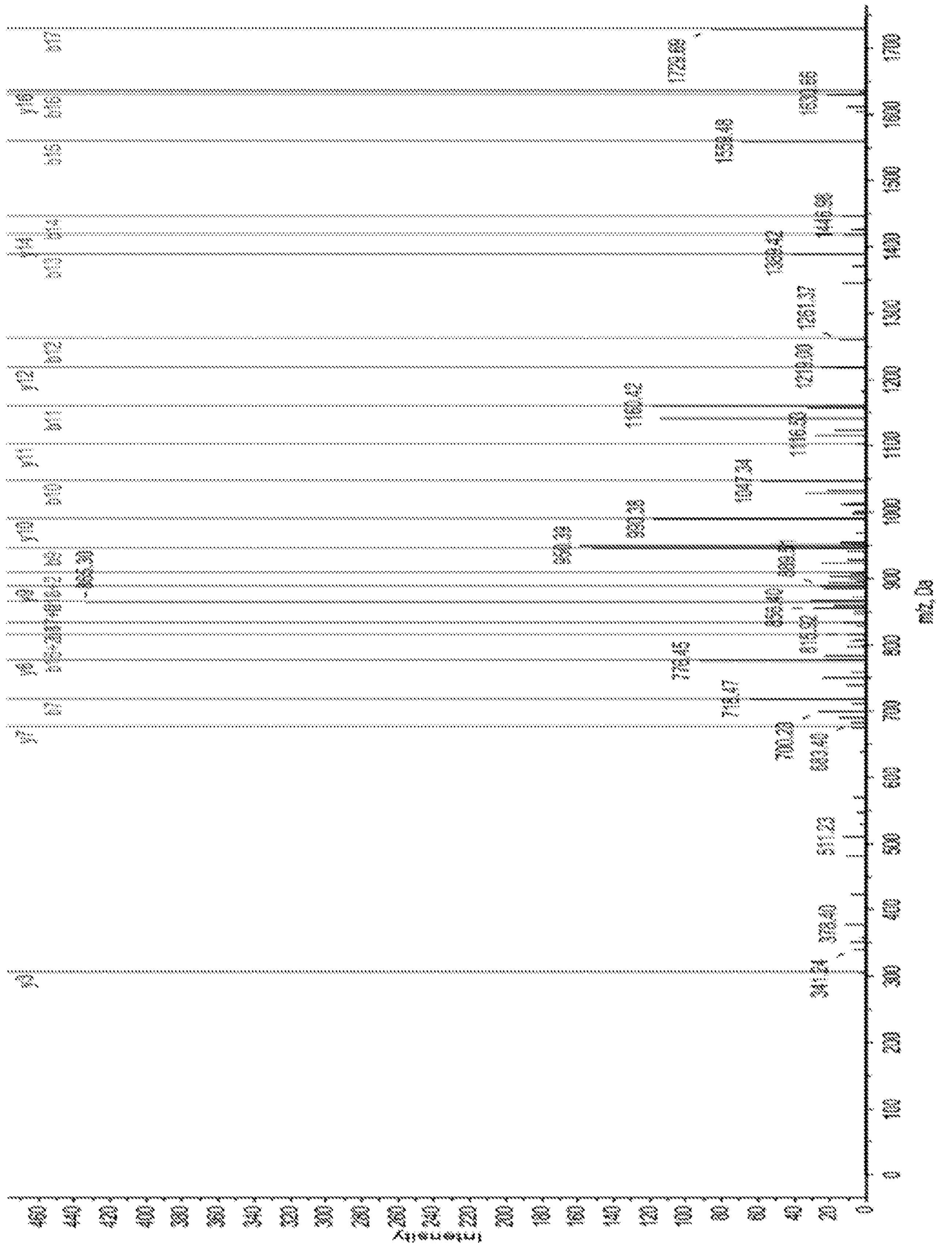

Intensity
m/z, Da
1374.67
657.35
1142.56
1039.54
758.42
930.50
873.40
487.35
287.13
230.11
143.12
171.11
129.10
1790.32
1644.77
1529.52

Fig. 1A-4



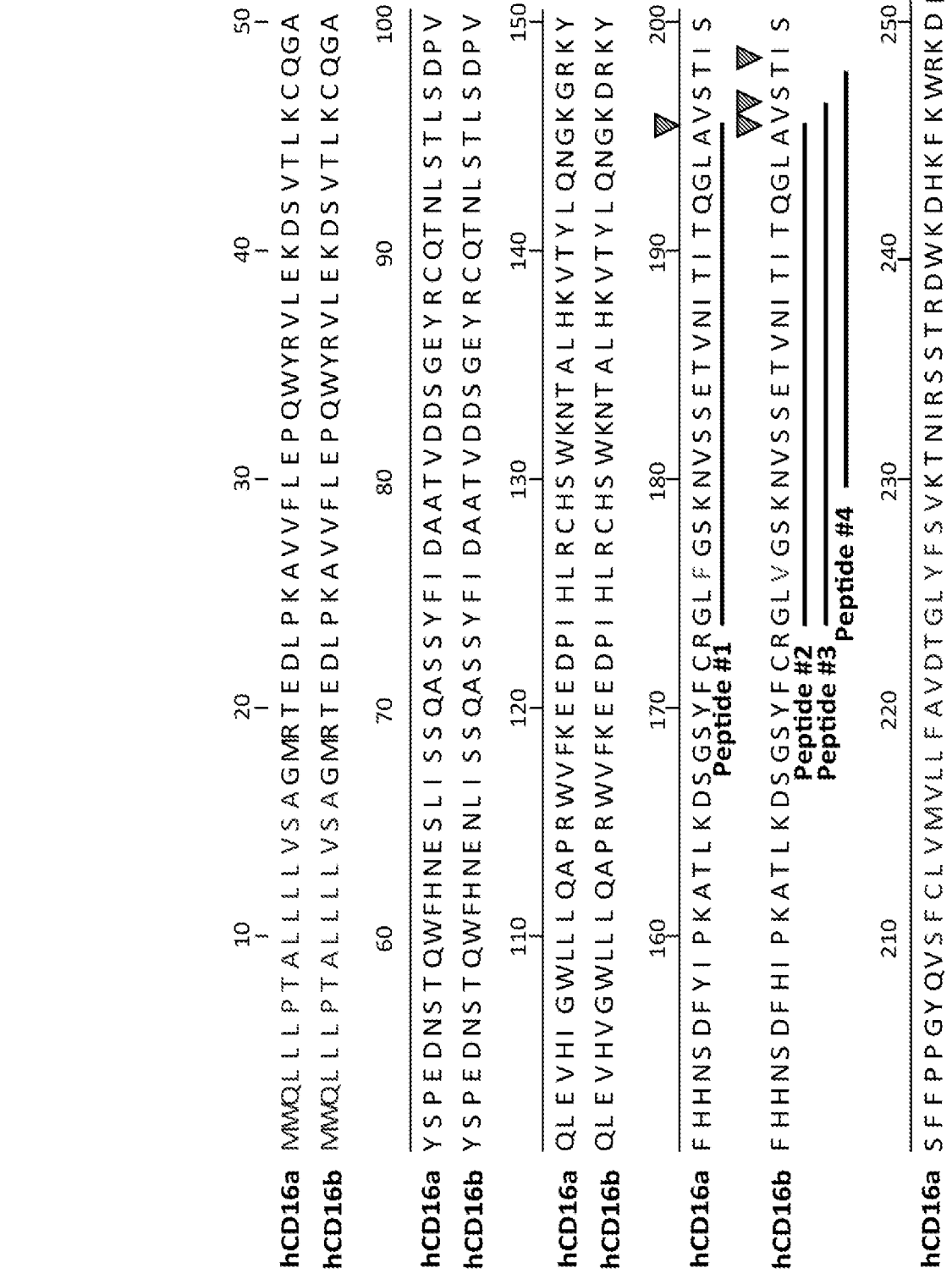

B
hCD16a MWQLLLPTALLLLVSAGMRTEDLPKAVVFLEPQWYRVLEKDSVTLKCQGA
hCD16b MWQLLLPTALLLLVSAGMRTEDLPKAVVFLEPQWYRVLEKDSVTLKCQGA
hCD16a YSPEDNSTQWFHNESLISSQASSYFIDAATVDDSGEYRCQTNLSTLSDPV
hCD16b YSPEDNSTQWFHNENLISSQASSYFIDAATVDDSGEYRCQTNLSTLSDPV
hCD16a QLEVHIGWLLLQAPRWVFKEEDPIHLRCHSWKNTALHKVTYLQNGKGRKY
hCD16b QLEVHVGWLLLQAPRWVFKEEDPIHLRCHSWKNTALHKVTYLQNGKDRKY
hCD16a FHHNSDFYIPKATLKDSGSYFCRGLFGSKNVSSETVNITITQGLAVSTISS
hCD16b FHHNSDFHIPKATLKDSGSYFCRGLVGSKNVSSETVNITITQGLAVSTISS
Peptide #1
Peptide #2
Peptide #3
Peptide #4
hCD16a SFFPPGYQVSFCLVMVLLFAVDTGLYFSVKTNIRSSTRDWKDHKFKWRKDPQDK
hCD16b SFS - GPI attaching site

FIG. 1

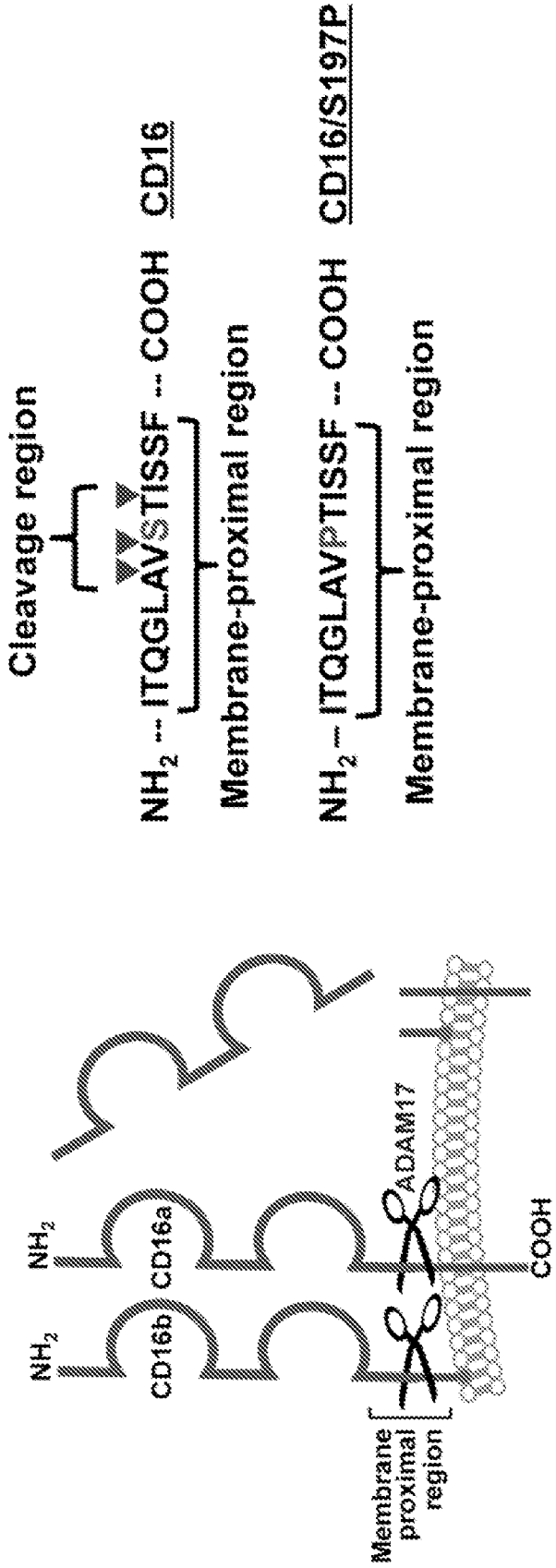

Cleavage region
NH2 -- ITQGLAVSTISSF -- COOH CD16
Membrane-proximal region
NH2 -- ITQGLAVPTISSF -- COOH CD16/S197P
Membrane-proximal region
NH2
CD16b
NH2
CD16a
ADAM17
COOH
Membrane proximal region

