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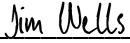Development of bispecific antibodies to retarget NK cell-mediated cytotoxicity

by
Emily Kang

DISSERTATION
Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Chemistry and Chemical Biology

in the

GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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**Statement concerning previously published materials**

The material in Chapter 2 has not been submitted for publication. Emily Kang performed most of the work described in this chapter. Helle Jensen developed the cell lines and advised on the experimental design. James Wells supervised the research.

Chapter 3 of this thesis is a reprint of the material as it appears in:

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**Development of bispecific antibodies to retarget NK cell-mediated cytotoxicity**

**Emily Kang**

**Abstract**

Cancer immunotherapies, or treatments that utilize a patient's own immune system to fight cancer, have been met with a great deal of success. Although most current immunotherapies have focused on boosting the adaptive immune system, there has been a growing interest in targeting components of the innate immune system. An important member of the innate immune system is the natural killer (NK) cell. Like T cells, NK cells are cytotoxic effector cells with the ability to distinguish and eliminate virally infected or transformed cells. They play a key role in the anti-tumor response and are able to identify target cells by recognizing stress-induced ligands that are frequently overexpressed on cancer cells. NK cells are best known for their ability to perform antibody-dependent cellular cytotoxicity (ADCC), a mechanism that is used by several therapeutic antibodies to eradicate tumor cells. Whereas all T cells can be targeted through the T cell receptor (TCR)/CD3 complex, NK cells have multiple activating, costimulatory, and inhibitory receptors that can be targeted to modulate their activity. This dissertation provides a functional mammalian display strategy used to identify antibodies that can target and stimulate NK cells. Four activating antibodies were identified and were developed into bispecific antibodies to redirect NK cell-mediated cytotoxicity towards tumor cells of interest and to stimulate NK cell activity. Chapter 1 discusses the background of NK cells and their role in anti-tumor immunity. Chapter 2 describes a mass spectrometry-based screening approach to identify cell surface proteins on NK cells. Chapter 3 provides the method designed to identify antibodies that are able to stimulate NK cell-mediated cytotoxicity and the generation of bispecific NK engagers.

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**Chapter 3**

**Chapter 1: Introduction**

**1.1 NK cells and their functions**

Natural killer (NK) cells are cytotoxic effector cells of the innate immune system and represent 5-20% of all lymphocytes found in the peripheral blood, spleen, and liver. They are one of three kinds of lymphocytes; the other two are known as B and T cells. They were first discovered in the 1970s and were noted for their unique ability to kill tumor and virally infected cells *in vitro* without prior immunization (1-4). Unlike cytotoxic T cells, which typically recognize specific major histocompatibility complex class I (MHC-I)-peptide complexes presented by virally infected or tumor cells, NK cells are able to recognize and kill transformed cells in the absence of antibodies or MHC (5). Instead, NK cells express a wide array germ-line encoded activating and inhibitory receptors that regulate their activity.

NK cells are now known to play a key role in tumor rejection and viral clearance. Upon target recognition, they are able to directly lyse the target cell and produce cytokines and chemokines to further shape the adaptive immune response (**Figure 1.1**). NK cells are able to kill susceptible target cells in two ways: they can release cytotoxic granules containing perforins and granzymes, or they can induce apoptosis in target cells via death receptor signaling (6). Once activated, NK cells are also able to rapidly produce and secrete cytokines, like IFN-γ, TNF-α, GM-CSF, and IL-10, and chemokines, like MIP-1α, MIP-1β, and RANTES (7-8). The secretion of these molecules is important for the recruitment of other components of the immune system and for modulating the adaptive immune response.
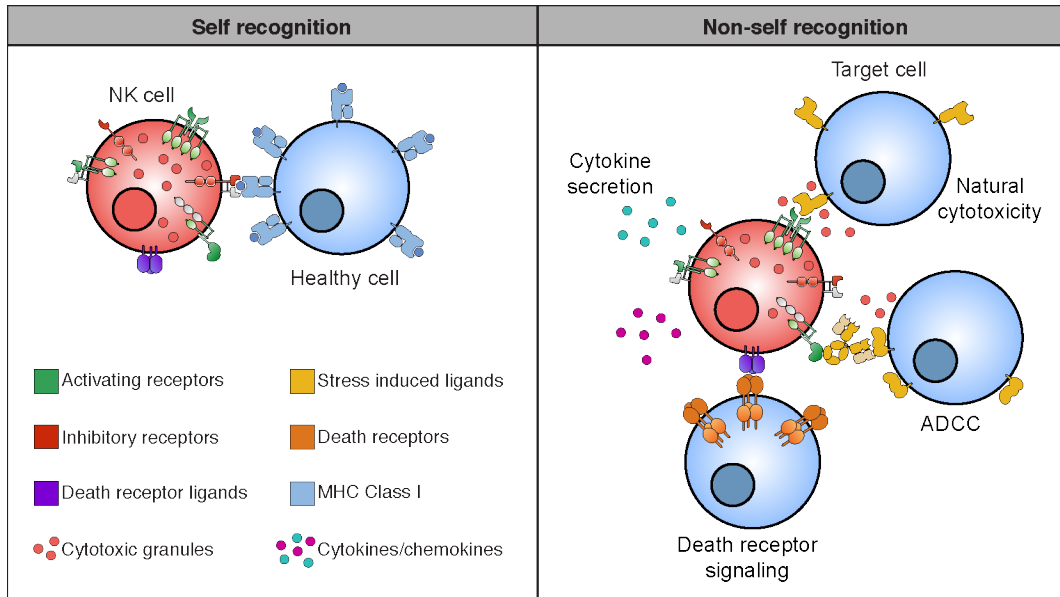
**Figure 1.1 NK cells are able to recognize self and non-self.** NK cells are able to recognize self through inhibitory receptors that can bind to MHC class I molecules. NK cells are able to recognize and kill non-self through a variety of mechanisms, including natural cytotoxicity, ADCC, and death receptor signaling. NK cells are also able to reshape the adaptive immune system through cytokine release.

## 1.2 NK cell surface receptors and tumor cell recognition

NK cells are able to recognize both self and non-self. They are able to do so through the expression of the multiple different activating and inhibitory receptors, which are able to recognize specific ligands on potential target cells. Inhibitory NK cell receptors bear immunoreceptor tyrosine-based inhibitory motif (ITIM) cytoplasmic tails, and contribute to self-recognition by detecting MHC-I molecules that are typically expressed at the cell surface of normal cells. The loss of self is an important mechanism by which NK cells target transformed or virally infected cells. Many transformed or infected cells down-regulate the expression of MHC-I molecules to escape T cell recognition. However, this decrease in MHC-I expression subsequently lowers the inhibitory signaling triggered by inhibitory NK cell receptors, which increases the susceptibility of these transformed or infected cells towards NK cell killing (9).

Although missing-self recognition contributes to the identification and targeting of transformed or virally infected cells, it is not the sole method by which NK cells identify targets.

NK cells also express many different activating and costimulatory receptors that can stimulate NK cell effector functions. Activating and costimulatory receptors can elicit function through different signaling pathways. Generally speaking, signals can be initiated by immunoreceptor tyrosine-based activation motifs (ITAM), YXXM motifs, or through other tyrosine-containing motifs. CD16A and the natural cytotoxicity receptors – NCR1/NKp46, NCR2/NKp44, and NCR3/NKp30 – all associate with adaptor proteins with ITAM-containing cytoplasmic tails. The C-type lectin-like receptor, natural-killer group2, member D, NKG2D, associates with YXXM-containing DAP10 adaptor proteins, and the costimulatory receptors, CD244 and TNFRSF9, signal through other tyrosine-containing motifs. Although different activating and costimulatory receptors can trigger different signaling pathways, they all ultimately regulate the activation and resultant cytotoxic effects of NK cells.

Several activating and costimulatory receptors have been characterized for their involvement in tumor rejection, and have been shown to recognize stress-induced ligands that are typically over-expressed on malignant cells. The activating receptors, NCR1, NCR2, and NCR3 are known to contribute to tumor cell recognition and to inducing NK cell-mediated cytotoxicity. Although the ligands for NCR1, NCR2, and NCR3, have not been fully identified or characterized, these receptors have been shown to bind to cancer cells and the presumed ligands for these receptors are suggested to contribute to NK cell activation (10-12). NKG2D is also known to play a key role in tumor cell recognition. Ligands for NKG2D, like MHC class I chain-related protein A (MICA) and B (MICB) and UL16-binding proteins (ULBP1-6) are up-regulated in a wide range of tumors of different tissue origin (13-14). Moreover, the shedding of NKG2D ligands has been shown to correlate with poor clinical outcome in cancer patients, suggesting that the expression of NK cell ligands is important for regulating a tumor immune response (15-16).

Another common mechanism by which NK cells recognize and kill tumor cells is through antibody-dependent cellular cytotoxicity (ADCC). Antibodies, like immunoglobulin G (IgG), that

bind to tumor antigens or to viral proteins can induce ADCC by binding to CD16A/FcγRIIIa. CD16A is an activating receptor that is commonly expressed on NK cells and is able to able to bind to the crystallizable fragment (Fc) region of an antibody. Many different clinically approved antibodies, like Rituximab (17), Trastuzumab (18), and Cetuximab (19-20), are known to induce ADCC, and this ability is known to contribute to the efficacy of these therapeutics.

**1.3 Engineering antibodies to improve ADCC**

ADCC is important for the clinical efficacy of therapeutic antibodies. As such, several methods have been developed to enhance ADCC. Modification of the Fc is a common method used to improve the binding interface between the Fc and CD16 and to enhance ADCC. Such methods include the afucosylation of the Fc-linked oligosaccharides and amino acid modification of the Fc. More recently, high affinity antibodies have been directly generated against CD16 to create bispecific antibodies and to redirect NK cells towards a target cell of interest.

**1.3.1 Antibodies and their effector functions**

Monoclonal antibodies (mAb) and antibody derivatives are a major drug class that can be used to treat a wide range of disease indications. The typical human IgG consists of two 50 kD heavy chains (HC) and two 25 kD light chains (LC). Human LCs can come from one of two different classes, κ or λ, whereas human HCs can come from one of five different isotypes, IgA, IgD, IgE, IgG, or IgM. Both the κ and λ light chain is composed of 2 domains, the constant domain (CL) and the variable domain (VL). IgAs, IgDs, and IgGs are composed of three constant (CH) and one variable (VH) domain. In contrast, IgEs and IgMs consist of four CH and one VH domain. The constant domains of the heavy chain determine the Fc effector function associated with the antibody isotype.

The common IgG is made up of two Fragment antigen-binding (Fab) arms and an Fc (**Figure 1.2A**). The heavy chains and light chains dimerize to form the Fab arms, and the heavy chains pair up to form the Fc. The Fv region of the Fab, which dictates antigen specificity, is

formed by the variable domains (VH and VL) of the HC and LC. The complementarity determining regions (CDR) are hypervariable regions within the Fv; there are six CDRs in total, three in the VL (CDR-L1, CDR-L2, and CDR-L3) and three in the VH (CDR-H1, CDR-H2, and CDR-H3) (**Figure 1.2B**). Together, these six CDRs form the antigen-binding site. Of all the CDRs, CDR-H3 is the most variable CDR and has the largest range in lengths and amino acid sequence diversity. It is thought to play a key role in antibody-antigen interactions.
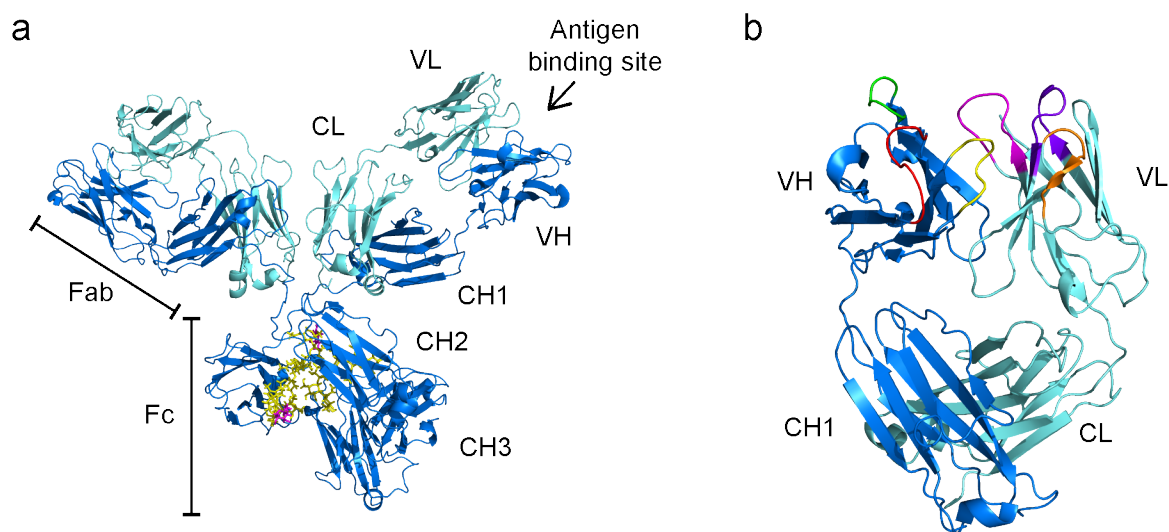


**Figure 1.2 The structure of an antibody, Protein Data Bank (PDB) ID: 1IGY** (21). (a) A ribbon representation of an intact mouse IgG1. The heavy chain is in blue and the light chain is in cyan. The glycan is shown as orange sticks and the fucose groups are shown in magenta. (b) a ribbon representation of the Fab. CDR-H1 is in red, CDR-H2 is in green, and CDR-H3 is in yellow. CDR-L1 is in purple, CDR-L2 is in orange, and CDR-L3 is in magenta.