FIG. 2

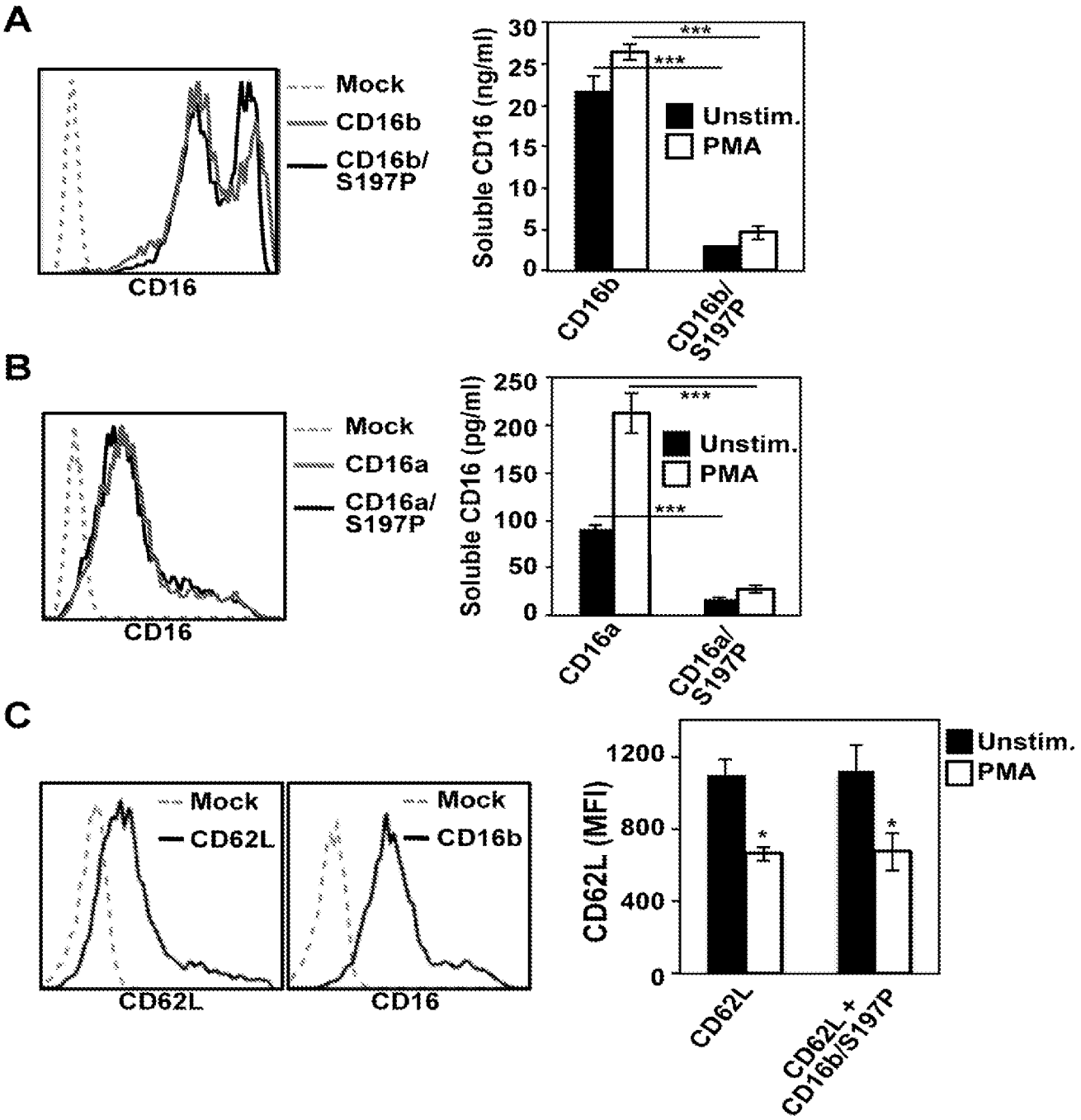

A
Mock
CD16b
CD16b/ S197P
CD16
Soluble CD16 (ng/ml)
30
25
20
15
10
5
0
***
***
Unstim.
PMA
CD16b
CD16b/ S197P
B
Mock
CD16a
CD16a/ S197P
CD16
Soluble CD16 (pg/ml)
250
200
150
100
50
0
***
***
Unstim.
PMA
CD16a
CD16a/ S197P
C
Mock
CD62L
CD62L
Mock
CD16b
CD16
CD62L (MFI)
1200
800
400
0
*
*
Unstim.
PMA
CD62L
CD62L + CD16b/S197P

FIG. 3

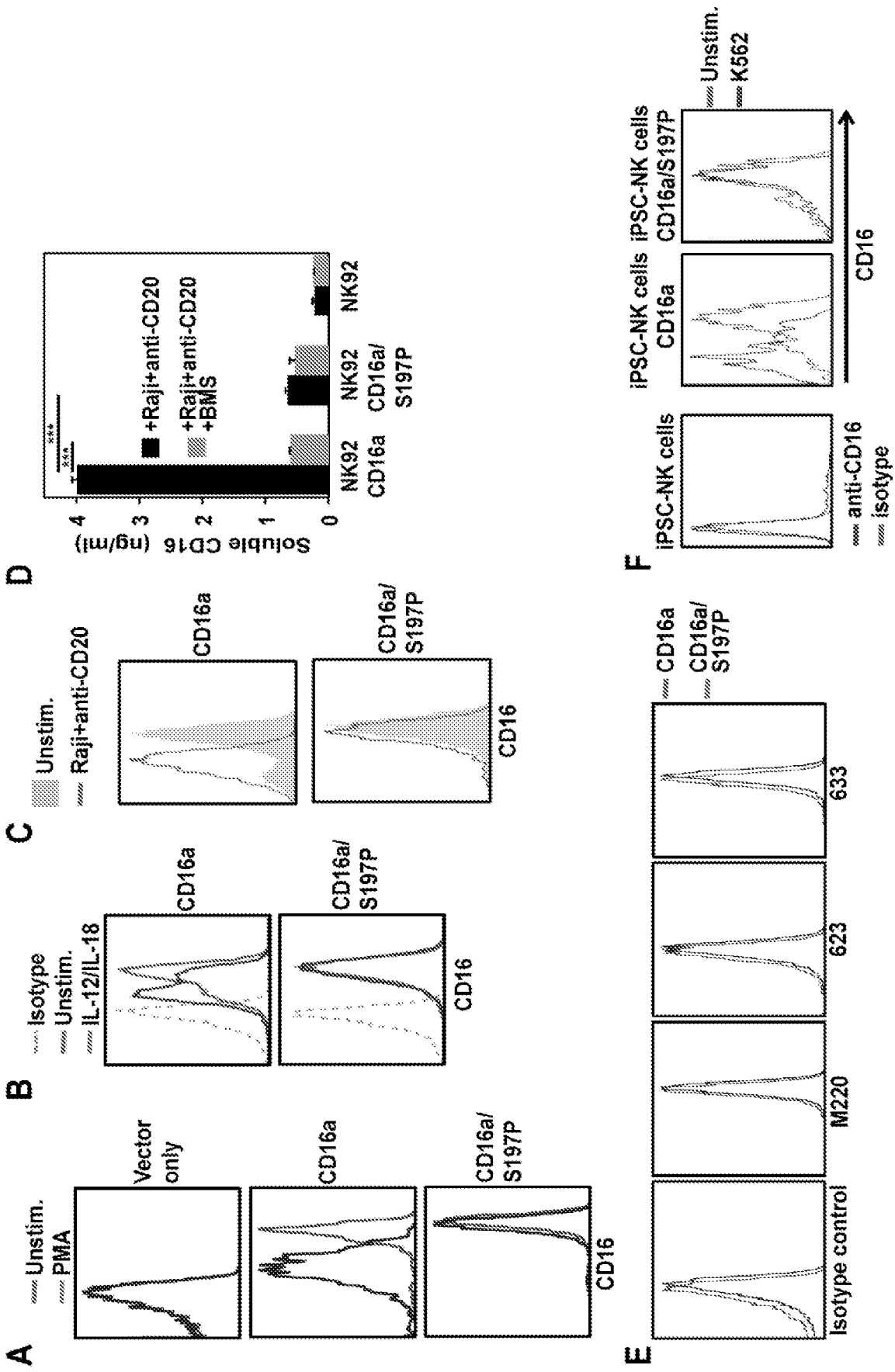

A
Unstim.
PMA
Vector only
CD16a
CD16a/ S197P
CD16
B
Isotype
Unstim.
IL-12/IL-18
CD16a
CD16a/ S197P
CD16
C
Unstim.
Raji+anti-CD20
CD16a
CD16a/ S197P
CD16
D
Soluble CD16 (ng/ml)
0
1
2
3
4
***
***
+Raji+anti-CD20
+Raji+anti-CD20 +BMS
NK92 CD16a
NK92 CD16a/ S197P
NK92
E
CD16a
CD16a/ S197P
633
623
M220
Isotype control
F
iPSC-NK cells
anti-CD16
isotype
iPSC-NK cells CD16a
iPSC-NK cells CD16a/S197P
Unstim.
K562
CD16

FIG. 4

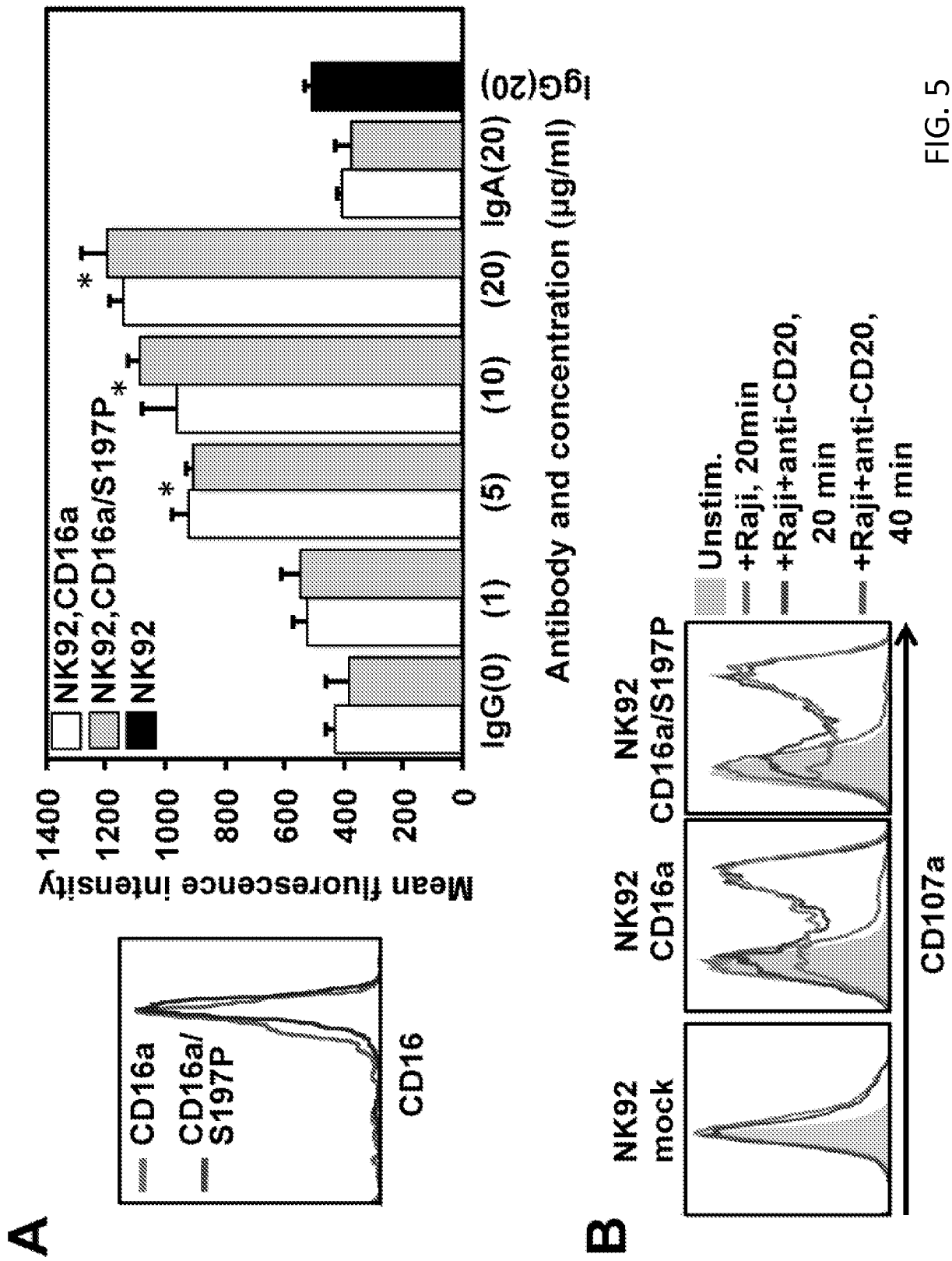

A
CD16a
CD16a/
S197P
CD16
Mean fluorescence intensity
1400
1200
1000
800
600
400
200
0
NK92,CD16a
NK92,CD16a/S197P
NK92
*
*
*
IgG(0) (1) (5) (10) (20) IgA(20) IgG(20)
Antibody and concentration (μg/ml)
B
NK92
mock
NK92
CD16a
NK92
CD16a/S197P
Unstim.
+Raji, 20min
+Raji+anti-CD20,
20 min
+Raji+anti-CD20,
40 min
CD107a

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/022998

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/735
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/196870 A1 (LEDBETTER JEFFREY A [US] ET AL) 6 August 2009 (2009-08-06)	1,2
Y	whole document esp. paragraphs [337,338,381] and seq id no 426	3-20
Y	WO 2010/040091 A1 (UNIV ARIZONA [US]; CHANG YUNG [US]; YAN HAO [US]) 8 April 2010 (2010-04-08) whole document esp. [5,165], seq id no 6, claims 43,50	3-20
X	EP 1 734 119 A2 (APPLIED RESEARCH SYSTEMS [AN] MERCK SERONO SA [CH]) 20 December 2006 (2006-12-20)	1,2
Y	the whole document esp. paragraphs [17,21]	3-20

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 June 2015	02/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brück, Marianne

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/022998

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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(19)中华人民共和国国家知识产权局

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61/971996 2014.03.28 US

(85)PCT国际申请进入国家阶段日
2016.11.28

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(87)PCT国际申请的公布数据
WO2015/148926 EN 2015.10.01

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代理人 罗文锋 鲁炜

(51)Int.Cl.
C07K 14/735(2006.01)
C12N 5/10(2006.01)
A61K 35/17(2015.01)
A61P 35/00(2006.01)

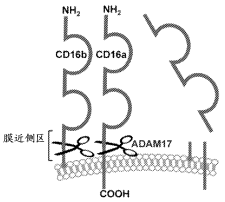
权利要求书1页 说明书13页
序列表4页 附图6页

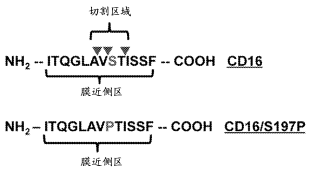
(54)发明名称

涉及经工程改造的CD16的多肽、细胞和方法

(57)摘要

本公开内容一般地描述修饰形式的CD16，表达修饰的CD16的遗传修饰的细胞，和涉及遗传修饰的细胞的方法。所述修饰形式的CD16可显示增加的抗肿瘤和/或抗病毒活性，这至少部分上是由于减少的对NK细胞刺激时ADAM17介导的脱落的易感性。





CN 106715467 A

1.一种经遗传修饰以表达包含膜近侧区和所述膜近侧区中的氨基酸修饰的CD16多肽的细胞。

2.一种细胞,包含:

编码包含膜近侧区和所述膜近侧区中的氨基酸修饰的CD16多肽的多核苷酸。

3.权利要求1或权利要求2的细胞,其中所述氨基酸修饰(medication)反映与CD16膜近侧区野生型氨基酸序列相比一个或多个氨基酸的添加、一个或多个氨基酸的缺失,或一个或多个氨基酸的置换。

4. 权利要求3的细胞,其中所述一个或多个氨基酸的置换包括SEQ ID NO:1的197位的丝氨酸残基的置换。

5.权利要求1或权利要求2的细胞,其中所述细胞为自然杀伤(NK)细胞。

6.权利要求1或权利要求2的细胞,其中所述细胞为中性粒细胞。

7.权利要求1或权利要求2的细胞,其中所述细胞为单核细胞。

8.权利要求1或权利要求2的细胞,其中与野生型CD16多肽相比所述修饰的CD16多肽显示减小的对ADAM17介导的脱落的敏感性。

9.权利要求1或权利要求2的细胞,其中与野生型CD16多肽相比所述修饰的CD16多肽显示减小的对NK细胞刺激时的切割的敏感性。

10.一种方法,包括:

给予需要这种处理的患者包括以下的治疗:

给予所述患者治疗性NK效应物;和

给予所述患者权利要求1或权利要求2的细胞。

11.权利要求10的方法,其中所述治疗性NK效应物包括治疗剂。

12.权利要求11的方法,其中所述治疗剂特异性识别肿瘤抗原。

13.权利要求12的方法,其中所述治疗剂包括特异性识别所述肿瘤抗原的抗体或抗体片段。

14.权利要求13的方法,其中所述肿瘤抗原包括HER2。

15.权利要求13或权利要求14的方法,其中所述抗体包括曲妥单抗(trastuzumab)或利妥昔单抗(rituximab)。

16. 权利要求10的方法,其中所述治疗性NK效应物包括双特异性杀伤衔接体(bi-specific killer engager, BiKE)。

17. 权利要求16的方法,其中所述BiKE包括CD16×CD33 BiKE、CD16×CD19 BiKE或CD16×EP-CAM BiKE。

18. 权利要求10的方法,其中所述治疗性NK效应物包括三特异性杀伤细胞衔接体(tri-specific killer cell engager, TriKE)。

19.权利要求11的方法,其中所述治疗剂特异性识别病毒靶。

20.用于改善对患者的治疗的方法,所述治疗包括给予所述患者治疗性NK效应物,所述方法包括:

给予所述患者权利要求1或权利要求2的所述细胞。

涉及经工程改造的CD16的多肽、细胞和方法

[0001] 与相关申请的交叉引用

本申请要求于2014年3月28日提交的美国临时专利申请序列号61/971,996的优先权，所述申请通过引用结合到本文中。

[0002] 发明概述

本公开内容一般地描述修饰形式的CD16，表达修饰的CD16的遗传修饰的细胞，和涉及遗传修饰的细胞的方法。所述修饰形式的CD16可显示增加的抗肿瘤和/或抗病毒活性，这至少部分上是由于减少的对NK细胞刺激时金属蛋白酶介导的脱落的敏感性。

[0003] 因此，在一个方面，本公开内容描述经遗传修饰以表达具有膜近侧区和膜近侧区中的氨基酸修饰的CD16多肽的细胞。

[0004] 在另一个方面，本公开内容描述包含编码具有膜近侧区和膜近侧区中的氨基酸修饰的CD16多肽的多核苷酸的细胞。

[0005] 在任何一个方面，所述氨基酸药物(medication)反映与CD16膜近侧区野生型氨基酸序列相比一个或多个氨基酸的添加、一个或多个氨基酸的缺失，或一个或多个氨基酸的置换。在这些实施方案的一些中，一个或多个氨基酸的置换包括SEQ ID NO:1的197位的丝氨酸残疾的置换。

[0006] 在任何一个方面，细胞可为自然杀伤(NK)细胞、中性粒细胞、单核细胞或T细胞。

[0007] 在任何一个方面，与野生型CD16多肽相比修饰的CD16多肽显示减小的对ADAM17介导的脱落的敏感性。

[0008] 在任何一个方面，与野生型CD1多肽相比修饰的CD16多肽显示减小的对NK细胞刺激时的切割的敏感性。

[0009] 在另一个方面，本公开内容描述一般地涉及给予需要这种处理的患者包括以下的治疗的方法：(a) 给予患者治疗性NK效应物，和(b) 给予患者上文概述的遗传修饰细胞的任何实施方案。

[0010] 在一些实施方案中，治疗性NK效应物包括治疗剂。在这些实施方案的一些中，治疗剂可包括抗体，或治疗性抗体片段。在这些实施方案的一些中，抗体或抗体片段与病毒抗原特异性结合。在其它实施方案中，抗体或抗体片段与肿瘤抗原特异性结合。

[0011] 在一些实施方案中，治疗剂可包括双特异性杀伤衔接体(BiKE)或三特异性杀伤细胞衔接体(TriKE)。

[0012] 在又一个方面，本公开内容描述用于改善对患者的免疫治疗的方法，其中所述免疫治疗涉及给予患者治疗性NK效应物。一般地所述方法包括进一步给予患者上文概述的遗传修饰细胞的任何实施方案。

[0013] 本发明的以上概述不意在描述本发明的每一公开的实施方案或每一实现。随后的说明书更具体地例示说明性的实施方案。在整个申请的若干位置，指导通过实施例的列表提供，这些实施例可以以不同的组合使用。在每种情况下，叙述的列表仅用作代表性群组，并且不应解释为排他的列表。

[0014] 附图简述

图1. 人CD16中胞外域切割位点的位置。(A) 从PMA-活化人NK细胞或中性粒细胞的细胞上清液免疫沉淀的可溶性CD16的胰蛋白酶肽(tryptic peptides)经受质谱分析。鉴定了四个具有非胰蛋白酶C末端的高置信度肽:1个肽来自NK细胞释放的可溶性CD16 (肽#1,左上),3个肽来自中性粒细胞释放的可溶性CD16 (肽#2,左下;肽#3,右上;和肽#4,右下)。(B)肽#1-4 (加下划线的)以及CD16a (SEQ ID NO:1)和CD16b (SEQ ID NO:2)中假定切割位点(箭头)的说明。所鉴定的肽中氨基酸176区分CD16a (F)与CD16b (V)。氨基酸1-16指示CD16a和CD16b的预测信号序列。氨基酸210-229指示CD16a的跨膜区域。氨基酸编号从信号序列中的甲硫氨酸开始。CD16a和CD16b的氨基酸序列分别来自NCBI参考序列NM_000569.6和NM_000570.4。

[0015] 图2. CD16胞外域脱落、切割区域和经工程改造的丝氨酸-197至脯氨酸突变的示意图。CD16a和CD16b通过ADAM17在膜近侧区内经受胞外域脱落,如所标明的。膜近侧区内的CD16切割区域基于质谱分析,其显示极为贴近的三个不同的切割位点(箭头)。进行定点突变以将CD16 (SEQ ID NO:1的氨基酸190-202)中的丝氨酸-197置换为脯氨酸(CD16/S197P)。

[0016] 图3. 经工程改造的S197P突变对CD16a和CD16b脱落的作用。转染的HEK293 (人胚胎肾)细胞以相似水平分别表达CD16b和CD16b/S197P (A)或CD16a和CD16a/S197P (B),如通过流式细胞术所测定的(左侧面板)。用或不用PMA处理(15 ng/ml在37℃持续30分钟)不同转染子并通过ELISA定量培养基上清液中的CD16的可溶性水平(右侧面板)。对于每一实验,每一处理条件重复三次,数据代表三个独立实验。条形图显示平均值± SD。统计显著性表示为***P<0.001。(C) 转染的HEK293细胞表达L-选择素(CD62L)或L-选择素和CD16b/S197P。转染和模拟转染细胞上L-选择素和CD16b/S197P的表面水平使用流式细胞术测量(直方图)。在PMA存在或不存在下37℃孵育表达L-选择素或L选择素和CD16b/S197P的转染子30分钟,并测定L-选择素染色的平均荧光强度(MFI) (条形图)。对于每一实验,每一处理条件重复三次,数据代表两个独立实验。条形图显示平均值± SD。统计显著性表示为*P<0.05。对于所有直方图,x-轴 = Log 10荧光,y-轴 = 细胞数目。

[0017] 图4. 经工程改造的S197P突变对NK细胞中CD16a脱落的作用。不用(Unstim.)或用PMA (100 ng/ml) 37℃处理用空载体(仅载体)、CD16a或CD16a/S197P转导的NK92细胞30分钟(A),用IL-12和IL-18 (分别100 ng/ml和400 ng/ml) 37℃处理24小时(B),或用Raji细胞和利妥昔单抗37℃处理60分钟(C)。CD16a的细胞表面水平通过流式细胞术测定。虚线指示同种型匹配的阴性对照抗体染色。(D) 在ADAM17抑制剂BMS566394 (5 μM)存在或不存在下用Raji细胞和利妥昔单抗37℃处理亲本NK92细胞和表达CD16a或CD16a/S197P的转导细胞60分钟。可溶性CD16a水平通过ELISA测定。每一处理条件重复三次,数据代表三个独立实验。条形图显示平均值 ± SD。统计显著性表示为***P<0.001。(E) 表达CD16a或CD16a/S197P的NK92细胞用抗-ADAM17 mAbs M220、623、633或同种型匹配的阴性对照抗体染色,如所标明的。(F) 从模拟转导iPSCs (左侧面板)或表达重组CD16a或CD16a/S197P的iPSCs(右侧面板)衍生的$CD56^{+}CD45^{+}$ NK细胞与或不与K562靶细胞在37℃孵育4小时。对于所有直方图,x-轴 = Log 10荧光,y-轴 = 细胞数目,并且数据代表至少3个独立实验。

[0018] 图5. 经工程改造的S197P突变对CD16a功能的作用。(A)以相等水平表达CD16a或CD16a/S197P的NK92细胞用单体人IgG (0-20 μg/ml)处理(左侧面板)。作为对照,细胞也用

单体人IgA (20 μg/ml)处理并用IgG (20 μg/ml)处理NK92亲本细胞(条)。抗体结合通过流式细胞术测定,如材料与方法中所描述的。条形图显示至少三个单独实验的平均值 ± SD。相对于IgG (0 μg/ml)、IgA或NK92亲本细胞 + IgG,统计显著性表示为*P<0.05。(B) 在Raji细胞不存在(Unstim.)或存在下用或不用抗CD20 利妥昔单抗37℃孵育模拟转导的NK92细胞或表达CD16a或CD16a/S197P的NK92细胞达到指定的时间点。NK92细胞活化通过流式细胞术由CD107a染色的上调评估。对于直方图,x-轴 = Log 10荧光,y-轴 = 细胞数目。数据代表至少3个独立实验。

[0019] 说明性实施方案的详述

本公开内容一般地描述修饰形式的CD16a,表达修饰的CD16a的遗传修饰的细胞,和涉及遗传修饰的细胞的方法。所述修饰形式的CD16a可显示增加的抗肿瘤和/或抗病毒活性,这至少部分上是由于减少的对NK细胞刺激时金属蛋白酶介导的脱落的敏感性。