The Fc region of an antibody is able to interact with a number of different Fc receptors. The Fc of the IgG isotype, in particular, is able to interact with the Fcγ receptor (FcγR) family. This family comprises CD64/FcγRI, CD32A/FcγRIIa and CD32B/FcγRIIb, and CD16A/FcγRIIIa and CD16B/FcγRIIIb. These receptors are variably expressed in a number of different immune cell types, and the interaction between the Fc and the different Fc receptors can mediate a number of different antibody effector functions, including ADCC, antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC). Fc and FcγR interactions can also modulate inflammation and immune cell maturation and activation. Additionally, Fc

interactions with FcRn contribute to modulating the half-life of IgGs. Since the Fc plays such an important role in regulating effector cell activity, significant efforts have been made to engineer the Fc to elicit specific effector functions.

## 1.3.2 Fc engineering to enhance ADCC

The Fc of an IgG typically contains two N-linked oligosaccharide at Asn297. The oligosaccharides in the Fc have a complex biantennary structure and are made up of N-acetylglucosamine (GlcNAc) and mannose groups. This core structure can be further modified to contain galactose, N-acetylneuraminic acid (sialic acid), fucose, and bisecting GlcNAc. The resulting sugar group is dependent on the expression levels of glycosyltransferases and glycosidases. To generate afucosylated antibodies, different Chinese Hamster Ovary (CHO) cell lines have been engineered. CHO cells have been engineered to overexpress the β(1,4)–*N*–acetylglucosaminyltransferase III (GnTIII) (22-23). GnTIII is a glycotransferase that introduces bisecting N-acetylglucosamine (GlcNAc) residues to N-linked oligosaccharides. Bisected oligosaccharides are poor substrates for core α1,6–fucosyltransferase. This allows for the generation of non-fucosylated products. FUT8 knock out CHO cell lines have also been created to generate afucosylated antibodies (24). FUT8 encodes the α1,6–fucosyltransferase, which catalyzes the transfer of fucose to a GlcNAc in an α1,6 linkage. This FUT8 knockout cell line produces a completely non-fucosylated product, and has been used to produce an afucosylated Rituximab variant that exhibited a 100-fold improvement in ADCC.

Amino acid modifications have also been made to increase the affinity of the Fc towards CD16. Alanine scanning of solvent exposed residues on the Fc revealed 27 mutations that increased binding to at least one FcγR (25). Three alanine mutations – S298A, E333A, and K334A – were shown to improve binding to CD16A, and the triple mutant increased the ability to induce ADCC by 40-100%. Computational design and high-throughput screening have also been used to identify Fc variants with improved Fcγ receptor binding and specificity (26). The S239D/I332E double mutant and the S239D/A330L/I332E triple mutant identified as higher

affinity Fc variants towards CD16A. The double mutant improved the affinity of Trastuzumab 100-fold and this greatly improved the ability of Trastuzumab to kill different HER2+ cell lines. Similar improvements in ADCC were demonstrated with generating similar Fc variants of Rituximab and Alemtuzumab.

### 1.3.3 Antibodies towards CD16A

Antibodies have also been generated to directly target CD16A and stimulate NK cell-mediated cytotoxicity. Bispecific antibodies generated through chemical conjugation or through somatic cell fusion of two hybridoma lines were first used to investigate the clinical relevance of ADCC in the 1990s, and were found to potentiate tumor cell lysis (27, 28). Such studies demonstrated that generating antibodies to directly target CD16A might be a promising approach to triggering ADCC in NK cells. Since then, several high affinity single chain Fvs (scFv) and diabodies have been developed to target CD16A on NK cells. Bi- and tri-specific antibodies that incorporate these scFvs or diabodies have been shown to effectively redirect NK cell-mediated cytotoxicity against several different tumors. A bispecific antibody targeting CD33 and CD16 was shown to lyse acute myeloid leukemia (AML) cell lines with a half maximum effective concentration (EC50) in the low picomolar range (29). Trispecific antibodies targeting CD19, CD22, and CD16 have also been shown to enhance NK cell function against primary acute lymphoblastic leukemia (ALL) and chronic lymphoblastic leukemia (CLL) tumor samples (30). Not only have bispecifics demonstrated efficacy in redirecting NK cell-mediated cytotoxicity towards liquid tumors, but bispecifics utilizing CD16 have also been shown to be useful in targeting solid tumors. A bispecific antibody targeting EpCAM and CD16 has been shown to be effective against several different EpCAM+ human carcinoma cell lines (31). Several different bispecific antibodies targeting EGFR and CD16 has been shown to lyse SW-982 sarcoma cells and A-431 carcinoma cells with an EC50 in the low picomolar range (32).

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**Chapter 2: Characterizing the cell surface proteome of natural killer cells**

**2.1 Abstract**

Natural Killer (NK) cells, like CD8+ T cells, are cytotoxic effector cells of the immune system. Both subsets are essential for the recognition and removal of cancer cells, but most cancer immunotherapies have been designed to target CD8+ T cells. Whereas all T cells express the CD3/TCR complex that can be exploited by immunomodulatory molecules to redirect T cell activity, NK cells express multiple activating, costimulatory, and inhibitory receptors that govern NK cell effector functions. Although NK cell activity is directly tied to the expression profile of NK cell receptors, the cell surface proteome of NK cells has not been fully assessed. To advance the development of novel NK-based therapies, we characterized the cell surface composition of an NK cell line to identify potential therapeutic targets

**2.2 Introduction**

NK cells are cytotoxic effector cells of the innate immune system. They were first characterized for their unique ability to recognize and kill malignant cells without prior immunization (1, 2). Since their discovery, NK cells have been shown to play a key role in host defenses against viruses and tumors. They are able to directly kill target cells by releasing cytotoxic granules containing perforins and granzymes, induce apoptosis in target cells by stimulating death receptor pathways, and shape the adaptive immune response by secreting cytokines and chemokines. These effector functions are tightly regulated by the activating, costimulatory, and inhibitory receptors that are expressed at the cell surface of NK cells (3, 4).

Activating and costimulatory NK cell receptors can initiate signaling cascades through several different sequence motifs, including the YINM motif, immunoreceptor tyrosine- based activation motif (ITAM), or immunoreceptor tyrosine-based switch motif (ITSM) (3). The stimulation of these pathways ultimately leads to the release of cytotoxic granules and cytokines. Although the stimulation of such pathways is important for the regulation of NK cell

activity, a comprehensive and unbiased analysis of the cell surface proteomic changes that occur upon receptor stimulation has not been evaluated.

Mass spectrometry (MS) based proteomic approaches allow for the unbiased identification and quantification of hundreds of different proteins. Cell surface proteomics methods have been developed to identify differentially expressed proteins in different cellular states (5-9), and can be used to find potential clinical biomarkers and therapeutic targets. Here, we perform cell surface proteomics on an NK cell line to identify potential markers of NK cell activation.

## 2.3 Materials and Methods

### Cells

NKL cells that were stably transduced with Ly49H were a kind gift from Lewis L. Lanier, UCSF. NKL cells were maintained in RPMI-1640 medium with 2 mM L-glutamine containing 10% FBS, and 100 IU/mL penicillin and 100 µg/mL streptomycin, supplemented with 200 U/mL human IL-2 (provided by the NCI Preclinical Repository).

### SILAC Mass Spectrometry

NKL cells were passaged 5 times in RPMI SILAC media (Thermo Fisher Scientific; Waltham, MA) containing L-[13 C6 ,15 N2 ]lysine and L-[13 C6 ,15 N4 ] arginine (heavy label; Cambridge isotope laboratories, Tewksbury, MA) or L-[12 C6 ,14 N2 ]lysine and L-[12 C6 ,14 N4 ]arginine (light label). Cells were then stimulated with plate-bound antibodies. In brief, Nunc maxisorp ELISA plates were washed twice with PBS and coated with 5 µg/mL anti-NKG2D mAb (1D11, BioLegend), 5 µg/mL anti-Ly49H (3D10, BioLegend), 5 µg/mL anti-CD244 mAb (C1.7, BioLegend), 5 µg/mL anti-NKG2D mAb and 5 µg/mL anti-CD244 mAb, or 5 µg/mL control mouse IgG1 (MOPC-21, BioLegend) in PBS for 24 h at 4 °C. The ELISA plates were then washed twice in PBS and blocked in culture medium for 10 min at room temperature (RT) before 250,000 cells were added to each well in the ELISA plate. A total of 25 million light-labeled and 25 million heavy labeled NKL cells were used per condition per experimental

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replicate. NKL cells were stimulated for 24 hours at 37 °C and 5% CO2. Cells were harvested and combined at a 1:1 cell count ratio and cell surface proteins were enriched using a modified CSC protocol as previously described (7-9).

Cell samples were washed and treated with 2 mM sodium periodate and then reacted with 1 mM biocytin hydrazide to label N-glycosylated cell surface proteins. Cell pellets were lysed with RIPA buffer (VWR) in the presence of a protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO) and biotinylated proteins were enriched with neutravidin beads. On-bead trypsin digestion was used to release bound proteins and a follow-up PNGase F digestion was used to release N-glycosylated peptides. The tryptic and PNGase F samples were desalted using SOLA HRP SPE columns (Thermo Fisher Scientific) and were run on a Q Exactive Plus (Thermo Fisher Scientific) mass spectrometer. Tryptic and PNGase F samples were separated with a linear gradient of 3-35% solvent B (Solvent A: 0.1% formic acid; solvent B: 80% acetonitrile, 0.1% formic acid) at 300 µL/min over 180 min or 120 min, respectively. Data were collected in data-dependent mode using a top 20 method with dynamic exclusion of 35 s and a charge exclusion setting that only sample peptides with a charge of 2, 3, or 4. Full (MS1) scan spectrums were collected as profile data with a resolution of 140,000 (at 200 m/z), AGC target of 3E6, maximum injection time of 120 ms, and scan range of 400–1800 m/z. Fragment ion (MS2) scans were collected as centroid data with a resolution of 17,500 (at 200 m/z), AGC target of 5E4, maximum injection time of 60 ms with a normalized collision energy at 27, and an isolation window of 1.5 m/z with an isolation offset of 0.5 m/z.

**Proteomic data analysis**

Peptide search for each experimental dataset was performed with ProteinProspector (v.5.13.2) against 20203 human proteins and the Ly49H mouse protein (Swiss-prot database, obtained March 5, 2015). MS1 peak area quantification was performed with Skyline software using the MS1 filtering function, and additional quantitative data analyses were performed using Python 2.7. Low confidence protein identifications were eliminated to ensure stringent

quantification of the cell surface proteome. SILAC datasets were then combined and reported as median log2(fold change) and p values were generated through a Wilcoxon ranked test for median log2(fold change).

## 2.4 Results and Discussion

**Differentially expressed genes identified upon receptor stimulation of NKL cells**

Activating and inhibitory signals initiated by germline-encoded receptors expressed at the cell surface regulate NK cell responses. To better characterize the phenotypic changes that occur upon NK cell stimulation, we performed cell surface proteomics on the human NK cell line, NKL, upon exposure to different stimuli. NK cells express a variety of activating receptors that can function through different signaling pathways. NKL cells stably transduced with the activating mouse Ly49H (KLRA8) receptor were used to explore the cell surface proteomic effects of different receptor stimuli. These cells overexpress the Ly49H receptor, which can associate with both the DAP10 and DAP12 (10) adapter proteins that carry a YINM motifs or ITAM motifs respectively (**Figure 2.1A**). NKL cells also constitutively express the activating receptor, NKG2D (KLRK1) (11, 12), which associates with the DAP10 adapter protein, and the costimulatory receptor, CD244 (2B4) (12, 13), which signals through its ITSM motifs. NKL cells stably transduced with the activating mouse Ly49H receptor were stimulated with saturating amounts of isotype IgG control or plate-bound antibodies against Ly49H, NKG2D, CD244, or the combination of NKG2D and CD244 for 24 hours. Cell surface proteins that were differentially expressed upon NKL cell activation were identified with a modified biocytin hydrazide cell surface capturing (CSC) method, and quantified using stable isotope labeling with amino acids in cell culture (SILAC). A total of 823 proteins were identified and quantified (**Supplemental Figure 2.1**); 42 proteins were differentially expressed in at least one of the conditions tested (**Figure 2.1B**). Although different signaling pathways were stimulated, there were many similarities in the overall changes in protein expression across all forms of stimuli (**Figure 2.1B**).
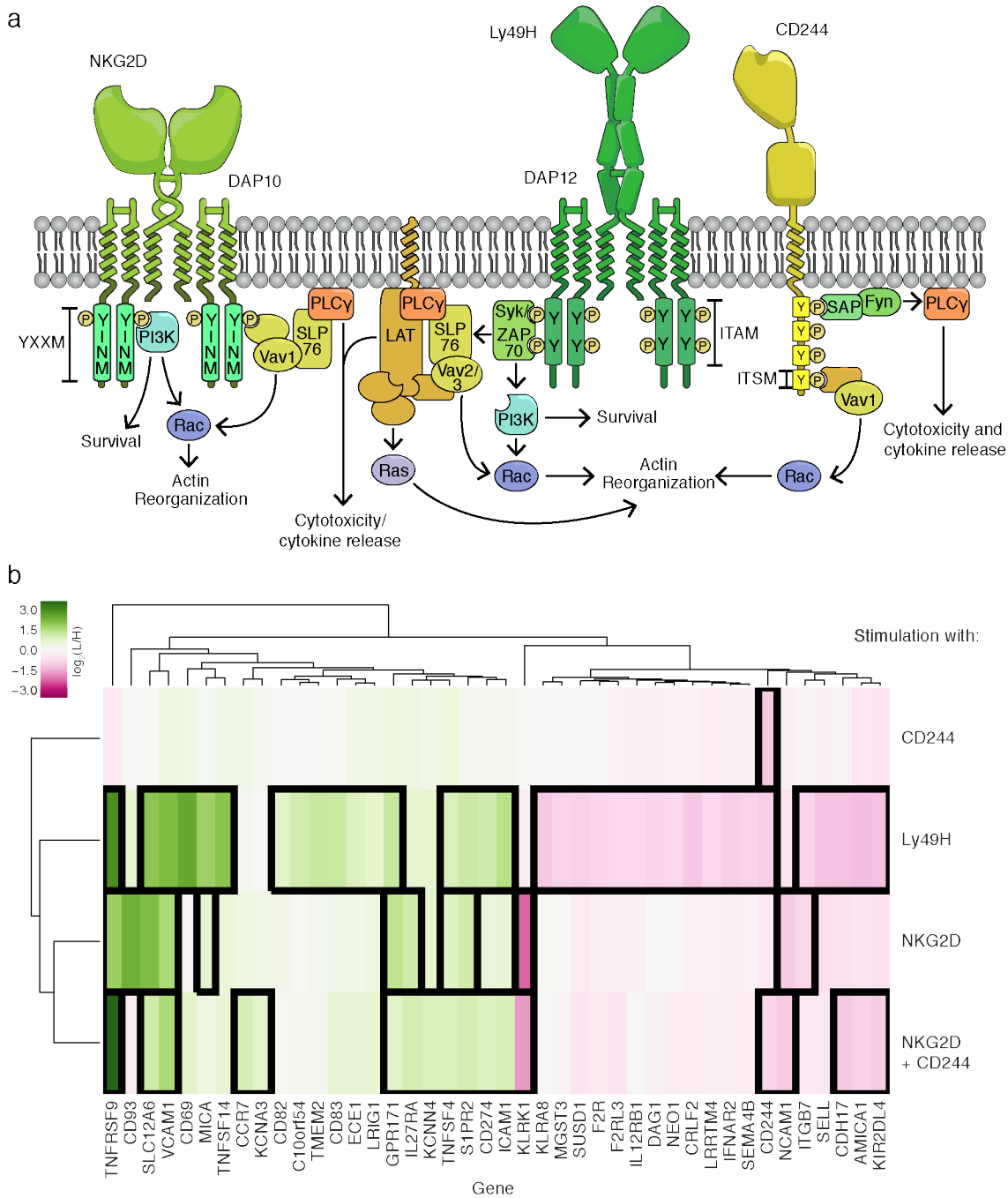
**Figure 2.1 Stimulation of different activating or costimulatory receptors induces changes in the cell surface proteome of Ly49H overexpression NKL cells.** (A) Different signaling cascades are initiated upon stimulation of different NK cell receptors. (B) Many of the same proteins are up-regulated (green) and down-regulated (magenta) upon stimulation of different activating or costimulatory receptors. Significantly up- or down-regulated proteins are boxed in black.

Stimulation of the costimulatory receptor, CD244, alone did not significantly alter the cell surface proteome of the NKL cell line. The only change observed upon CD244 stimulation was the down- regulation of the stimulated receptor, which was to be expected as previous studies have found that receptor engagement results in receptor internalization and a decrease in CD244 cell surface expression. Changes in the cell surface proteome were only observed when the activating receptors, Ly49H or NKG2D, were stimulated. Stimulating the NKL cell line with Ly49H, NKG2D, or the combination of NKG2D and CD244 significantly up- or down-regulated the expression (log2FC = ±1.5) of 12 to 35 proteins. Stimulation with both NKG2D and CD244 produced a larger difference in protein expression than stimulation with either NKG2D or CD244 alone, suggesting that the integration of different signaling pathways enhances the phenotypic change induced by activation. However, stimulation via the Ly49H receptor resulted in the greatest number of changes and produced alterations of the greatest magnitude, suggesting that ITAM signaling induces the largest changes to the cell surface proteome. TNFRSF9, SLC12A6, VCAM1, GPR171, TNFSF4, and S1PR2 were commonly up-regulated in all three of these activation conditions. Interestingly, TNFRSF9, and TNFSF4 have previously been reported to be up- regulated on activated NK cells.

Since activating receptor stimulation also results in the secretion of various cytokines and chemokines, we also tested the effects of these soluble factors on the NKL cell surface proteome. Media from NKL cells that had been stimulated with CD244, Ly49H, or the combination of NKG2D and CD244 for 24 hours was collected. Fresh NKL cells were cultured in the presence of the conditioned media for 24 hours to determine how soluble factors that are secreted upon NK activation alter the cell surface proteome. Exposure to these soluble factors resulted in very few changes in protein expression (**Supplemental Figure 2.2**), suggesting that most of the proteins that were differentially expressed upon receptor stimulation were likely a direct result of receptor engagement rather than exposure to soluble factors that were secreted after NK activation.

**Immune cell processes are regulated by activating receptor stimulation**

To determine which cellular processes were affected by activating receptor stimulation, we used DAVID (14, 15) to performed Gene Ontology (GO) analysis on the up- and down-regulated proteins identified from each dataset (**Figure 2.2**). In the Ly49H, the NKG2D, and the combination stimulation of NKG2D and CD244 datasets, gene clusters involving immunoglobulin (Ig)-like domains were highly enriched. Additionally, gene clusters involving C-type lectin-like domains were highly enriched in the Ly49H and NKG2D datasets. This is in alignment with what is known about NK receptor gene complexes. NK cells are known to express many activating and inhibitory receptors that are classified within the Ig superfamily (IgSF) or the C-type lectin-like proteins (16). NK cell receptors that belong to the IgSF include activating and inhibitory killer-cell Ig like receptors (KIRs), leukocyte Ig-like receptors (LILRs), leukocyte-associated Ig-like receptors (LAIRs), and natural cytotoxicity receptors (NCRs). NK cell receptors that belong to the C-type lectin-like family of proteins include the CD94 and NKG2 receptor family (17, 18).

For the Ly49H dataset, 13 gene clusters were identified. Interestingly, one of the most prominently enriched gene clusters involved virus receptor activity. NK cells are known to play a role in host defenses against viral infection. Moreover, Ly49H is known to interact with m157, a glycoprotein on murine cytomegalovirus (MCMV) (19). Clusters involved in lymphocyte adhesion were also highly enriched. NK cells express a wide array of integrins and other adhesion molecules, and the expression and activation of these adhesion molecules are important for mediating NK cell activation (20-22). In the NKG2D dataset, 8 gene clusters were identified; and in the combination stimulation of NKG2D and CD244, 3 gene clusters were identified. The most prominent gene cluster identified by these two datasets was related to membrane proteins. This is to be expected seeing as cell surface proteins were enriched for these datasets.
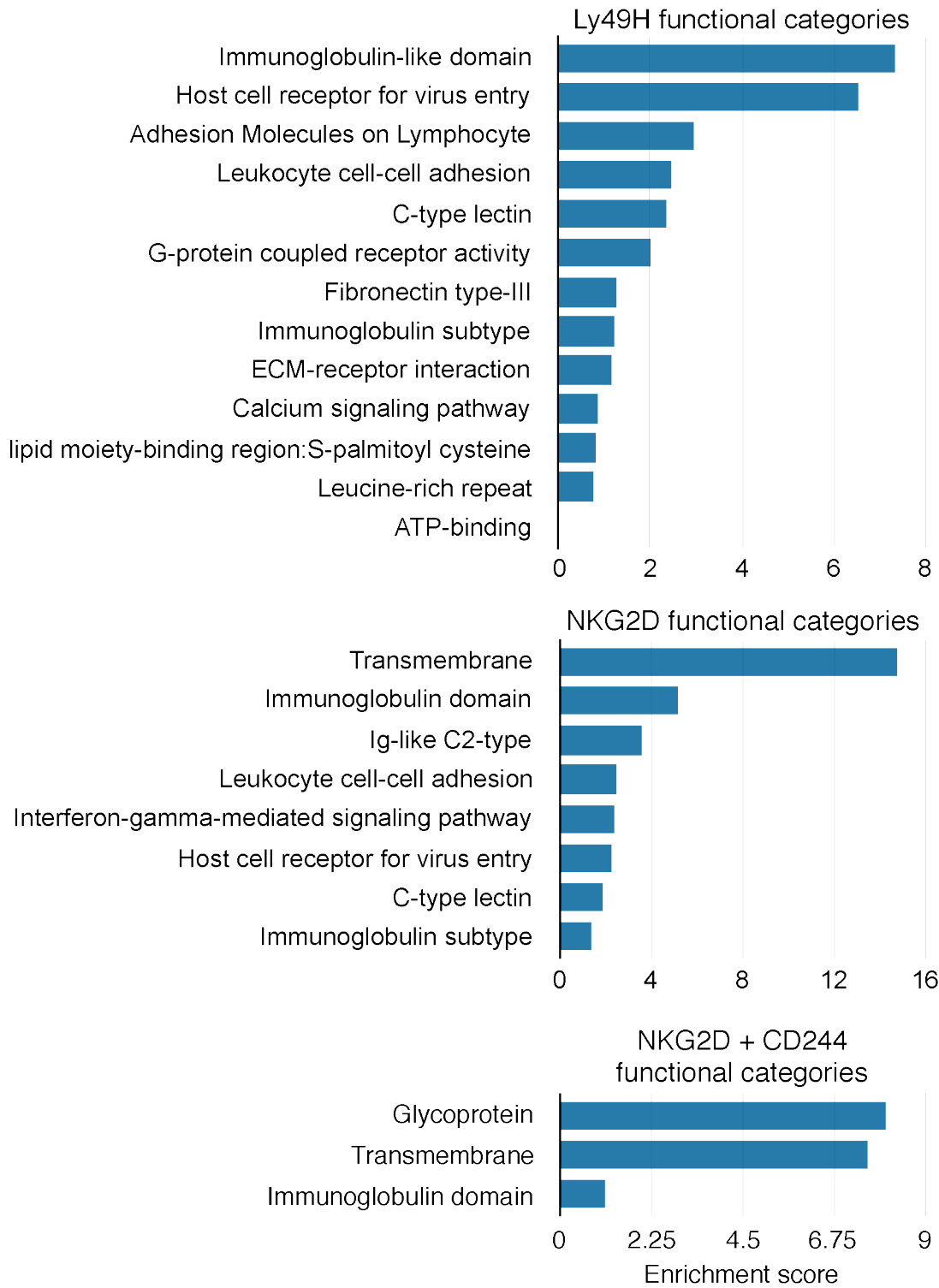
**Figure 2.2 Results from DAVID functional annotation cluster analysis of up- and down-regulated proteins from MS datasets.**

Additionally, STRING analysis (23), which identifies known and predicted protein-protein interactions, identified several functionally related protein clusters (**Supplemental Figure 2.3** – **Supplemental Figure 2.5**). Protein clusters characterized as integral component of the membrane were highly enriched in both the Ly49H and NKG2D datasets. This is to be expected as proteins that are typically expressed at the plasma membrane were specifically enriched for proteomic analysis. Like the GO analysis, protein clusters involved in immune system processes and in cell adhesion were highly enriched in the Ly49H and NKG2D datasets.