[0020] 与许多实体肿瘤类型相比,在过去的30年间具有上皮性卵巢癌的妇女的存活率几乎未改变。此外,当前对复发性卵巢癌的标准治疗提供低(<20%)响应率。尽管卵巢癌样品中普遍的HER2过表达,在晚期卵巢癌患者中用抗-HER2抗体曲妥单抗处理仅提供有限的响应。这一对曲妥单抗的抗性可能由功能失调的NK细胞介导的抗体-依赖性细胞毒性引起。因此,存在对创新治疗策略的迫切需求。我们描述了一种用于提供治疗处理策略的新途径。

[0021] 卵巢癌的一个关注为周围环境,其中肿瘤细胞发展可为高度促炎的因此很可能促进浸润NK细胞上CD16a切割从而减少抗体-依赖性细胞毒性。数种抗体已经作为有效的用于治疗人恶性肿瘤的靶向治疗而出现。其功效部分上是由于与自然杀伤(NK)细胞上Fcγ RIIIa/CD16a的抗体相互作用和通过抗体-依赖性细胞毒性诱导杀死癌细胞。人IgG Fc受体CD16 (FcγRIII)由两种同种型组成:CD16a (FcγRIIIa)和CD16b (FcγRIIIb)。CD16a由自然杀伤(NK)细胞表达,CD16b由中性粒细胞表达。NK细胞活化通过称为胞外域脱落(涉及金属蛋白酶ADAM17和发生在接近质膜的单一胞外区域的蛋白水解事件)的过程导致两种CD16同种型的表面水平的快速下调(图1A)。

[0022] 如上文所指出的,卵巢癌患者可能抵抗NK细胞介导的免疫治疗——即,肿瘤对NK细胞介导的治疗不敏感。例如,卵巢癌细胞通常表达表皮生长因子受体HER2,但用治疗抗体曲妥单抗靶向其仅提供有限的临床响应。该抗性至少部分上可由胞外域脱落造成——即,细胞因子对NK细胞的活化、靶细胞相互作用和/或肿瘤浸润可导致CD16切割和损伤抗体-依赖性细胞毒性。因此,阻断胞外域脱落过程具有临床意义。

[0023] 我们已经使用质谱法测定CD16a和CD16b的切割位点并从人血白细胞克隆CD16a和CD16b的cDNA。以定向方式突变每一cDNA以诱导单个氨基酸改变。197位的丝氨酸变为脯氨酸(图1B)。该突变阻断CD16a和CD16b的切割,防止细胞活化时其下调。体外扩增的NK细胞中抗切割CD16a的表达维持该IgG Fc受体的高表面水平,这增强NK细胞刺激、治疗抗体功效和癌细胞杀伤。

[0024] ADAM17具有许多细胞表面底物,但不具有可用于预测CD16a切割位点的蛋白质水解共有序列。因此,我们使用LC-MS-MS测定从活化的人外周血白细胞释放的可溶性CD16中的C-端切割位点。我们在CD16的膜近侧区(CD16a与CD16b之间相同的区域)观察到极为接近的三个假定切割位点(图2,箭头)。尽管ADAM17蛋白质水解不需要共有序列,切割区域的二级结构很重要。试图阻断CD16a切割,我们用脯氨酸置换丝氨酸-197 ($CD16a^{197P}$)以引入构

象变化。

[0025] 我们通过从活化NK细胞的培养基上清液，并分开地从中性粒细胞的培养基上清液免疫沉淀CD16而鉴定CD16切割的位置。用PNGaseF处理免疫沉淀的CD16以移除N-聚糖，胰蛋白酶消化，对所产生的肽进行质谱分析。鉴定到包含非胰蛋白酶C端的高置信度的四个不同肽谱(peptide patterns)(图1A)。

[0026] 对于从活化NK细胞的培养基上清液富集的CD16，我们仅观察到一个肽谱，其对应于SEQ ID NO:1的氨基酸甘氨酸-174至丙氨酸-195 (肽#1, 图1A)。CD16a和CD16b的膜近侧区，除了残基176之外，具有相同的氨基酸序列。该位置处苯丙氨酸表示CD16a，其存在于肽#1中(图1A和B)。该肽在丙氨酸-195/缬氨酸-196处显示非胰蛋白酶P1/P1’切割位点(图1B)。

[0027] 对于从活化的中性粒细胞的培养基上清富集的CD16，我们检测到具有非胰蛋白酶C端的三个不同肽谱(肽#2-4，图1A和1B)。肽#2对应于SEQ ID NO:2的氨基酸甘氨酸-174至丙氨酸-195，肽#3对应于SEQ ID NO:2的氨基酸甘氨酸-174至缬氨酸-196，肽#4对应于SEQ ID NO:2的氨基酸天冬酰胺-180至苏氨酸-198。肽#2和肽#3在176位包含缬氨酸，表示CD16b，并在丙氨酸-195/缬氨酸-196和缬氨酸-196/丝氨酸-197处显示P1/P1’位点(图1B)。肽#4在苏氨酸-198/异亮氨酸-199处具有P1/P1’位点(图1B)。尽管该肽从来自富集的中性粒细胞的可溶性CD16衍生，但其不包含176位的氨基酸来鉴定同种型(图1B)。无论如何，高置信度肽显示在CD16中的第三个切割位点。总之，这些发现证明CD16中存在切割区域而不是单个特异性切割位点。

[0028] 我们通过使用定点突变进一步检查CD16的切割区域以确定在基于细胞的测定中是否可破坏CD16a和CD16b切割。ADAM17倾向于优选在底物区域中与其催化位点相互作用的α-螺旋构象。此外，ADAM17切割位点特异性的蛋白质组学研究显示对在P1’、P2’或P3’位置处的脯氨酸残基非常低的偏好性。因此我们用脯氨酸置换CD16a和CD16b切割区域中的丝氨酸-197 (S197P，如图2中所标明的)。

[0029] 在人肾细胞系HEK293中分别表达CD16b和CD16b/S197P，该细胞系不表达内源性CD16。HEK293转染子在其表面以相似水平表达CD16b或CD16b/S197P (图3A)。从转染HEK293释放高水平的CD16b，当用PMA处理它们时所述水平进一步增加，如通过ELISA所测定的(图3A)。然而，未处理或PMA处理的HEK293细胞所产生的CD16b/S197P的可溶性水平与CD16b相比显著更低(图3A)。

[0030] 我们还使用相同方法检查了S197P突变对CD16a切割的作用。CD16a的表面表达需要与γ链二聚体联合。我们因此使用稳定表达人γ链的HEK293细胞。比较表达相等表面水平CD16a或CD16a/S197P的HEK293转染子(图3B)，我们测定未处理和PMA处理细胞的培养基上清液中每一受体的可溶性水平。再次，当与CD16a相比时观察到显著更低水平的可溶性CD16a/S197P (图3B)。

[0031] 为了评估CD16中经工程改造的S197P突变是否可破坏ADAM17活性，我们还用L-选择素，一个白细胞正常表达的很好地描述的ADAM17底物，转染表达或缺乏CD16b/S197P的HEK293细胞。两种转染子表达相等水平的L-选择素，所述L-选择素水平在用PMA活化后相似地下调(图3C)，证明S197P突变影响CD16脱落但不影响ADAM17活性。

[0032] 为了评估S197P突变对NK细胞中CD16a脱落的作用，我们使用人NK细胞系NK92(Gong等人，1994, *Leukemia* 8:652-658)。这些细胞缺乏内源性CD16a的表达，但可稳定表

达重组CD16a。我们转导NK92细胞以分别表达CD16a和CD16a/S197P。用PMA活化表达相等水平这些受体的细胞并通过流式细胞术检查细胞表面CD16水平。CD16a，而非CD16a/S197P，经历细胞表面表达的显著下调(图4A)。IL-12和IL-18为NK细胞的生理刺激物，其单独或组合可诱导CD16a脱落。用IL-12和IL-18处理的NK92细胞显示CD16a而非CD16a/S197P在其细胞表面表达中相当可观的下调(图4B)。通过CD16a的结合IgG的细胞的直接参与(engagement)也可诱导其脱落，此处我们通过在抗-CD20 mAb利妥昔单抗存在或不存在下将表达CD16a或CD16a/S197P的NK92细胞与CD20阳性Burkitt's淋巴瘤细胞系Raji孵育来对其进行检查。用利妥昔单抗处理的Raji细胞诱导CD16a而非CD16a/S197P的下调(图4C)。

[0033] BMS566394为高选择性的ADAM17抑制剂，其具有对于ADAM17比对于其它金属蛋白酶更高的效能数量级。BMS566394以与S197P突变相似的效率阻断CD16a脱落，但对活化的表达CD16a/S197P的NK92细胞没有额外的阻断作用(图4D)。这些发现提供ADAM17是在其切割区域内切割CD16a的主要脱落酶的进一步证据。然而，有可能ADAM17表达水平在表达CD16a或CD16a/S197P的NK92细胞中不相等，导致其不同的脱落。我们因此用多种抗-ADAM17 mAbs染色表达CD16a或CD16a/S197P的NK92细胞并观察到相同的细胞表面水平(图4E)。

[0034] 为了确定S197P突变对原代NK细胞的CD16a脱落的作用，我们使用人iPSCs产生经工程改造的NK细胞。我们先前已经报道从iPSCs衍生功能NK细胞及其与外周血NK细胞的相似性(Knorr等人，2013 *Stem Cells Transl Med*. 2:274-283; Ni等人，2014, *Stem Cells* 32:1021-1031)。将CD16a和CD16a/S197P cDNA克隆入*Sleeping Beauty*转座子质粒用于基因插入和在iPSC细胞中稳定表达，所述iPSC细胞随后分化为成熟NK细胞。从模拟转导的iPSC细胞衍生的NK细胞表达低水平的内源性CD16a，而转导的CD16a和CD16a/S197P以较高的水平表达(图4F)。当与K562细胞相互作用时NK细胞活化通过多种受体(包括BY55/CD160)发生，导致ADAM17活化和CD16a脱落。我们用K562细胞刺激iPSC衍生的NK细胞，发现CD16a经历显著的细胞表面表达下调，而CD16a/S197P的表达保持稳定(图4F)。

[0035] 内源和重组CD16a具有足够的亲和力以结合单体IgG。为了检查S197P突变对CD16a功能的作用，我们比较CD16a和CD16a/S197P的IgG结合能力。以相等水平表达CD16a或CD16a/S197P的NK92细胞以相似的剂量依赖方式结合IgG (图5A)。对照由结合到表达CD16a或CD16a/S197P的NK92细胞的IgA和结合到NK92亲本细胞的IgG组成。二者均以基本的背景水平发生(图5A)。这些发现证明通过CD16a和CD16a/S197P的特异和相等的IgG结合。

[0036] CD16a是NK细胞上有效的活化受体，我们检查了当抗体处理的肿瘤细胞参与时经工程改造的S197P突变是否影响CD16a诱导细胞活化的能力。NK92细胞活化通过测量CD107a的上调评估，其在脱粒时非常迅速地发生并且是NK细胞活化的灵敏标志。与用或不用利妥昔单抗处理的Raji细胞孵育的模拟转导NK92细胞显示低水平和相似上调的CD107a (图5B)。当单独与Raji细胞孵育时，以相等水平表达CD16a或CD16a/S197P的NK92细胞也少量上调CD107a，而其与用利妥昔单抗处理的Raji细胞的孵育导致CD107a的相当大上调(图5B)。总之，上述发现表明CD16a中经工程改造的S197P突变不损伤其功能。

[0037] 因此，我们证明CD16a和CD16b中经工程改造的S197P突变在涉及天然ADAM17的基于细胞的测定中有效阻断其脱落。CD16a中的S197P突变还阻断人NK细胞系NK92中的受体脱落，但其不损伤受体功能。表达相等水平CD16a或CD16a/S197P的NK92细胞在一系列抗体浓度中以相似的效率结合单体IgG。另外，当结合到Raji细胞的利妥昔单抗参与时，表达CD16a

或CD16a/S197P的NK92细胞以可比较的方式上调活化标志CD107a。

[0038] 多能干细胞允许遗传操作以产生经工程改造的NK细胞。本公开内容描述从表达野生型CD16a或CD16a/S197P的转导iSPCs产生经工程改造的NK细胞。与NK92细胞同样，CD16a在iPSCs衍生的NK细胞中经历脱落，证明当细胞活化时正常的ADAM17活性，而CD16a/S197P不脱落。

[0039] CD16a和NK细胞细胞毒性功能在癌症患者中可经历相当大的下调。编码CD16a/S197P的cDNA可用于产生稳定的人诱导多能干细胞(iSPCs)和胚胎干细胞(ESCs)。这些干细胞可然后分化为表达CD16a/S197P的原代NK细胞。表达抗切割CD16a/S197P (例如，单核细胞)或CD16b/S197P (例如，中性粒细胞)的其它细胞群也可从hESCs/iPSCs衍生。