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## 2.6 Supplemental Figures
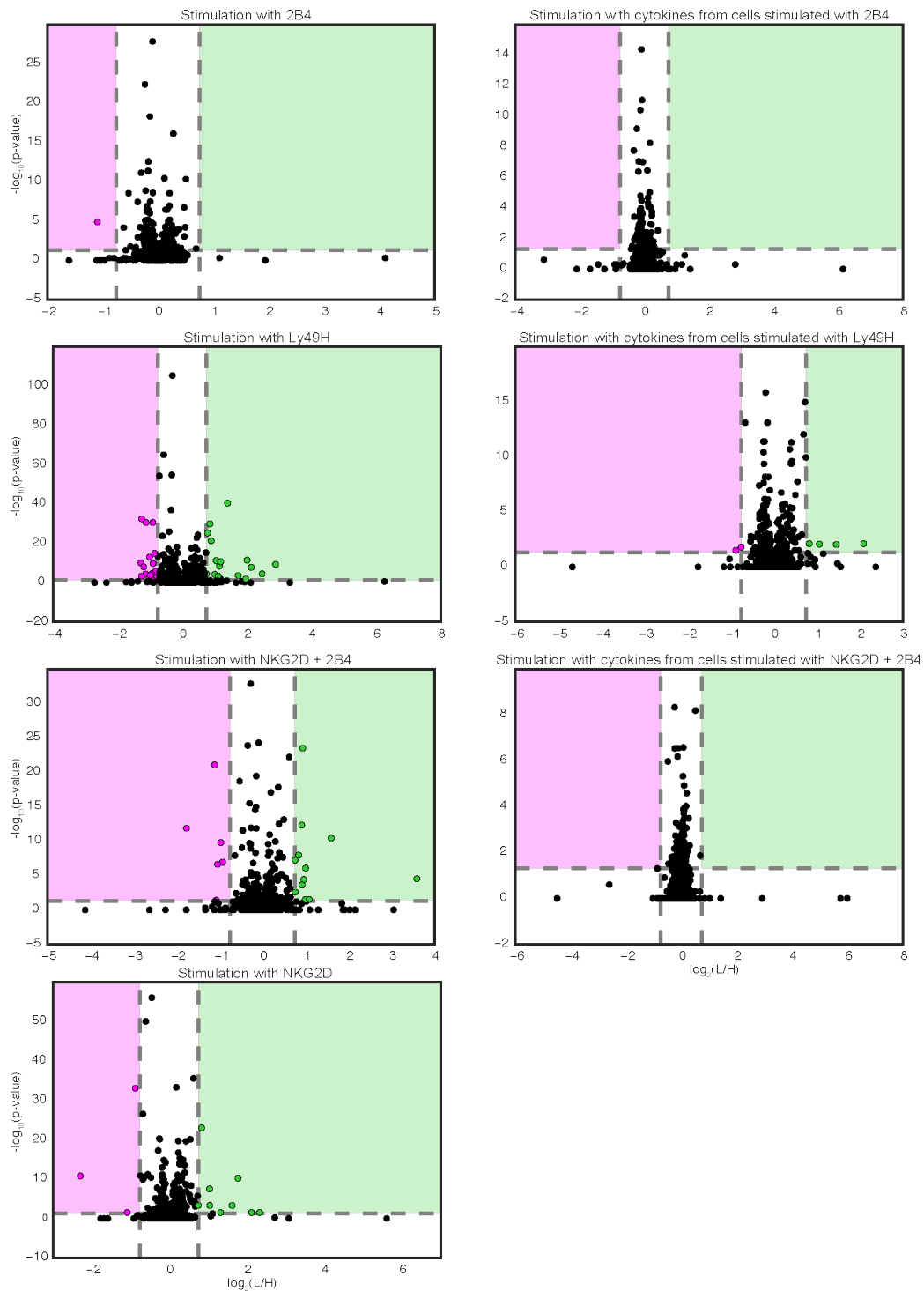


**Supplemental Figure 2.1 Volcano plots of each experiment.** (A, C, E, F) Volcano plots of Ly49H overexpression NKL cell lines that were stimulated with agonistic antibodies against activating or costimulatory receptors. (B, D, G) Volcano plots of Ly49H overexpression NKL cell lines that were stimulated with cytokines that were secreted by cells that had previously been

stimulated with agonistic antibodies against activating or costimulatory receptors. Green dots represent significantly upregulated proteins and magenta dots represent significantly downregulated proteins.
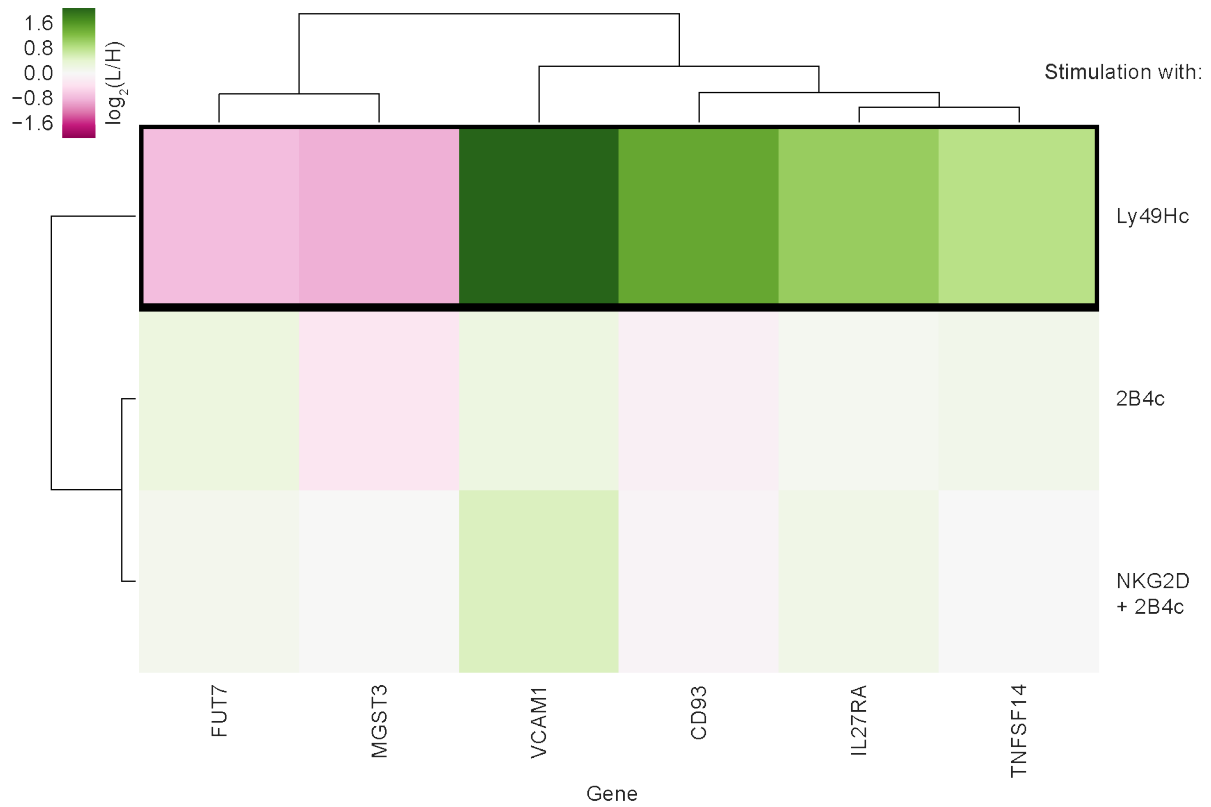
**Supplemental Figure 2.2 Activation with cytokines that were secreted by cells that were stimulated with different activating or costimulatory receptors induces few changes in the cell surface proteome of Ly49H overexpression NKL cells.** Most of these changes are the same as those found from direct stimulation of activating or costimulatory receptors. Significantly up- or down-regulated proteins are boxed in black.
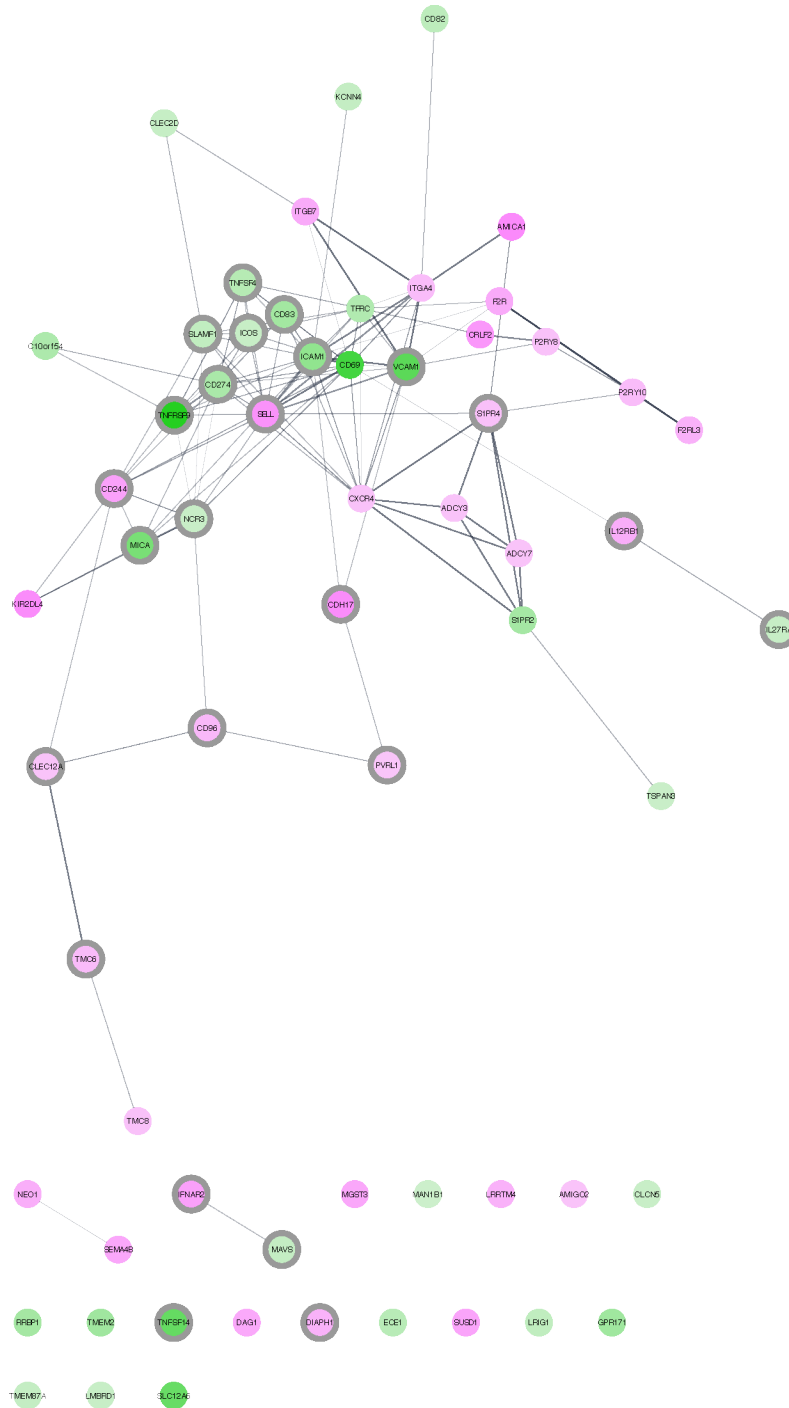
**Supplemental Figure 2.3 STRING analysis of up- and down-regulated proteins identified from Ly49H stimulation of NKL cells.** Up-regulated proteins are in green. Down-regulated proteins are in magenta. Nodes outlined in thick grey lines are involved in immune system processes.
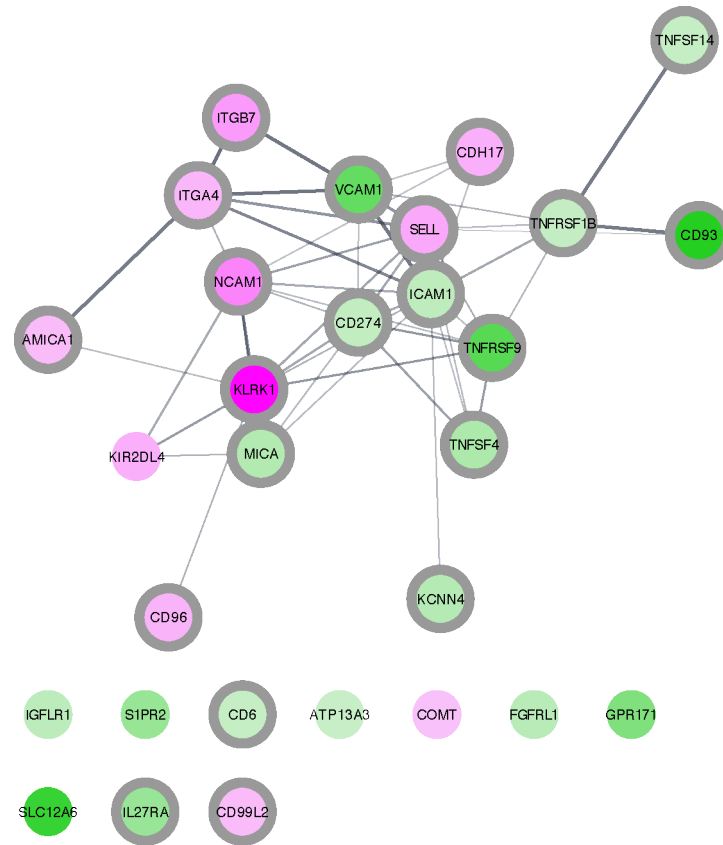
**Supplemental Figure 2.4 STRING analysis of up- and down-regulated proteins identified from NKG2D stimulation of NKL cells.** Up-regulated proteins are in green. Down-regulated proteins are in magenta. Nodes outlined in thick grey lines are involved in immune system processes.
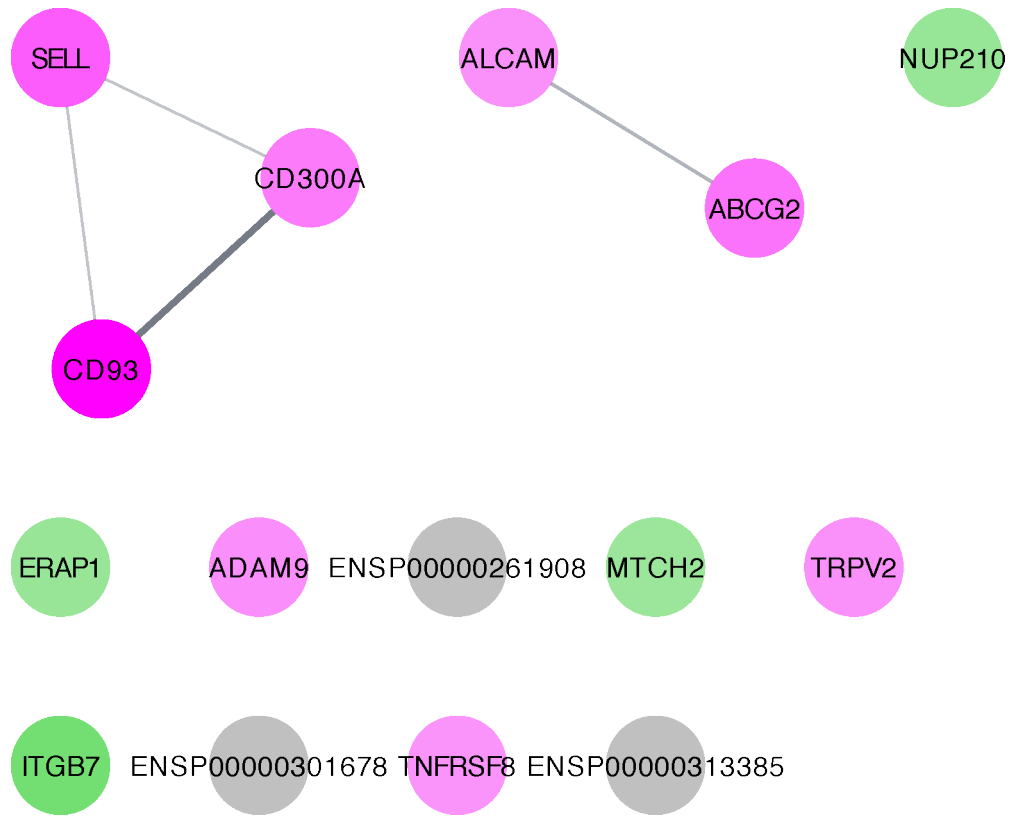
**Supplemental Figure 2.5 STRING analysis of up- and down-regulated proteins identified from combination NKG2D and CD244 stimulation of NKL cells.** Up-regulated proteins are in green. Down-regulated proteins are in magenta.