[0040] 为了产生用在人类患者中对抗不同形式癌症或感染的NK细胞免疫治疗，CD16a/S197P-表达NK细胞可介导增加的抗体-依赖性细胞毒性(ADCC)活性或其它CD16a介导的活性(例如，IFNγ和TNFα产生)。例如，CD16a/S197P-表达NK细胞可与治疗抗体(例如，曲妥单抗或利妥昔单抗)、双特异性杀伤衔接体(BiKE，例如，CD16×CD33、CD16×CD19或CD16×EP-CAM双特异性杀伤细胞衔接体)或三特异性杀伤细胞衔接体(TriKE)组合。也可产生具有增加的CD16-介导活性的其它治疗细胞群(例如，中性粒细胞、单核细胞、T细胞，等)。

[0041] CD16a/S197P在人iPSCs或人ESCs中的表达可产生具有增强的抗瘤病况例如，HER2卵巢癌的ADCC活性的NK细胞群。在一些情况下，瘤病况可用治疗抗体例如，曲妥单抗处理。成熟NK细胞可从人胚胎干细胞和iPSCs衍生。

[0042] 可克隆野生型CD16a和/或CD16a/S197P以产生表达单独的CD16a受体的稳定iPSC细胞系或稳定ESC细胞系。可使用任何合适的克隆方法。示例性克隆方法包括，例如，基于病毒的方法、转座子载体(例如，*Sleeping Beauty*)或核转染。在一个实例中，iPSCs可使用*Sleeping Beauty*转座子载体来修饰。载体可包含选择系统，例如，GFP/博来霉素(zeocin)抗性融合蛋白，其允许双选择系统(博来霉素抗性和流式细胞术分选)。iPSCs可分化为成熟NK细胞，如先前所描述的(Ni等人，2011，*J. Virol*. 85:43-50; Knorr等人，2013，*Stem Cells Transl Med* 2:274-283; Woll等人，2009，*Blood* 113:6094-6101)。iPSCs中转基因受体的表达可导致衍生NK细胞中高水平表达。未分化iSPCs中CD16表达可破坏NK细胞分化。在这种情况下，CD16表达可使用，例如，CD56或天然CD16a启动子推延，以致CD16a表达更好地与正常NK细胞分化一致。

[0043] 可比较表达相等水平野生型CD16a与CD16a/S197P的NK细胞。CD16构建体的表达水平可通过FACS分选基于GFP表达来匹配，所述GFP表达以与CD16a构建体成比例的方式发生。匹配的CD16a水平可通过FACS验证。NK细胞抗HER2-表达卵巢癌细胞的细胞毒性可在治疗抗体，例如，曲妥单抗存在或不存在下通过标准铬释放测定评估。可评估用非铬标记卵巢癌细胞的抗体-依赖性细胞毒性。可通过ELISA评估NK细胞的细胞因子(例如，IFNγ、TNFα)产生和CD16a的可溶性水平，通过FACS评估CD16a和其它活化标志(例如，CD107a、CD62L)的细胞表面水平。

[0044] 实施例3中描述的人肿瘤异种移植模型可用于体内评估表达不可切割CD16a的NK细胞的抗癌活性。与人CD16不同，当细胞刺激时小鼠CD16不经历胞外域脱落，因此确定CD16a脱落对NK细胞介导的ADCC的作用不能在正常小鼠中建立模型。表1提供实验群组和处理的代表性集合。

[0045] 表1. 肿瘤异种移植模型

群组	n	处理#
1	5	无处理
2	5	仅OVCAR3细胞
3	5	OVCAR3 + NK细胞/WT-CD16a
4	5	OVCAR3 + NK细胞/ WT-CD16a + 曲妥单抗
5	5	OVCAR3 + NK细胞/$CD16a^{197P}$
6	5	OVCAR3 + NK细胞/ $CD16a^{197P}$ + 曲妥单抗
7	5	OVCAR3 + NK细胞/仅载体
8	5	OVCAR3 + NK细胞/载体 + 曲妥单抗

#处理至少进行两次并汇总数据。

[0046] 肿瘤生长和/或消退可通过常规方法每周监测,包括,例如,生物发光成像、超声、CT、MRI、另一种成像技术和/或称重小鼠(Woll等人, 2009, *Blood* 113:6094-6101)。也可对小鼠采血(例如,每周)以定量人NK细胞存活。各种效应物功能标志(例如,IFNγ、CD16a)的表达和/或细胞表面水平可使用常规技术例如,通过FACS评估。小鼠可在任何适当时期,例如,60天期间随访。处死时,可检查内脏(例如,脾、肝、肺、肾和/或卵巢)的转移证据(例如,通过生物发光),如先前所描述的(Woll等人, 2009, *Blood* 113:6094-6101)。

[0047] 我们的分析允许定义和比较表达野生型CD16a与CD16a/S197P的iPSC-衍生NK细胞的抗体-依赖性细胞毒性活性和体内效能。因此,我们在本文中描述修饰形式的CD16a、表达修饰的CD16a的遗传修饰的细胞(例如,NK细胞、中性粒细胞、单核细胞、T细胞等)和涉及遗传修饰的细胞的方法。例如,表达修饰形式的CD16a,CD16a/S197P的NK细胞显示增加的抗卵巢癌活性,这至少部分上是由于减少的对NK细胞刺激时ADAM17-介导的脱落的敏感性。这继而,在接合(engage)抗体-标记的癌细胞(例如用治疗抗体标记的癌细胞)时,增加抗体-依赖性细胞毒性活性。此外,通过NK细胞的抗体识别增加与肿瘤细胞的接触稳定性并通过其它活化受体(例如NKG2D)支持NK细胞活性。

[0048] 术语“和/或”意指一个或所有所列元素或两个或更多个所列元素的组合;术语“包括”及其变体在说明书和权利要求中这些术语出现处不具有限制性含义;除非另外说明,“一个”、“一种”、“所述”和“至少一个”可交换地使用,意指一个或一个以上;通过端点叙述的数字范围包括该范围内包含的所有数字(例如,1-5包括1、1.5、2、2.75、3、3.80、4、5,等)。

[0049] 在前述说明中,为了清楚起见可能分开描述具体的实施方案。除非另外清楚指明一个具体实施方案的特征与另一个实施方案的特征不相容,否则某些实施方案可包括与一个或多个实施方案有关的本文所描述的相容特征的组合。

[0050] 对于本文所公开的包括不连续步骤的任何方法,所述步骤可以以任何可行次序进行。并且,适当时,可同时进行两个或更多个步骤的任何组合。

[0051] 本发明通过下列实施例说明。应理解的是具体的实施例、材料、量和程序应根据如本文所阐述的本发明的范围和精神广义解读。

实施例

[0052] 实施例1

质谱法

从健康个体采集外周血依照尼苏达大学研究审查委员会(University of Minnesota Institutional Review Board)批准的方案根据方案# 9708M00134进行。人中性粒细胞和NK细胞分离如先前所描述的进行(Wang等人, 2013, *Biochim Biophys Acta*. 1833:680-685; Long等人, 2010, *J Leukoc Biol*. 87:1097-1101; Long等人, 2012, *J Leukoc Biol*. 92:667-672)。富集的中性粒细胞或NK细胞 (在PBS中1 × 10^7/ml; Mediatech, Inc. Manassas, VA)用PMA (分别15 ng/ml或50 ng/ml; Sigma-Aldrich, St. Louis, MO)在37℃活化30分钟。过滤细胞上清液(0.45 μm孔径)并使用mAb 3G8 (BioLegend, Inc., San Diego, CA)和Pierce直接免疫沉淀试剂盒(Thermo Fisher Scientific, Rockford, IL)按照制造商的说明免疫沉淀CD16。纯化的CD16通过靶向几丁质结合结构域的Remove-iT PNGase F (New England BioLabs, Inc., Ipswich, MA)按照制造商的说明脱糖基化。简言之,将10-20 μg纯化的CD16在40 mM DTT存在下55℃变性10分钟,然后用3 μl Remove-iT PNGase F (New England BioLabs, inc., Ipswich, MA)在37℃孵育1小时。然后使用几丁质磁珠从反应中去除Remove-iT PNGase F。

[0053] 对CD16进行SDS-PAGE并通过氪荧光蛋白染色(Thermo Fisher Scientific, Rockford, IL)检测可溶性CD16对应的凝胶带,通过CD16免疫印迹分析同一凝胶中邻近的泳道而验证,然后将其切下并用胰蛋白酶对其进行标准凝胶内消化。将从凝胶提取的消化的肽干燥并在98:2:0.01的水:乙腈:甲酸中复溶用于液相色谱-质谱法分析,通过质谱法(VELOS OPBITRAP, Thermo Fisher Scientific, Rockford, IL)以数据依赖扫描模式分析≤1 μg的等分试样,如先前所描述的(Lin-Moshier等人, 2013, *J Biol Chem*. 288:355-367)。数据库检索用Protein Pilot 4.5 (AB Sciex, Framingham, MA)针对附加污染物数据库(thegpm.org/cRAP/index,109蛋白质)的NCBI参考序列人蛋白质FASTA数据库进行,所述Protein Pilot 4.5使用Paragon评分算法(Shilov等人, 2007, *Mol Cell Proteomics* 6:1638-1655)。检索参数为:半胱氨酸碘乙酰胺;胰蛋白酶;仪器Orbi MS (1-3ppm) Orbi MS/MS;生物修饰ID焦点,其包括天冬酰胺脱酰胺;彻底的检索努力;和假发现率分析(用相反的数据库)。

[0054] *cDNA表达构建体的产生*

CD16b作为命名为NA1和NA2的两个等位基因变体存在,其差异为在其胞外区域的N端部分的四个氨基酸。ADAM17以相似的效率切割两个CD16b等位基因变体。对于本研究,我们仅检查NA1变体。CD16a也存在两个等位基因变体,它们在176位具有缬氨酸或苯丙氨酸残基。ADAM17以相似的效率切割CD16a的这两个等位基因变体。对于本研究,我们仅检查缬氨酸等位基因变体CD16a。

[0055] CD16a和CD16b从人白细胞cDNA扩增,如先前所描述的(Wang等人, 2013, *Biochim Biophys Acta*. 1833:680-685; Dong等人, 2014, *Arthritis Rheumatol*. 66:1291-1299)在*Bam*HI和*Eco*RI限制酶位点单独克隆入pcDNA3.1质粒(Invitrogen, Carlsbad, CA)。构建体然后按照制造商的说明进行Quik-Change Site-directed Mutagenesis (快速改变定点诱变) (Agilent Technologies, Santa Clara, CA)以将CD16a和CD16b中197位的丝氨酸转换为脯氨酸。所有构建体经测序证实存在预期突变并且不存在任何自发突变。

[0056] 随后将CD16a cDNA在*Bam*HI和*Eco*RI限制酶位点处克隆入Dr. G. Nolan

(Stanford University, Stanford, CA)提供的双顺反子逆转录病毒表达载体pBMN-IRES-EGFP。CD16a构建体还如先前所述(Wilber等人, 2007, *Stem Cells* 25:2919-2927; Tian等人, 2009, *Stem Cells* 27:2675-2685)克隆入双顺反子*Sleeping Beauty*转座子质粒(pKT2-IRES-GFP:zeo)中。简言之,使用引物:5'-CCG GAA TTC CAG TGT GGC ATC ATG TGG CAG CTG CTC-3' (正向, SEQ ID NO:XX)和5'-CCG GAA TTC TCA TTT GTC TTG AGG GTC CTT TCT-3' (反向, SEQ ID NO:YY) PCR扩增野生型CD16a和CD16a/S197P。有下划线的是*Eco*RI位点。将*Eco*RI-消化的CD16a和CD16a/S197P PCR片段单独克隆入pKT2-IRES-GFP:zeo中。正确的CD16a定向和序列通过PCR和测序分析确认。我们先前已经克隆了全长人L选择素(CD62L) cDNA (Feehan等人, 1996, *J Biol Chem*. 271:7019-7024; Matala等人, 2001, *J Immunol*. 167:1617-1623),将所述全长人L选择素(CD62L) cDNA在限制酶位点*Xba*I处转移至pcDNA3.1载体中。如先前所述(Dong等人, 2014, *Arthritis Rheumatol*. 66:1291-1299)克隆全长人FcRγ cDNA,并修改为使用pcDNA3.1载体。

[0057] *表达重组L-选择素、CD16a和CD16b的细胞系的产生*

HEK293细胞(人胚胎肾细胞系)和NK92细胞(人NK细胞系) (ATCC, Manassas, VA)按照贮藏所的说明培养。HEK293细胞使用Lipofectamine 2000 (Invitrogen, Carlsbad, CA)按照制造商的说明用含有或不含CD16b、CD16b/S197P和/或L-选择素的pcDNA3.1瞬时转染。稳定表达人FcRγ的HEK293细胞通过同一方法用含有或不含CD16a或CD16a/S197P的pcDNA3.1瞬时转染。NK92细胞通过先前所描述的逆转录病毒产生和感染程序(Matala等人, 2001, *J Immunol*. 167:1617-1623; Walcheck等人, 2003, *J Leukoc Biol*. 74:389-394; Wang等人, 2009, *J Immunol*. 182:2449-2457)用含有或不含CD16a或CD16a/S197P的pBMN-IRES-EGFP稳定转导。构建体表达通过EGFP荧光和CD16染色评估,如通过流式细胞术所测定的。人iPSCs (UCBiPS7, 衍生自脐带血CD34细胞)培养(maintained)在小鼠胚胎成纤维细胞上(Knorr等人, 2013, *Stem Cells Transl Med*. 2:274-283; Ni等人, 2014, *Stem Cells* 32:1021-1031)。CD16a或CD16a/S197P的稳定表达如先前所述使用*Sleeping Beauty*转座子系统来进行(Wilber等人, 2007, *Stem Cells* 25:2919-2927; Tian等人, 2009, *Stem Cells* 27:2675-2685)。简言之,用pKT2-IRES-GFP:zeo与在核转染仪(nucleofector)溶液V中的转座酶DNA的组合 (Lonza Inc., Gaithersburg, MD)使用程序设置B16核转染(nucleofect)iPSCs。核转染的细胞立即悬浮在包含博来霉素(50 μg/ml)的iPSC生长培养基中并接种到小鼠胚胎成纤维细胞上。