**Chapter 3: A functional mammalian display screen identifies rare antibodies that stimulate NK cell–mediated cytotoxicity**

## 3.1 Abstract

Therapies that boost the antitumor immune response have shown a great deal of success. Although most of these therapies have focused on enhancing T cell functions, there is a growing interest in developing therapies that can target other immune cell subsets. Like T cells, natural killer (NK) cells are cytotoxic effector cells that play a key role in the antitumor response. To advance the development of NK-based therapies, we developed a functional screen to rapidly identify antibodies that can activate NK cells. We displayed antibodies on a mammalian target cell line and probed their ability to stimulate NK cell–mediated cytotoxicity. From this screen, we identified five antibodies that bound with high affinity to NK cells and stimulated NK cell–mediated cytotoxicity and interferon-γ (IFN-γ) secretion. We demonstrate that these antibodies can be further developed into bispecific antibodies to redirect NK cell–mediated cytotoxicity toward CD20+ B cell lymphoma cells and HER2+ breast cancer cells. While antibodies to two of the receptors, CD16 and NCR1, have previously been targeted as bispecific antibodies to redirect NK cell–mediated cytotoxicity, we demonstrate that bispecific antibodies targeting NCR3 can also potently activate NK cells. These results show that this screen can be used to directly identify antibodies that can enhance antitumor immune responses.

## 3.2 Significance

We have developed a method to screen for antibodies that can induce natural killer (NK) cell–mediated cytotoxicity. NK cells have the innate ability to identify and kill target cells. Antibodies that bind to NK cell surface proteins are anchored to the cell surface of a target cell line and probed for their abilities to stimulate NK cytotoxicity. Target cells displaying antibodies that induce NK cell–mediated cytotoxicity are depleted from the antibody pool. Because the antibodies are based on the same scaffold, antibodies on surviving target cells can be identified

through next generation sequencing of complementarity determining region H3. This method facilitates the identification of antibodies that can stimulate immune cell activation and may be used to design immunotherapies.

### 3.3 Introduction

Cancer immunotherapies have garnered a great deal of success over the last decade. Much of this success has been driven by the development of antibody-based therapeutics that redirect and enhance the cytotoxic potential of CD8+ T cells via immune checkpoint blockade or CD3/T cell receptor (TCR) complex stimulation. Like CD8+ T cells, natural killer (NK) cells are cytotoxic effector cells that mediate antitumor responses (1-3). They play a key role in tumor immunosurveillance and are able to identify and remove target cells by recognizing stress-induced ligands that are frequently overexpressed on cancer cells. NK cells are also known to perform antibody-dependent cellular cytotoxicity (ADCC), a mechanism that is used by multiple current therapeutic monoclonal antibodies to eradicate tumor cells (4-6). Given the crucial role that NK cells play in tumor immunosurveillance, the identification of novel immunotherapies that can target and redirect NK cell cytotoxicity merits further investigation.

Whereas all T cells express the CD3/TCR complex that can be exploited by immunomodulatory molecules to redirect T cell activity, NK cells express multiple activating, costimulatory, and inhibitory receptors that govern NK cell activity (7, 8). Moreover, the NK cell repertoire is highly diverse, and the expression of these activating and inhibitory receptors among different cell subsets varies greatly within and among individuals (9, 10). These factors make it difficult to develop antibodies that can recruit and stimulate NK cells. Here, we report an approach to directly evaluate the ability of a curated set of antibodies to induce NK cell–mediated cytotoxicity.

We couple a mammalian display screen to a next generation sequencing (NGS) readout to characterize antibodies that bind to and activate NK cells. Antibodies were selected against six NK cell receptors from an antigen-binding fragment (Fab)-phage library that was based on

the trastuzumab scaffold and were displayed on a target cell line to generate a mammalian display library. NK cells have the innate ability to recognize and kill unhealthy cells. We reasoned that an antibody against an NK cell surface protein that was displayed on a target cell could drive the interaction between an NK cell and a target cell. If the antibody was also able to activate NK cells, then the cell displaying the antibody would be killed and deselected. All of our antibodies are constructed on the same scaffold, allowing the use of the same set of primers to amplify and sequence the complementarity determining region (CDR) H3 of each clone. Thus, we rationalized that we should be able to screen these antibodies in a pooled manner and quantify the depletion of specific antibody clones through NGS of CDR H3. Indeed, antibody binders that were depleted in our functional screen were able to stimulate NK cell cytotoxicity and interferon-γ (IFN-γ) secretion. We found that the most potent stimulators of NK cell–mediated cytotoxicity were high-affinity binders to previously identified activating NK receptors, like CD16, NCR1, and NCR3, and that binding to an upregulated NK cell surface protein, TNFSF4, or costimulator NK receptors, TNFRSF9 and CD244, were unable to stimulate NK activity. These activating antibodies were applied to the generation of bispecific antibodies to redirect NK cells toward CD20+ B cell lymphoma cells and HER2+ breast cancer cells. We believe this method can facilitate the discovery of rare antibodies that can stimulate immune cell activation and promote the design of immunotherapies.

## 3.4 Results

### Development of Antibodies against NK Cells.

In order to determine how to best target NK cells for the generation of NK cell–based immunotherapies, we sought to generate antibodies toward NK cell antigens with well-understood roles in NK cell activation. We chose to develop antibodies against CD16A (11, 12), NCR1 (13, 14), and NCR3 (15), three well-characterized activating receptors that are known to initiate NK cell–mediated cytotoxicity. We also chose to generate antibodies against the costimulatory receptors, CD244 (16) and TNFRSF9 (4-1BB) (17), because costimulatory

receptors can synergize with other activating receptors and signals (18, 19) to stimulate NK cells. Lastly, we chose to develop antibodies against TNFSF4 (OX40L), a ligand that can be up-regulated upon NK stimulation (20) but is not known to regulate NK cell–mediated cytotoxicity.

To generate antibodies against these antigens, we expressed the extracellular domains (ECDs) of these proteins as tobacco etch virus (TEV)-cleavable crystallizable fragment (Fc) fusions and performed Fab-phage display selections to enrich for high-affinity antibody binders (**Supplemental Figure 3.1**), as previously described (21). After each selection, Fab-phage enzyme-linked immunosorbent assays (ELISAs) were performed to determine the relative affinity and selectivity of these binders for their antigen targets (**Supplemental Figure 3.2**). Multiple antibodies were generated against each receptor, with a total of 69 antibodies isolated against six NK cell receptors (**Supplemental Table 3.1**).