[0058] *从CD16a-hESC和CD16a-iPSC细胞衍生NK细胞*

hESCs和iPSCs的造血分化如先前所述(Ng等人, 2005, *Blood* 106: 1601-1603; Ng等人, 2008, *Nat Protoc* 3:768-776; Le Garff-Tavernier等人, 2010, *Aging Cell* 9: 527-535)进行。简言之,在含有干细胞因子(SCF, 40 ng/ml)、血管内皮生长因子(VEGF, 20 ng/ml)和骨形态发生蛋白4 (BMP4, 20 ng/ml)的BPEL培养基中在96孔圆底板的每孔中接种3000个单细胞。BPEL培养基包含Iscove's Modified Dulbecco's Medium (IMDM, 86 ml, Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA)、含Glutmax I的F12营养混合液(Nutrient Mixture) (86 mL, Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA)、10%去离子牛血清白蛋白(BSA, 5 ml, Sigma-Aldrich, St. Louis, MO)、5%聚乙烯醇(10 ml, Sigma-Aldrich, St. Louis, MO)、亚麻酸(20 μl 1 gm/ml溶液,

Sigma-Aldrich, St. Louis, MO)、亚油酸(20 μl 1 gm/ml溶液, Sigma)、Synthecol 500x溶液(Sigma-Aldrich, St. Louis, MO)、a-monothioglyceral (3.9 μl/100 ml, Sigma-Aldrich, St. Louis, MO)、无蛋白杂交瘤混合物II (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA)、抗坏血酸(5 mg/ml, Sigma)、Glutamax I (Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA)、胰岛素-转铁蛋白-硒100x溶液(Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA)、青霉素/链霉素(Invitrogen, Thermo Fisher Scientific, Inc., Waltham. MA)。

[0059] 在造血分化的第11天,将旋转拟胚体(spin embryoid bodies)直接转移至具有或不具EL08-1D2间质细胞的24孔板的提供有细胞因子的NK培养基中(Le Garff-Tavernier等人, 2010, *Aging Cell* 9:527-535)。培养4-5周后,用APC-、PE-、FITC-和PerCP-cy5.5-偶联的IgG或抗人血液表面抗原的特异性抗体:CD45-PE、CD56-APC、CD56-PE、CD16-PerCP-cy5.5、NKG2D-PE、NKp44-PE、NKp46-PE、CD158b-FITC、CD158e1/2-FITC (BD Pharmingen, San Jose, CA)、CD158a/h-PE和CD158i-PE (Beckman Coulter, Inc., Pasadena, CA)染色单细胞悬液。抗体染色通过流式细胞术评估。

[0060] *细胞刺激*

分别用15 ng/ml和100 ng/ml PMA在37℃活化RPMI 1640培养基(Mediatech, Inc., Manassas, VA)中的HEK293和NK92细胞30分钟。NK92细胞用IL-12 (PeproTech Inc, Rocky Hill, NJ)和IL-18 (R&D Systems, Inc., Minneapolis, MN)分别以100 ng/ml和400 ng/ml活化达到标明的时间点。通过CD16a的NK92细胞活化通过与用抗-CD20 mAb 利妥昔单抗(1 μg/ml) (Genentech, Inc., South San Francisco, CA)处理的CD20-阳性Burkitt's淋巴瘤细胞系Raji (ATCC,按照贮藏所的说明生长) (1:1比率)孵育而介导,如先前所描述的(Romee等人, 2013, *Blood* 121:3599-3608)。通过洗涤Raji细胞去除过量的利妥昔单抗。在一些实验中,NK92细胞用选择性ADAM17抑制剂BMS566394 (5 μM) (Bristol-Myers Squibb Company, Princeton, NJ)预孵育30分钟。从iPSCs衍生的NK细胞用人红白血病细胞系K562 (ATCC,按照贮藏所的说明生长)刺激,如先前所描述的 (Romee等人, 2013, *Blood* 121:3599-3608)。简言之,与K562靶细胞(2:1比例)在37℃孵育iPSC-衍生的NK细胞4小时。

[0061] *抗体结合测定*

细胞与单体人IgG和IgA (Sigma-Aldrich, St. Louis, MO)的结合如先前所述(Dong等人, 2014, *Arthritis Rheumatol*. 66:1291-1299)实施,并进行了一些修改。PBS中5 × 10^6/ml的NK92亲本细胞或表达CD16a或CD16a/S197P的转导细胞在4℃用IgG或IgA以标明的浓度一式三份孵育1小时。充分洗涤细胞并用APC-缀合的驴抗人Fc(重链和轻链)抗体(Jackson Immunoresearch, West Grove, PA)按照制造商的说明进行孵育。洗涤细胞,然后立即通过流式细胞术分析。

[0062] *流式细胞术和ELISA*

对于细胞染色,阻断非特异性抗体结合位点,细胞用标明的抗体染色并通过流式细胞术检查,如先前所描述的(Wang等人, 2013, *Biochim Biophys Acta*. 1833:680-685; Romee等人, 2013, *Blood* 121:3599-3608)。流式细胞术分析在FACSCanto和LS RII仪器(BD Biosciences, San Jose, CA)上进行。人CD16通过mAbs 3G8 (BioLegend, Inc., San

Diego, CA)和DJ130c (Santa Cruz Biotech, Santa Cruz, CA)检测。CD107a通过mAb H4A3 (Biolegend, Inc., San Diego, CA)检测。ADAM17通过mAbs M220 (Doedens等人, 2000, *J Biol Chem*. 275:14598-14607)、111633和111623 (R&D Systems, Inc., Minneapolis, MN)检测。人L-选择素通过mAb LAM1-116 (Ancell Corp., Stillwater, MN)检测。同种型匹配的阴性对照mAbs用于评估非特异性染色水平。CD16 ELISA通过定制的流式微球测定(cytometric bead assay)进行,如先前所描述的(Wang等人, 2013, *Biochim Biophys Acta*. 1833:680-685)。

[0063] *统计分析*

统计分析使用Prism软件(GraphPad, San Diego, CA)适当时使用ANOVA和学生t检验进行。认为P值< 0.05是显著的。

[0064] 实施例2

表达相等水平WT CD16a和CD16a^{197P} (CD16a/S197P)的NK细胞的比较

CD16构建体的表达水平通过FACS分选基于GFP表达匹配(如上述对于NK92细胞进行的,图2),所述GFP表达以与CD16构建体成比例的方式发生。对于所有测定匹配的CD16a水平通过FACS验证。作为对照,评估用空*Sleeping Beauty*转座子载体修饰的iPSC-衍生NK细胞(仅表达GFP)。iPSC-衍生的NK细胞表达低水平的内源性CD16a (数据未示出)。NK细胞对HER2-表达卵巢癌细胞的细胞毒性在曲妥单抗的存在或不存在下通过标准铬释放测定评估。也进行了用非铬标记卵巢癌细胞的抗体-依赖性细胞毒性。NK细胞的细胞因子(例如,IFNγ、TNFα)产生和CD16a的可溶性水平通过ELISA评估。CD16a和其它活化标志(例如,CD107a、CD62L)的细胞表面水平通过FACS评估。

[0065] 实施例3

人肿瘤异种移植模型,其用于检验表达CD16a^{197P} (CD16a/S197P)的iPSC-衍生NK细胞在曲妥单抗存在下是否具有增加的体内抗卵巢癌活性。

[0066] 使用NOD/SCID/γc$^{-/-}$ (NSG)小鼠和经稳定工程改造而表达用于生物发光成像的萤火虫荧光素酶的人卵巢癌细胞系的异种移植模型(Geller等人, 2013, *Cytotherapy* 15:1297-1306)用于检验腹膜内(ip)递送NK细胞的抗卵巢癌细胞活性。过表达HER2的OVCAR3卵巢癌细胞系用作体内靶(Hellstrom等人, 2001, *Cancer Res* 61:2420-2423)。亚致死照射的(225 cGY) NSG雌性小鼠腹膜内注射表达荧光素酶用于生物发光成像而产生的OVCAR3 (2 × 10^{5}细胞)以定量肿瘤生长或消退(Geller等人, 2013, *Cytotherapy* 15:1297-1306)。在小鼠得到单次腹膜内注射20 × 10^6 NK细胞前,让肿瘤生长7天。然后每隔一天给予小鼠IL-2 (5 μg/小鼠),持续4周,如先前所描述的(Woll等人, 2009, *Blood* 113: 6094-6101),以促进NK细胞的体内存活。曲妥单抗以50 μg的剂量腹膜内给予,每周一次,持续4周(所述剂量为先前在该模型中使用的剂量) (Warburton等人, 2004, *Clinical cancer research* 10:2512-2524)。比较表达相等水平WT CD16或CD16a^{197P} (CDa6a/S197P)的iPSC-衍生NK细胞的体内效能。对照包括仅表达GFP (仅载体)的iPSC-衍生NK细胞,和仅接受卵巢癌细胞的小鼠同龄组。所有小鼠得到相同的IL-2处理。

[0067] 肿瘤生长/消退每周通过生物发光成像和称重小鼠监测,如先前所描述的(Woll等人, 2009, *Blood* 113: 6094-6101)。每周还对小鼠采血以定量人NK细胞存活。各种效应物功能标志(例如,IFNγ、CD16a)的表达/细胞表面水平通过FACS评估。对小鼠随访达~60天。

处死时，通过生物发光检查内脏(例如，脾、肝、肺、肾和/或卵巢)的转移证据，如先前所描述的(Woll等人，2009，*Blood* 113：6094-6101)。

[0068] 示例性实施方案

实施方案1．一种经遗传修饰以表达包含膜近侧区和膜近侧区中的氨基酸修饰的CD16多肽的细胞。

[0069] 实施方案2．一种细胞，包含：

编码包含膜近侧区和膜近侧区中的氨基酸修饰的CD16多肽的多核苷酸。

[0070] 实施方案3．实施方案1或实施方案2的细胞，其中所述氨基酸药物(medication)反映与CD16膜近侧区野生型氨基酸序列相比一个或多个氨基酸的添加、一个或多个氨基酸的缺失，或一个或多个氨基酸的置换。

[0071] 实施方案4．实施方案3的细胞，其中一个或多个氨基酸的置换包括SEQ ID NO:1的197位的丝氨酸残基的置换。

[0072] 实施方案5．任何前述实施方案的细胞，其中所述细胞为自然杀伤(NK)细胞。

[0073] 实施方案6．任何前述实施方案的细胞，其中所述细胞为中性粒细胞。

[0074] 实施方案7．任何前述实施方案的细胞，其中所述细胞为单核细胞。

[0075] 实施方案8．任何前述实施方案的细胞，其中与野生型CD16多肽相比所述修饰的CD16多肽显示减小的对ADAM17介导的脱落的敏感性。

[0076] 实施方案9．任何前述实施方案的细胞，其中与野生型CD16多肽相比所述修饰的CD16多肽显示减小的对NK细胞刺激时的切割的敏感性。

[0077] 实施方案10．一种方法，其包括给予需要这种处理的患者包括以下的治疗：

给予患者治疗性NK效应物，和

给予患者权利要求1-9中任一项的细胞。

[0078] 实施方案11．实施方案10的方法，其中所述治疗性NK效应物包括治疗剂。

[0079] 实施方案12．实施方案11的方法，其中所述治疗剂特异性识别肿瘤抗原。

[0080] 实施方案13．实施方案12的方法，其中所述治疗剂包括特异性识别肿瘤抗原的抗体或抗体片段。

[0081] 实施方案14．实施方案13的方法，其中所述肿瘤抗原包括HER2。

[0082] 实施方案15．实施方案13或实施方案14的方法，其中所述抗体包括曲妥单抗或利妥昔单抗。

[0083] 实施方案16．实施方案10的方法，其中所述治疗性NK效应物包括双特异性杀伤衔接体(BiKE)。

[0084] 实施方案17．实施方案16的方法，其中所述BiKE包括CD16×CD33 BiKE、CD16×CD19 BiKE或CD16×EP-CAM BiKE。

[0085] 实施方案18．实施方案10的方法，其中所述治疗性NK效应物包括三特异性杀伤细胞衔接体(TriKE)。

[0086] 实施方案19．实施方案11或16-18中任一项的方法，其中所述治疗剂特异性识别病毒靶。

[0087] 实施方案20．用于改善对患者的治疗的方法，所述治疗包括给予患者治疗性NK效应物，所述方法包括：

给予患者权利要求1-9中任一项的细胞。

[0088] 本文所引用的所有专利、专利申请和出版的完整公开内容以及电子可得的材料(包括,例如,核苷酸序列提交(例如GenBank和RefSeq中的)以及氨基酸序列提交(例如SwissProt、PIR、PRF、PDB中的)和来信GenBank和RefSeq中注释的编码区域的翻译)通过引用以其整体结合。如果本申请的公开内容与本文通过引用结合的任何文献的公开内容之间存在任何不一致,应以本申请的公开内容为准。前面的详述和实施例仅为了清楚理解给出。不应从其理解不必要的限制。本发明不限于所显示和描述的确切细节,因为对本领域技术人员显而易见的变更将包含在通过权利要求书界定的本发明内。

[0089] 除非另外说明,说明书和权利要求书中使用的表述组分的量、分子量等的所有数字在所有情况下应理解为被术语“约”修饰。相应地,除非另外指示相反,说明书和权利要求书中列出的数字参数为近似值,其可根据欲通过本发明获得的期需性质而改变。至少,并且不试图将等同物的教义限制在权利要求书的范围中,每一数字参数应至少根据所报道的有效数字的数并通过应用普通舍入技术来解释。

[0090] 尽管阐述本发明的广泛范围的数字范围和参数为近似值,但具体实施例中列出的数值仍尽可能准确地报告。然而,所有数值固有地包含由其各自的检验测量结果中存在的标准偏差所必然得出的范围。

[0091] 所有的标题均为了方便读者并且不应用于限制标题之后的正文的含义,除非如此说明。

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100 105 110

Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys
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His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn
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1 5 10 15

Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro
20 25 30

Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln
35 40 45

Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu
50 55 60

[0003] Asn Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr
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85 90 95

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100 105 110

Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys
115 120 125

His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn
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Gly Lys Asp Arg Lys Tyr Phe His His Asn Ser Asp Phe His Ile Pro
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Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu Val
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[0004]

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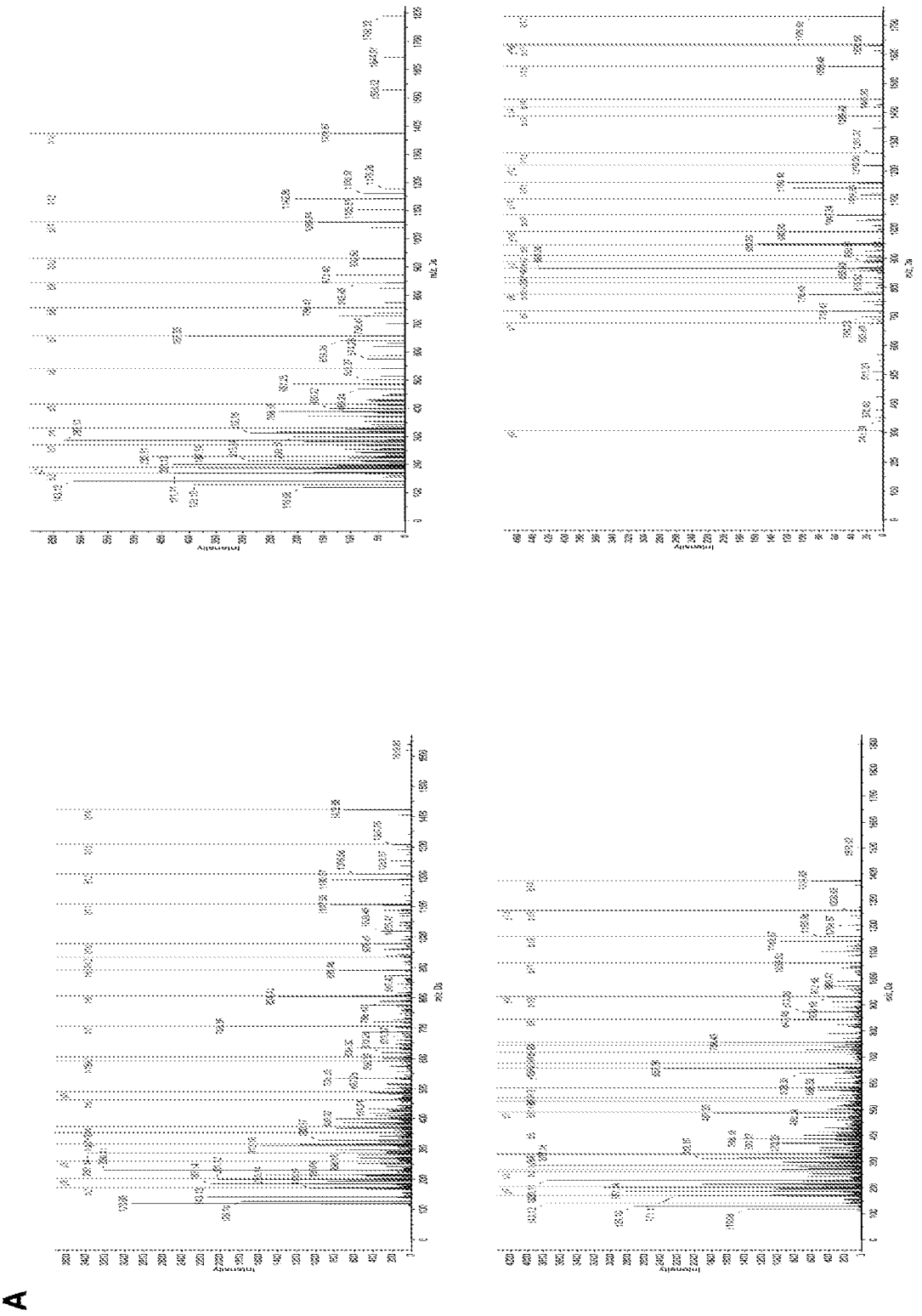

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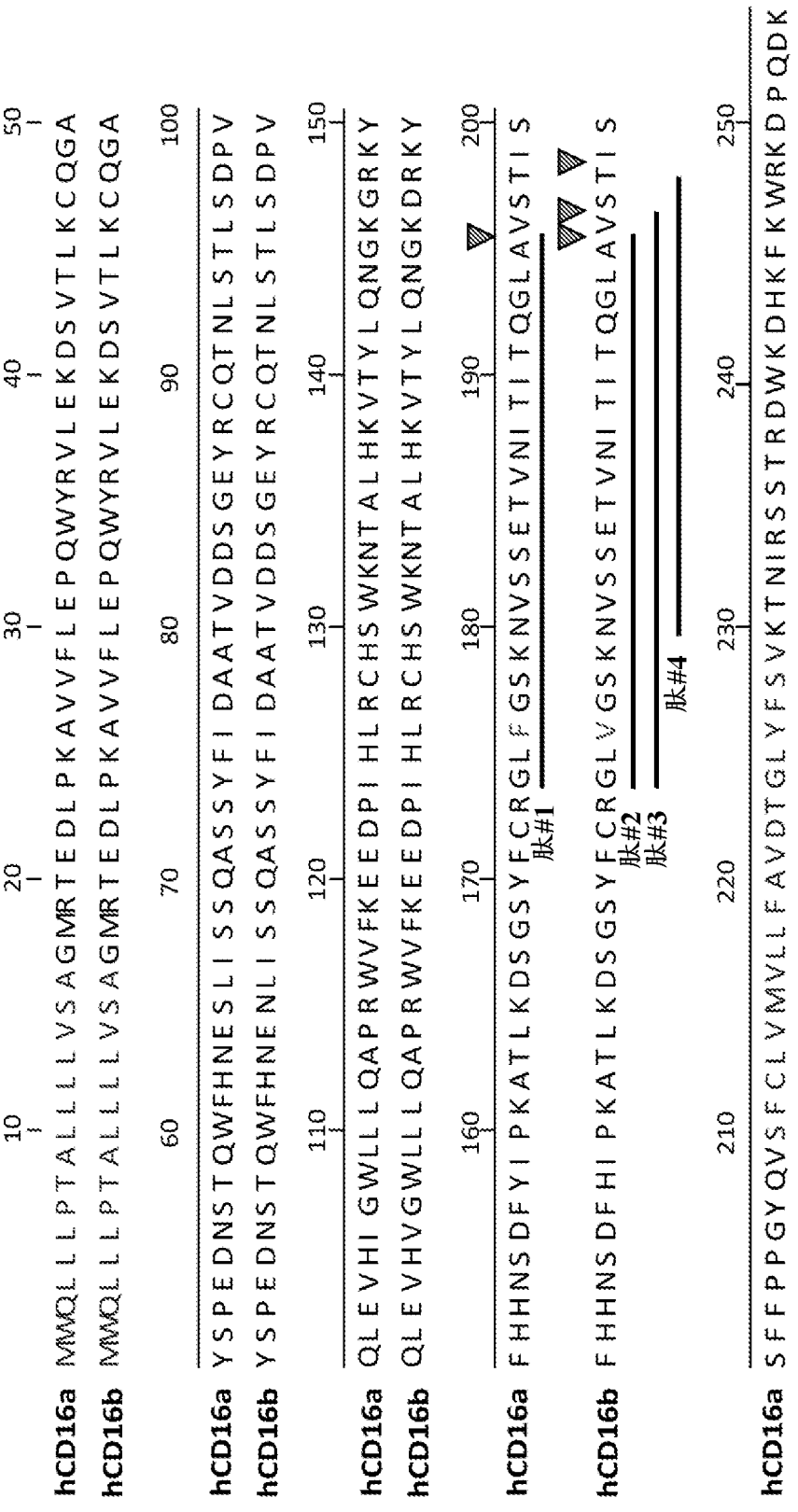