**Functional Screen Identifies Activating Antibodies.**

To evaluate the properties that are needed to generate effective NK cell engagers, we developed a pooled functional screen to assess the abilities of the selected antibody clones to induce NK cytotoxicity. The 69 antibodies that were generated, along with an anti-green fluorescent protein (GFP) control, were pooled and converted into single-chain Fabs (scFabs). These were displayed on a Jurkat cell line (**Figure 3.1A**) to generate a small mammalian display library. The scFabs were robustly expressed at the cell surface, as determined by staining for human-specific Fab (**Supplemental Figure 3.3**). This scFab mammalian display library was incubated for either 4 or 24 h in the presence or absence of either resting or interleukin-2 (IL-2)–stimulated purified peripheral blood NK cells. We hypothesized that Jurkat cells displaying antibody clones that could stimulate NK cytotoxicity would be depleted from the library when in the presence of NK cells. This depletion could be quantified through NGS of the most unique CDR of the antibodies, CDR H3 (**Figure 3.1B**).
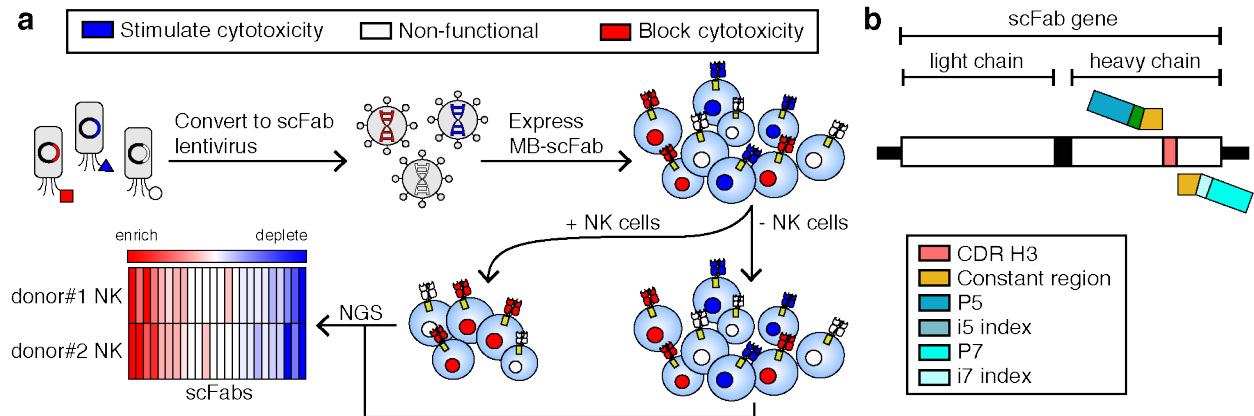
**Figure 3.1 Schematic of the functional screen.** (A) The phage from NK cell antigen selections were screened via ELISA, and Fabs with unique CDRs were converted into scFabs. Jurkat cells were transduced with membrane-bound (MB) scFabs to generate the mammalian display library. The library was incubated in the presence or absence of peripheral blood NK cells, and surviving cells were subjected to next generation DNA sequencing to identify scFabs that were depleted by NK cells. (B) The scFab gene was integrated into the Jurkat genome. P5 and P7 were Illumina adapters sequences and the i5 and i7 indices were used to distinguish different samples. Sequencing of CDR H3 was used to distinguish different scFabs.

Because NK cells are highly heterogeneous and can vary greatly between individuals (9, 10), we performed separate experiments using NK cells isolated from two different blood donors. Although the results of the functional screen were not completely reproducible, particularly for the 4-h time point by donor 2, pairwise comparisons of the normalized NGS signals for the biological replicates generally showed good reproducibility, particularly at the 24-h time point (*Supplemental Figure 3.4*). Surprisingly, although all of the antibodies were generated to target NK cell surface proteins, only four antibodies, CD16.03, NCR1.11, NCR3.18, and NCR3.19, were depleted in the presence of NK cells (**Figure 2** and **Dataset S1**). Interestingly, only antibodies that targeted known activating receptors appeared to induce NK cell–mediated cytotoxicity. Many tumors have been known to overexpress stress-induced ligands that can stimulate NK cell activity (2, 3). Like many of these tumors, Jurkat cells have been shown to express NK cell ligands (22, 23). These ligands could potentially synergize with costimulatory receptor signaling to promote NK cell–mediated cytotoxicity. However, antibodies targeting costimulatory receptors, like TNFRSF9 and CD244, or other cell surface proteins, like

TNFSF4, on NK cells were not depleted. This implies that even if tumors overexpress NK cell ligands, the recruitment of NK cells through costimulatory receptors or other NK cell surface antigens may not be sufficient to drive tumor cell lysis.
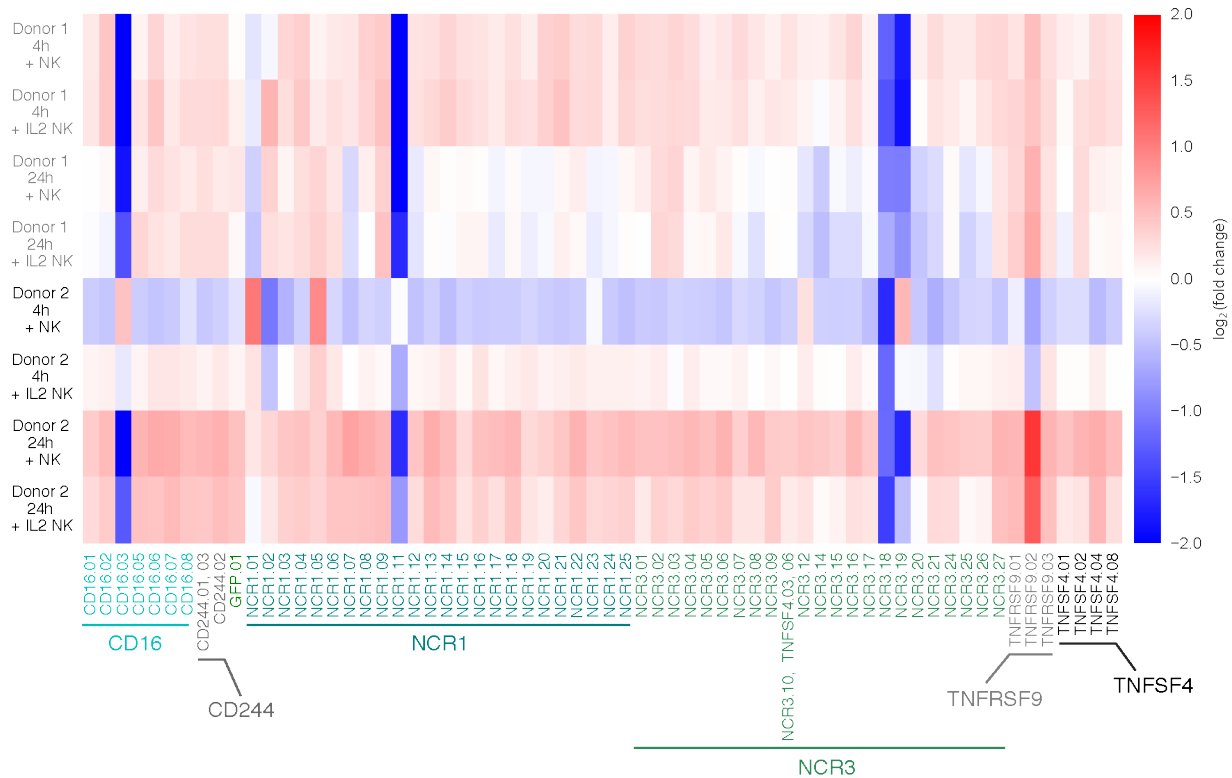


**Figure 3.2 A functional mammalian display screen identifies antibodies that stimulate NK cytotoxicity.** Sixty-nine scFabs targeting six NK cell antigens were displayed on Jurkat cells to generate the mammalian display library. The library was incubated with resting or IL-2–stimulated peripheral blood NK cells from two different donors for either 4 or 24 h. NGS counts were normalized to the mammalian display library that was cultured for 4 or 24 h in the absence of NK cells. Only four scFabs, CD16.03, NCR1.11, NCR3.18, and NCR3.19, were depleted by NK cells. Positive NGS signals (enriched) are shown in red, and negative NGS signals (depleted) are shown in blue.

**Validation of Antibody Hits Identified in Screen.**

To validate the observations made by the functional screen, we chose to characterize the activity of nine antibody clones, four that were identified as activating and five that were identified as nonfunctional. We generated the immunoglobulin G (IgG) versions of these antibodies and tested their abilities to stimulate NK cell cytotoxicity in an antibody-redirected lysis assay (**Figure 3A** and **Dataset S2**). In this assay, FcγR+ THP-1 cells would bind to the Fc

portion of the IgGs, and the Fab arms would bind the effector NK cells. If the Fab arms were able to stimulate NK cell–mediated cytotoxicity, then the THP-1 target cells would be lysed. Gratifyingly, all four antibodies that were identified as activating, CD16.03, NCR1.11, NCR3.18, and NCR3.19, were able to induce NK cytotoxicity. Moreover, four of the five antibodies that were identified as nonfunctional, CD16.08, NCR1.01, NCR1.05, and TNFRSF9.01, did not appear to stimulate NK cytotoxicity. However, one of the antibodies that was identified as nonfunctional, NCR3.12, appeared to stimulate NK cell–mediated cytotoxicity.
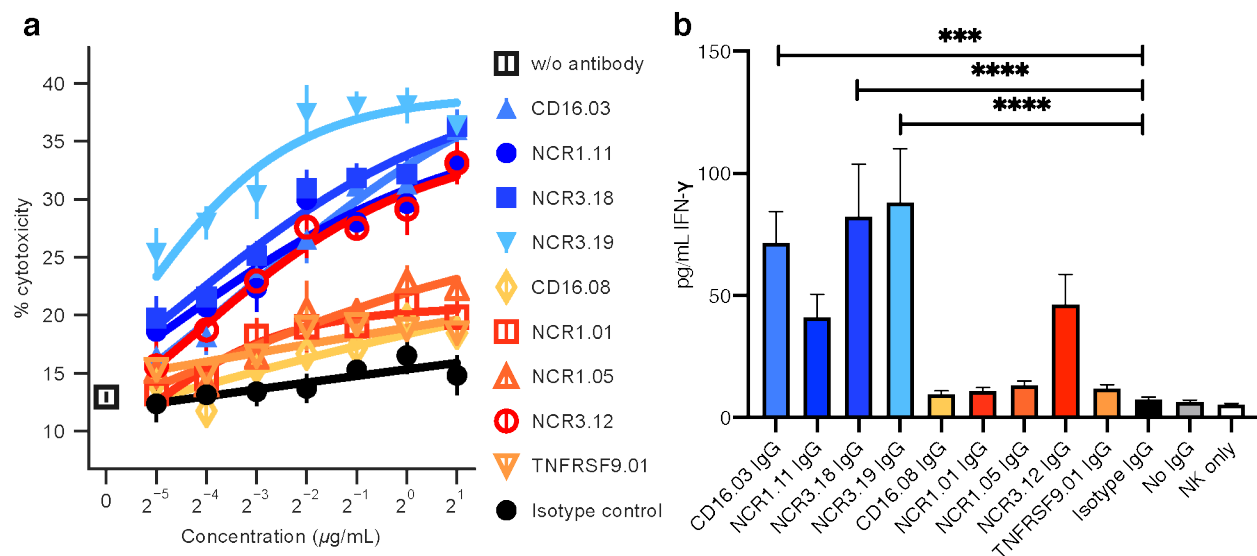


**Figure 3.3 In vitro activity of antibodies identified from a functional screen against FcγR+ cell lines.** (A) A redirected lysis assay using peripheral blood NK cells against FcγR+ THP-1 cells in the presence of varying concentrations of antibodies was used to assay the ability of antibodies to stimulate NK cell-mediated cytotoxicity. Data are representative of five independent experiments. (B) An IFN- secretion assay using peripheral blood NK cells against FcγR+ P815 cells in the presence or absence of varying concentrations of antibodies was used to assay the ability to activate NK cells. All four antibodies that were identified as activating in the functional screen were able to elicit NK cytotoxicity and IFN-γ secretion. Only one antibody that was identified as nonstimulatory in the functional screen was able to elicit NK cytotoxicity and IFN- secretion. Values represent mean ± SEM of eight different donors. ***P < 0.001; ****P < 0.0001.

We also sought to determine if other NK cell effector functions, like cytokine secretion, could be stimulated with our antibodies. To determine if our antibodies were also able to induce cytokine secretion, we measured the amount of IFN-γ produced by NK cells that were coincubated with FcγR+ P815 cells and IgG. Only the activating antibodies, CD16.03, NCR1.11,

NCR3.18, and NCR3.19, and the putative nonfunctional antibody, NCR3.12, were able to significantly increase the amount of IFN-γ secreted (**Figure 3*B*** and **Dataset S3**). Although NCR3.12 was able to stimulate NK cell activity, it does not stimulate as much cytotoxicity or IFN-γ secretion as the other activating antibodies.

**Activating Antibodies Have High Affinity for Their Receptor Targets.**

Although many of the antibodies target the same cell surface receptors, not every antibody was able to stimulate NK cell activity. To better understand the differences between activating and nonfunctional antibodies, we determined the specificity and affinity of the antibodies. To investigate the specificity of both activating and nonfunctional antibodies for their receptor targets, we developed a tetracycline-inducible cell line for each protein target—CD16, NCR1, NCR3, CD244, TNFRSF9, and TNFSF4. The ECDs of these proteins were fused to a generic transmembrane domain and were expressed upon tetracycline addition. Both activating and nonfunctional antibody clones bound exclusively to cells that overexpressed their respective receptor targets. No off-target binding was observed (***Supplemental Figure 3.5***), demonstrating that both activating and nonfunctional antibodies were highly selective for the receptor targets that they had been selected against.

We also determined if antibody affinity played a role in the difference between activating and nonfunctional antibodies. To evaluate the affinity of the selected antibodies for NK cells, we titrated the nine Fab clones on peripheral blood NK cells. For antibodies that bound to the activating receptors, CD16, NCR1, and NCR3, activating antibodies were found to bind more tightly to NK cells than nonfunctional antibodies (***Supplemental Figure 3.6*** and **Dataset S4**). Additionally, NCR3.12, the putative nonfunctional antibody that was able to stimulate NK cell activity, had a much lower affinity than the activating antibodies, NCR3.18 and NCR3.19. This suggests that high-affinity antibodies to activating receptors were better able to induce NK cell-mediated cytotoxicity. Importantly, the nonfunctional antibody that bound to the costimulatory receptor, TNFRSF9, bound with high affinity yet displayed little to no NK cell activity. This further

supported our functional screen results, which suggested that activating receptors should be targeted to induce NK cell-mediated cytotoxicity.

**Generation of Bispecific Antibodies toward CD20+ B Cell Lymphoma Cells and HER2+ Breast Cancer Cells.**

To demonstrate that these antibodies may be used to further the development of NK therapeutics, we generated CD20-targeting bispecific antibodies. CD16.03, NCR1.11, NCR3.12, and NCR3.19 were converted into single-chain variable fragments (scFvs) and associated with the anti-CD20 Rituximab Fab with a flexible linker. Additionally, to test if scFv domain ordering or Fab arm linkage has an effect on binding or stimulating cytotoxicity, we generated constructs with different variable heavy (VH) and variable light (VL) domain orders, whether VH-VL (HL) or VL-VH (LH), and attached the scFv to either the heavy or light chain of the CD20 Fab (**Figure 4A**). We then evaluated their ability to redirect NK cell–mediated cytotoxicity toward CD20+ Daudi B cell lymphoma cells. All of the bispecific constructs generated were able to bind to their respective antigens in a dose-dependent manner (***Supplemental Figure 3.7***) and promote the lysis of Daudi cells (**Figure 4 *B–E***). Moreover, CD20xNCR3.12_B was as effective as the anti-CD20 human IgG1 monoclonal antibody (mAb), and CD20xCD16.03_D, CD20xNCR1.11_B, and CD20xNCR1.11_D were found to be even more effective than the anti-CD20 human IgG1 mAb (**Figure 4*F*** and **Datasets S5–S9**). This suggests that designing high-affinity bispecific antibodies that target other activating receptors, like NCR1 or NCR3, may be as effective as, if not more effective than, antibodies inducing ADCC.
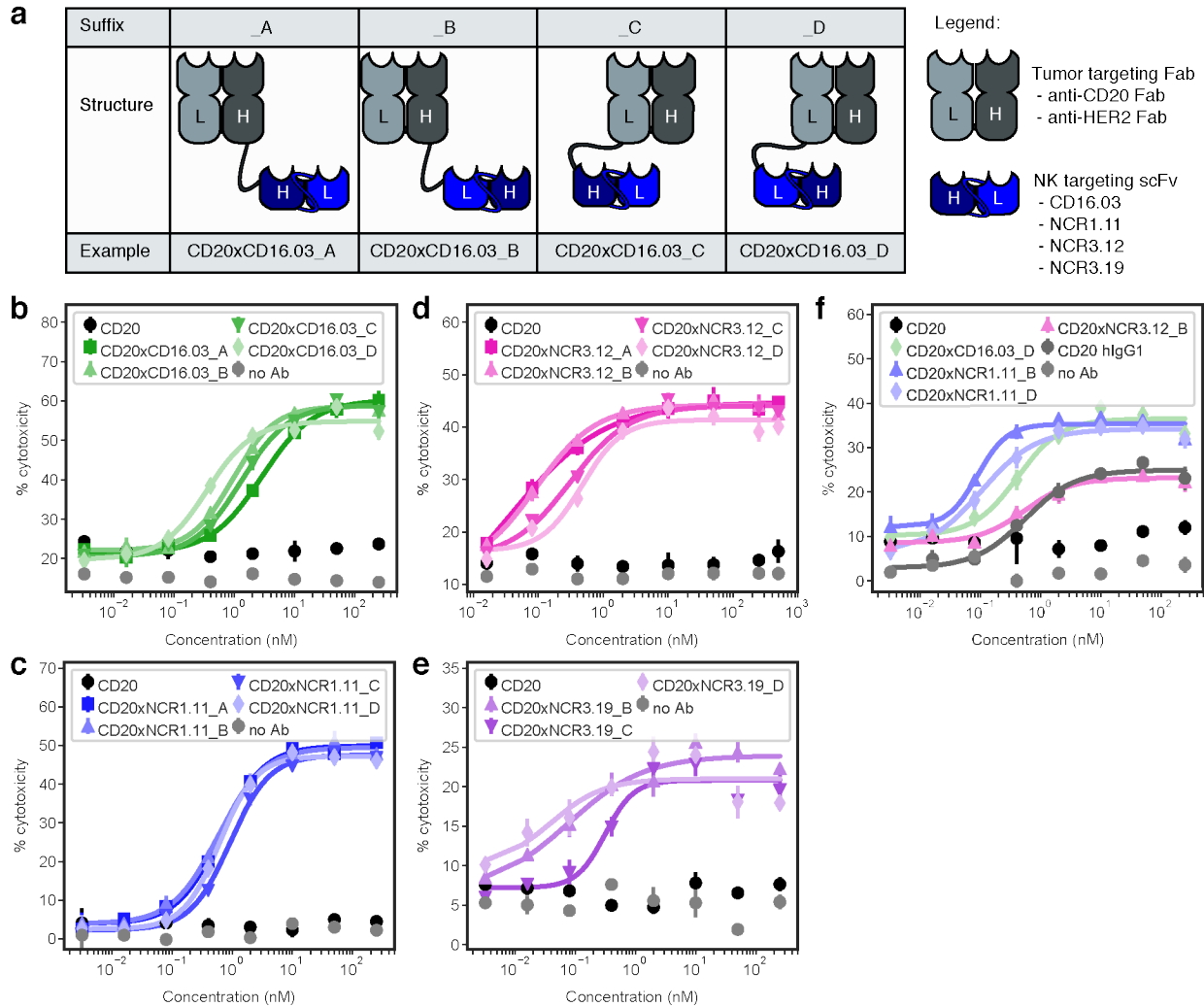
**Figure 3.4 Bispecific constructs generated and cytotoxicity induced by bispecific constructs against CD20+ Daudi.** (A) Each NK-targeting antibody was converted into an scFv (in blue) and attached to either the light chain (L) or heavy chain (H) of the tumor-targeting Fab (in gray). The tumor antigen was either CD20 or HER2. Cytotoxicity induced by bispecific constructs against CD20+ Daudi. (B) Cytotoxicity induced by PBMCs in the presence of anti-CD20–scFv CD16.03 bispecifics at an Effector-to-target ratio (E:T) of 10:1. (C) Cytotoxicity induced by NCR1+ NKL cells in the presence of anti-CD20–scFv NCR1.11 bispecifics at an E:T of 3:1. (D) Cytotoxicity induced by NCR3+ NK92MI cells in the presence of anti-CD20–scFv NCR3.12 bispecifics at an E:T of 1:9. (E) Cytotoxicity induced by NCR3+ NK92MI cells in the presence of anti-CD20–scFv NCR3.19 bispecifics at an E:T of 1:9. (F) Comparison of cytotoxicity induced by PBMCs to anti-CD20 human IgG1 mAb.

While all constructs were able to stimulate NK cytotoxicity, some subtle but consistent differences were observed. Whereas almost all of the NCR1.11-based bispecific antibodies appeared to be of somewhat equal efficacy, certain CD16.03-, NCR3.12-, and NCR3.19-based bispecific antibodies were more effective than others. Of the CD16.03-based bispecific

antibodies, the LH domain ordering was more potent than their HL counterparts. Additionally, linkage of the CD16.03 scFv to the anti-CD20 light chain was more effective than linkage to the heavy chain. In comparison, the CD20xNCR3.12_B bispecific antibody stimulated NK cytotoxicity better than any of the other NCR3.12-based bispecific antibodies. Furthermore, of the NCR3.19-based bispecific antibodies, the bispecifics with the LH-based domain order appeared to outperform the bispecific with an HL-based domain order. Overall, LH ordering in the scFv induced NK cell–mediated cytotoxicity more robustly than the HL ordering. However, differences in efficacy due to the linkage of the scFv to either the light chain or the heavy chain of the tumor-targeting Fab may be dependent on the NK cell targeting scFv.

To demonstrate the versatility of these constructs, we also generated HER2-targeting bispecific antibodies from NCR1.11. The NCR1.11 antibody was converted into an scFv and associated with the anti-HER2 Trastuzumab Fab. Again, constructs with different domain orders and attachment to either chain of the Fab were generated, and their ability to lyse HER2+ SK-BR3 breast cancer cells was evaluated. All of the constructs were able to redirect NK cell–mediated cytotoxicity toward SK-BR3 cells (*Supplemental Figure* **3.8** and **Dataset S10**), demonstrating that these antibodies can be reformatted to target different tumor cell types.

**Bispecific Antibodies Promote Cytotoxicity in Rituximab-Refractory B Cell Lymphoma Cells.**

To determine if the bispecific antibodies generated would be able to redirect NK cell–mediated cytotoxicity toward primary B cell lymphomas, we tested the efficacy of three of the most potent bispecific antibodies, CD20xCD16.03_D, CD20xNCR1.11_B, and CD20xNCR3.12_B, against the SC1 lymphoma line. The SC1 cell line was derived from a patient with a highly refractory CD79-mutated diffuse large B cell lymphoma, originating in skin and metastasizing to the brain and cerebrospinal fluid. The tumor was refractory to a combination rituximab plus cyclophosphamide, vincristine, adriamycin and prednisone, as well as to high-dose methotrexate plus rituximab. It was also refractory to combination etoposide plus cytarabine and

to irradiation. All of the bispecific antibodies tested were able to redirect NK cell–mediated cytotoxicity toward SC1 lymphoma cells (**Figure 5**). While the bispecific antibodies generated were more potent than the anti-CD20 human IgG1 mAb against the CD20+ Daudi cell line, the anti-CD20 human IgG1 mAb was slightly more effective against the SC1 lymphoma cells than the bispecific antibodies. The different cytotoxicities observed between Daudi and SC1 lymphoma cells could be due to the different binding affinities of the bispecific antibodies and the anti-CD20 human IgG1 mAb toward the two different lymphoma cell lines. Indeed, CD20 expression levels are lower and more variable in the SC1 lymphoma cells than in the CD20+ Daudi cell line (***Supplemental Figure 3.9***). In contrast to the bispecific antibodies that only have a single CD20-binding arm, the bivalent nature of the anti-CD20 human IgG1 mAb may allow the IgG to better bind to SC1 lymphoma cells.
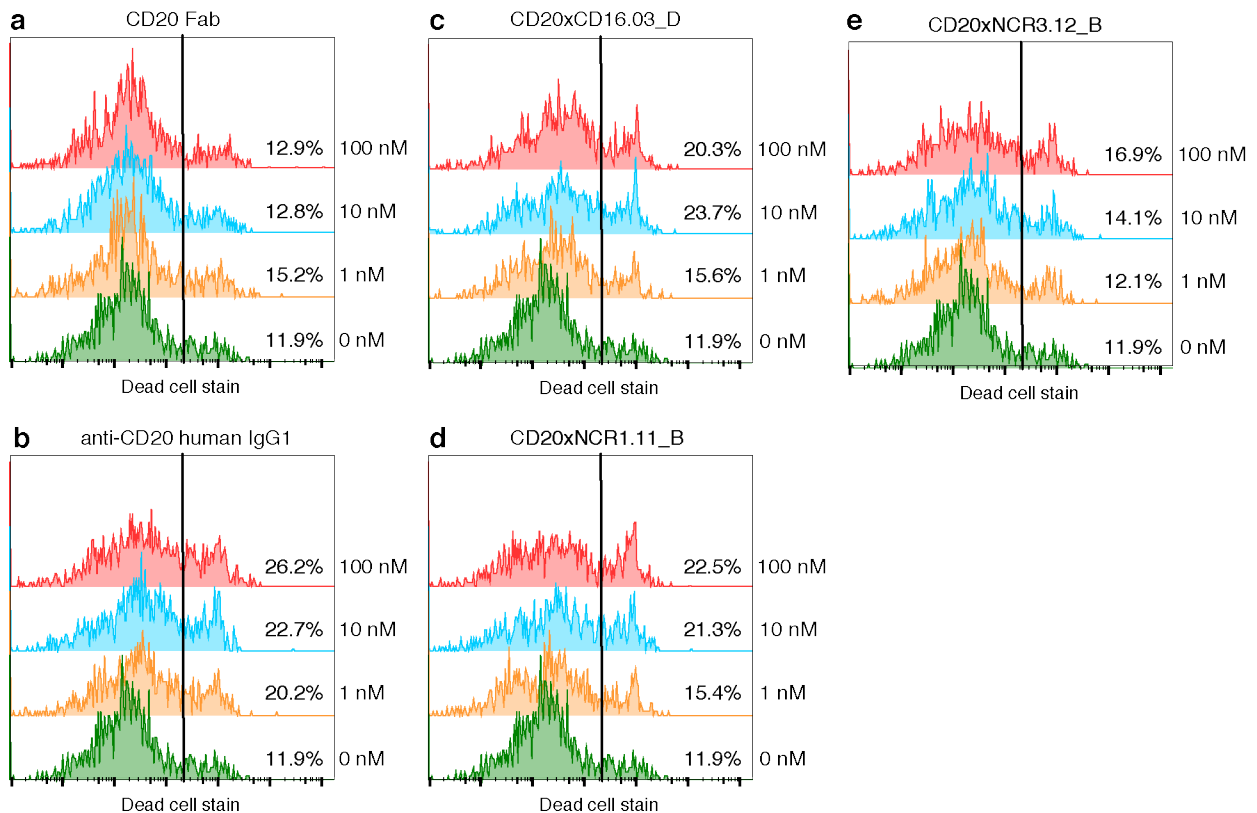


**Figure 3.5 Cytotoxicity of SC1 lymphoma cells by the following antibodies:** (A) Anti-CD20 Fab. (B) Anti-CD20 human IgG1 mAb. (C) Anti-CD20–scFv CD16.03 bispecifics. (D) Anti-CD20–scFv NCR1.11 bispecifics. (E) Anti-CD20–scFv NCR3.12 bispecifics.