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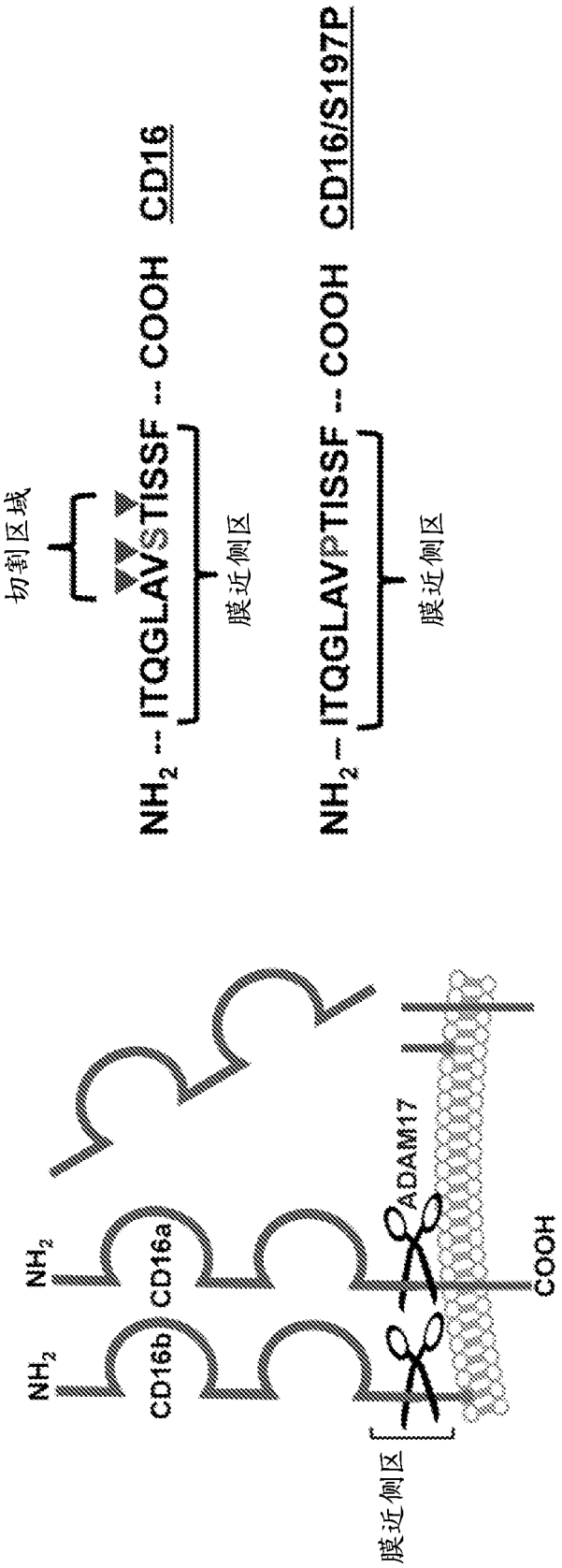
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膜近侧区
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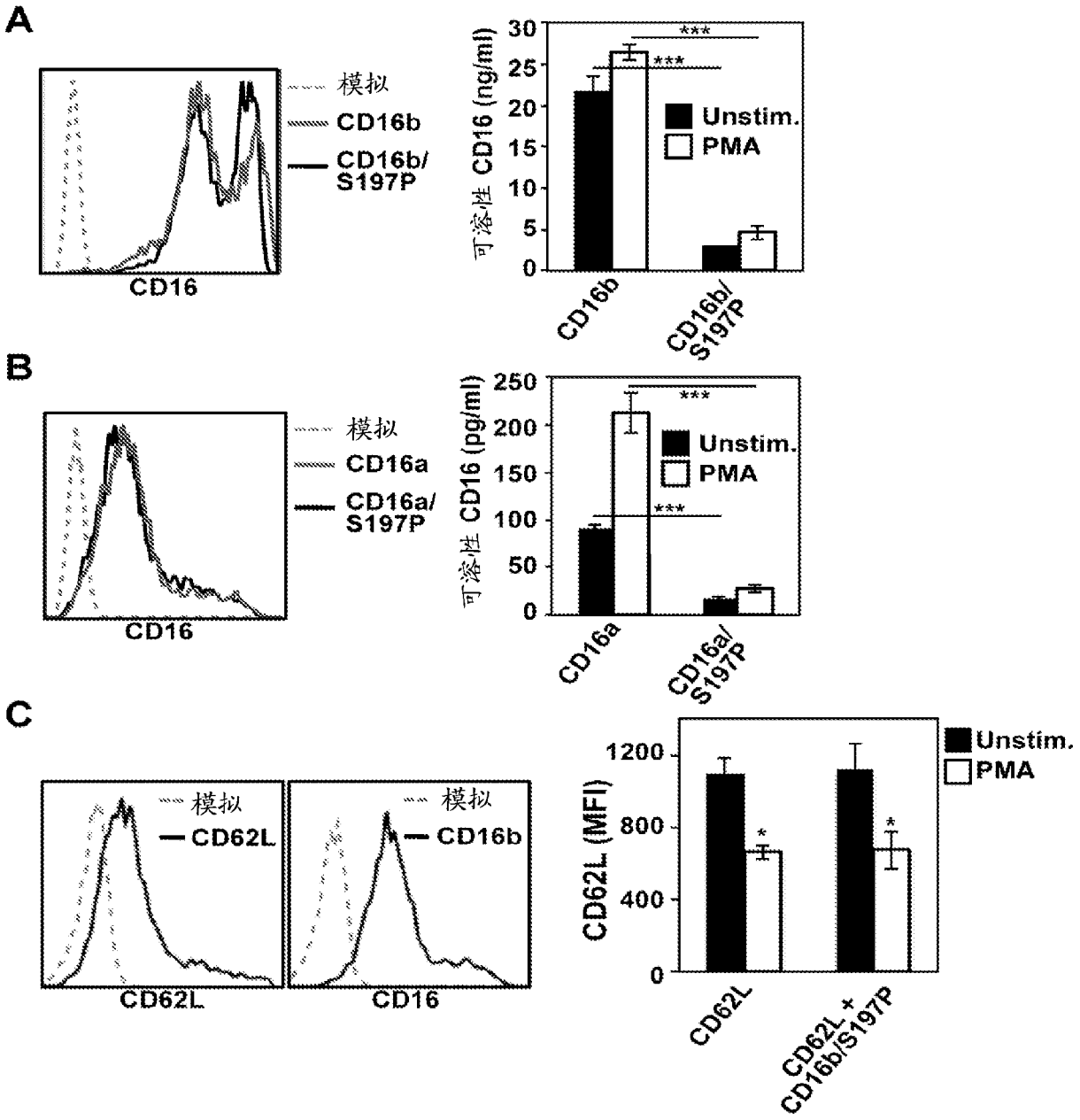
CD16b
NH2
CD16a
ADAM17
COOH
膜近侧区

图 2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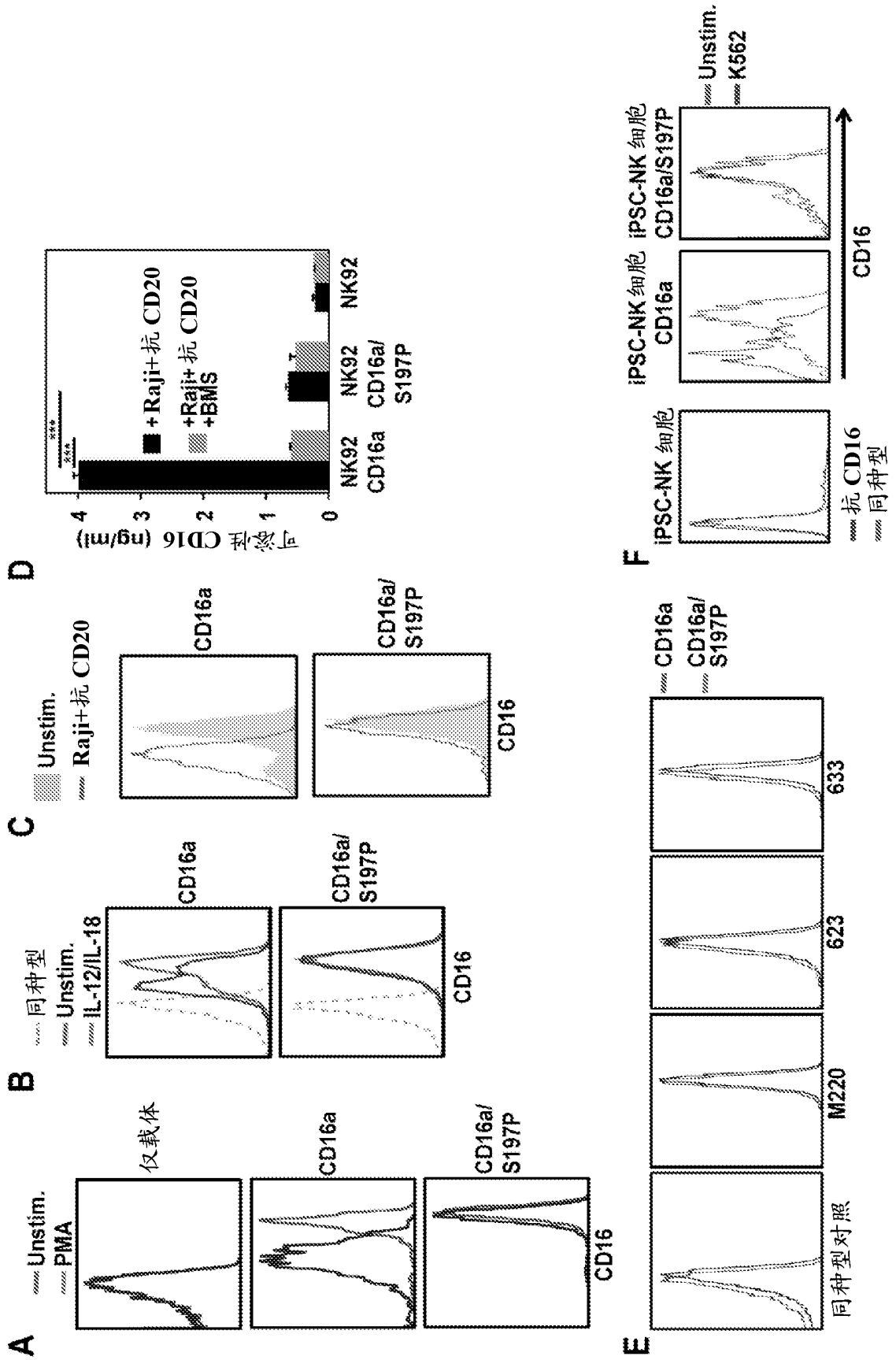
模拟
CD16b
CD16b/S197P
CD16
可溶性 CD16 (ng/ml)
30
25
20
15
10
5
0
***
***
Unstim.
PMA
CD16b
CD16b/S197P
B
模拟
CD16a
CD16a/S197P
CD16
可溶性 CD16 (pg/ml)
250
200
150
100
50
0
***
***
Unstim.
PMA
CD16a
CD16a/S197P
C
模拟
CD62L
CD62L
模拟
CD16b
CD16
CD62L (MFI)
1200
800
400
0
*
*
Unstim.
PMA
CD62L
CD62L + CD16b/S197P

图 3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Unstim.
PMA
仅载体
CD16a
CD16a/S197P
CD16
B
同种型
Unstim.
IL-12/IL-18
CD16a
CD16a/S197P
CD16
C
Unstim.
Raji+抗 CD20
CD16a
CD16a/S197P
CD16
D
可溶性 CD16 (ng/ml)
+Raji+抗 CD20
+Raji+抗 CD20 +BMS
NK92 CD16a
NK92 CD16a/S197P
E
同种型对照
M220
623
633
CD16a
CD16a/S197P
F
iPSC-NK 细胞
抗 CD16
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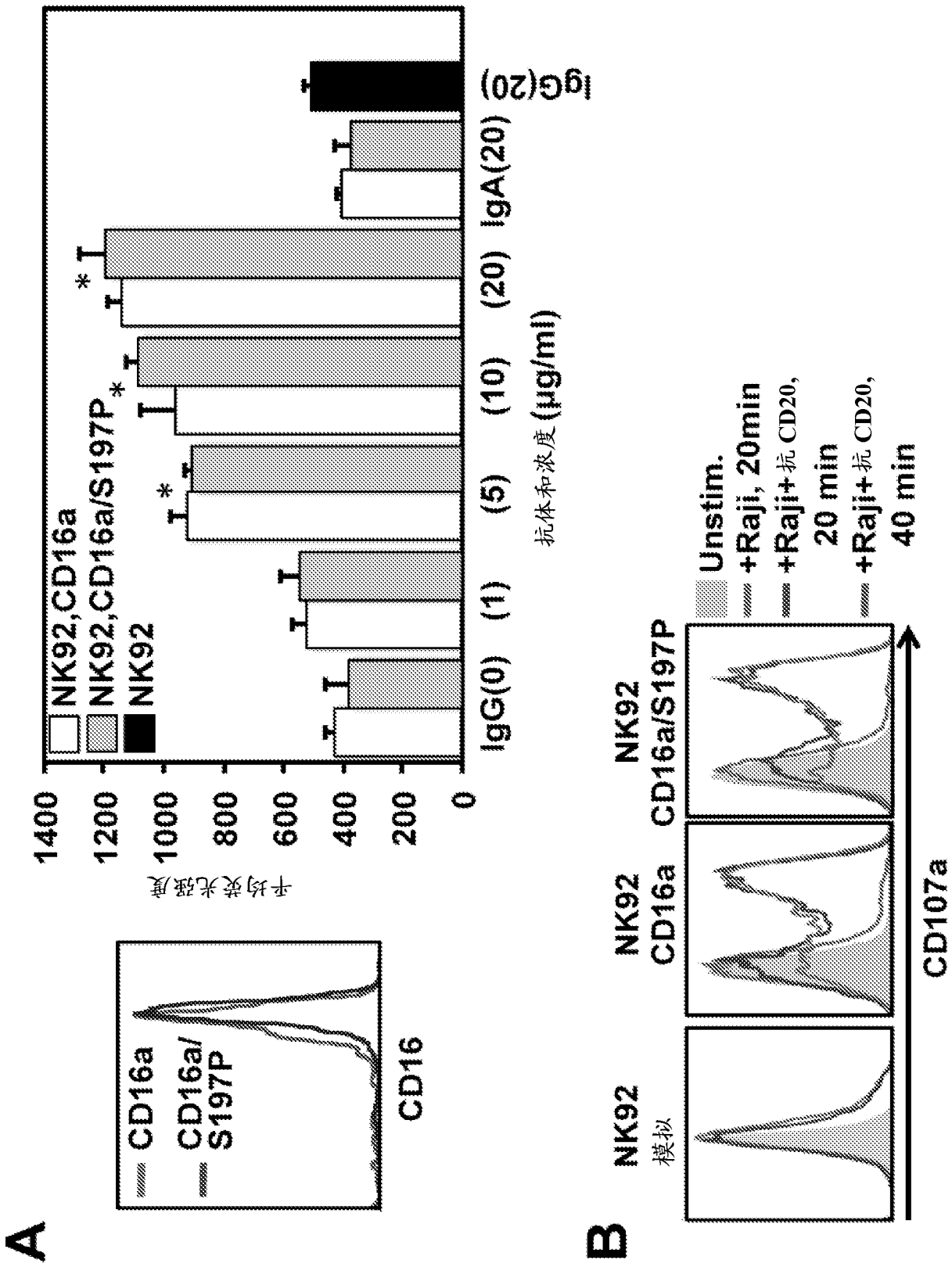
iPSC-NK 细胞 CD16a
iPSC-NK 细胞 CD16a/S197P
Unstim.
K562
CD16

图 4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CD16a/
S197P
CD16
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1200
1000
800
600
400
200
0
NK92,CD16a
NK92,CD16a/S197P
NK92
*
*
*
IgG(0)
(1)
(5)
(10)
(20)
IgA(20)
IgG(20)
抗体和浓度(μg/ml)
B
NK92
模拟
NK92
CD16a
NK92
CD16a/S197P
Unstim.
+Raji, 20min
+Raji+抗 CD20,
20 min
+Raji+抗 CD20,
40 min
CD107a

图 5