## 3.5 Discussion

NK cells have the unique ability to recognize and kill unhealthy cells and are known to play a key role in cancer immunosurveillance. As such, they have become an attractive target for developing new cancer immunotherapies. In this study, we describe an approach to identify functional antibodies that can recruit and stimulate NK cell activity. From the hits identified from our mammalian display screen, we demonstrated the potential of generating various NK cell–targeting therapeutics by constructing bispecific antibodies to redirect NK cell–mediated cytotoxicity toward CD20+ lymphoma cells, as well as HER2+ breast cancer cells.

To facilitate the advancement of NK cell–targeting therapeutics, we developed a functional mammalian display screen to directly assess the ability of a curated set of 69 antibodies to stimulate NK cell–mediated cytotoxicity. Others have previously used phage display (24) and hybridoma technology (25) against purified antigens to identify NK cell binders. Using mammalian display, we are able to assess these unique functional effects and rapidly identify clones for further investigation. Indeed, other groups have used mammalian display to successfully identify individual antibody or peptide clones that induce unique phenotypes (26, 27) or stimulate specific functional effects (28). Inspired by such work, we created a functional screen to assess the unique cytotoxic effects of NK cells. Moreover, as our desired phenotype was amenable to the large sequencing capabilities of NGS, we were able to quantify the functional effects of all of our clones in parallel.

We developed multiple antibodies to target different NK cell surface proteins, including known activating receptors CD16, NCR1, and NCR3; costimulatory receptors TNFRSF9 and CD244; and an NK cell receptor with no known regulatory role TNFSF4. Surprisingly, only 4 of 69 antibodies were depleted from the mammalian display library by the introduction of NK cells, demonstrating the effectiveness of our screening method. Interestingly, all of these antibodies targeted known NK-activating receptors, like CD16, NCR1, and NCR3. However, it should be

noted that the majority of our antibodies in the functional screen target NCR1 and NCR3. This could potentially bias our functional screen toward antibodies that stimulate NCR1 or NCR3.

Upon further analysis, we determined that high-affinity antibodies targeting activating receptors were able to stimulate NK cell–mediated cytotoxicity and IFN-γ secretion, whereas low-affinity antibodies targeting the same receptors were not able to stimulate NK cell activity. This is consistent with previous findings that demonstrated that higher-affinity CD16 polymorphisms were better able to mediate ADCC (29, 30) and were associated with a higher response rate to rituximab, trastuzumab, and cetuximab treatment (4-6). Although only a select number of antibodies were chosen for additional testing, the correlation that we found between antibody affinity and NK cell activity agrees with previous reports describing binders toward CD16 and NCR1. Others have previously shown that antibodies that bind to epitopes outside of the Fc-binding site of CD16 can stimulate ADCC and that higher-affinity CD16 binders are more potent than their lower-affinity counterparts (31, 32). Additionally, NCR1-binding antibodies are able to stimulate NK cell–mediated cytotoxicity, regardless of which domain on NCR1 is targeted (25). This suggests that high-affinity antibodies are needed to stimulate NK cell activity.

Although developing high-affinity antibodies toward NK cell receptors appears to be needed to stimulate NK cell activity, we have found that high-affinity antibodies targeting other NK cell receptors, outside of known activating NK cell receptors, were not able to stimulate NK cell–mediated cytotoxicity. It is not entirely surprising that targeting costimulatory receptors did not result in NK cell activation as others have previously shown that NK cell activation typically requires coengagement of different activating and costimulatory NK cell receptors (18, 19).

To demonstrate the utility of the antibodies identified by the functional screen, we converted four of the activating antibodies, CD16.03, NCR1.11, NCR3.12, and NCR3.19, into NK-targeting bispecific antibodies. All of the CD20-targeting bispecific and the Her2-targeting bispecific antibodies generated were able to redirect NK cell–mediated cytotoxicity toward CD20+ Daudi B cell lymphoma cells and Her2+ SK-BR3 breast cancer cells, respectively. This

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suggests that the antibodies identified by the screen may be used to further develop different NK cell–targeting therapeutics. Indeed, high-affinity antibodies targeting CD16 (31, 32) and NCR1 (25) have previously been developed to create bispecific and trispecific NK cell engagers and redirect NK cell–mediated cytotoxicity, and they appeared to have good efficacy in vitro and in vivo. In this study, several of the bispecific antibodies, including an NCR3-targeting bispecific antibody, were at least as potent as the anti-CD20 human IgG1 mAb, suggesting that developing antibodies against NCR3 may also be an effective way to recruit and stimulate NK activity.

In addition to promoting robust lysis of the well-established CD20+ Daudi B cell lymphoma cell line, our bispecific antibodies were also able to redirect NK cell–mediated cytotoxicity toward the highly refractory SC1 B cell lymphoma line. However, our bispecific antibodies were not any more efficacious than the anti-CD20 human IgG1 mAb in promoting the lysis of SC1 B cell lymphoma cells. This may be due to the avidity effect that the anti-CD20 human IgG1 mAb has toward CD20+ cells. Although our bispecific antibodies were not any more efficacious than the anti-CD20 human IgG1 mAb in this case, additional engineering to improve the affinity of the tumor-targeting moiety may further promote the cytotoxic potential of the bispecific antibodies developed. More importantly, the antibodies identified via our functional screen appear to be amenable toward the development of additional NK cell–targeting engagers.

Given the growing interest in developing antibodies to target other immune cell types to the tumor microenvironment, we believe that this method may be useful in identifying targets and antibodies that can redirect the cytotoxic or phagocytic functions of other immune cell types. The size of the mammalian display library can be increased to probe a larger set of immune cell receptors. Additionally, the same mammalian display library may be used to screen the functions of multiple immune cell types, so as to determine if certain subsets of antibodies may be used to cross-react with different cell types. Moreover, since all of these antibodies are

based on the same scaffold, the desired antibody can be easily cloned and converted into different multispecific formats. We believe that this work provides important insights into the design of NK cell–targeting antibodies and illustrates a method that may be useful for identifying immunotherapeutic antibodies.

## 3.6 Materials and Methods

Peripheral blood mononuclear cells (PBMCs) and human NK cells were isolated from peripheral blood of deidentified healthy donors (Blood Centers of the Pacific or Vitalant). Details of the cells used, as well as the methods used for the phage display selections, ELISAs, lentiviral production, functional screen implementation and analysis, antibody expression and purification, flow cytometry, and cytotoxicity assays, are presented in **_Supplemental Materials and Methods_**. Additional questions pertaining to methods, protocols, and reagents are available upon request.

## 3.7 Data availability

Python scripts used to analyze the NGS data, plot Fab-phage ELISAs, and plot cytotoxicity and on cell titration curves have been deposited in GitHub (https://github.com/e6kang/EK_screen). All other study data (Datasets S1–S9) are included in the article and/or supporting information.

## 3.8 Acknowledgements

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### 3.10 Supplemental Materials and Methods

**Cells**

HEK293T cells were cultured in DMEM supplemented with 10% FBS and 100 IU/mL penicillin and 100 μg/mL streptomycin. Jurkat and Raji cells were cultured in RPMI-1640 containing 2mM L-glutamine containing 10% FBS and 100 IU/mL penicillin and 100 μg/mL streptomycin. NK92MI cells were cultured in α-MEM without ribonucleosides and deoxyribonucleosides, but containing 2mM L-glutamine, and supplemented with 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 12.5% horse serum, 12.5% FBS, and 100 IU/mL penicillin and 100 μg/mL streptomycin. NKL cells that were stably transduced with NCR1 were maintained in RPMI-1640 containing 2mM L-glutamine containing 10% FBS and 100 IU/mL penicillin and 100 μg/mL streptomycin, supplemented with 200U/mL IL-2 (National Cancer Institute BRB Preclinical Repository). SK-BR3 cells were cultured in McCoy's 5a supplemented with 10% FBS and 100 IU/mL penicillin and 100 μg/mL streptomycin.

PBMCs were isolated by Ficoll-Paque and maintained in RPMI-1640 containing 2mM L-glutamine containing 10% FBS and 100 IU/mL penicillin and 100 μg/mL streptomycin. Primary human NK cells were isolated from peripheral blood of de-identified, healthy donors (Blood Centers of the Pacific or Vitalant) using RosetteSep (StemCell) followed by Ficoll-Paque. The cells were maintained in RPMI-1640 containing 2mM L-glutamine containing 10% FBS and 100 IU/mL penicillin and 100 μg/mL streptomycin.

Tetracycline inducible cell lines overexpressing NK cell surface protein ECDs were generated by co-transfecting pOG44 vector with a construct encoding each ECD fused to the transmembrane domain of platelet-derived growth factor with a HA tag in the pcDNA5/FRT mammalian expression vector.

**Phage display selections**

TEV-cleavable Fc-fusion proteins were expressed and biotinylated in Expi293F cells using the standard expression protocol. Media were harvested after 4 days of expression and

protein was purified by protein A affinity chromatography. Phage selections were performed according to previously established protocols[1] with the Fab-phage Library E[2]. Non-specific binders were depleted from the library by incubating the phage pool with Fc-domain immobilized on streptavidin beads. Fab phage were selected using biotinylated Fc-fusions that were captured on streptavidin-coated magnetic beads, and were released through TEV elution. Each selection consists of four rounds. With each round, decreasing amounts of Fc-fusions (1 μM, 100 nM, 10 nM, and 10 nM) were used. ELISAs were performed for 96 individual Fab-phage clones from the third or fourth rounds of selection to evaluate for affinity and selectivity. The best clones were pooled and converted to scFabs and subcloned to a lentiviral expression vector for further characterization with our functional screen.

**Fab Phage ELISAs**

ELISAs were performed as previously described[1]. In brief, Maxisorp plates were coated with 10 μg/mL of Neutravidin overnight at 4 °C. Biotinylated target antigen (20 nM) was captured on the Neutravidin coated plates for 30 min, and then exposed to a 1:5 dilution of phage supernatants for 30 min. Bound phage were detected via a horseradish-peroxidase-conjugated anti-phage monoclonal antibody (GE Lifesciences).

**Production of lentivirus in HEK293T cells**

Lentivirus was produced by the transfection of $2.2×10^6$ HEK 293T cells in T-25 flask, using 3 μg of lentiviral expression vector from the pooled scFab NK cell binders, 0.33 μg of pMD2.G, and 2.7 μg of pCMV-dR8.91, and 15 μL of FuGENE HD transfection reagent (Promega). After 48 hr, cell supernatant was collected and cellular debris was removed by a 45-μm pore filter. Jurkat cells were transduced at an MOI < 0.3.

**Functional screen of scFab mammalian display system**

Freshly isolated NK cells were cultured in the presence or absence of 400U/mL IL-2 for 16 hours. The scFab mammalian display library was washed and was incubated for 4 hr or 24 hr with 10μg/mL DNase I while in the presence or absence of resting or IL-2-stimulated NK

cells. Surviving cells were collected and genomic DNA was isolated and used as a PCR template for NGS. The H3 sequence was amplified from the genomic DNA with flanking primers using Q5 DNA polymerase (NEB). The mix was thermocycled for 20 cycles. The amplicon was gel purified and submitted to the CZBiohub for analysis on a NextSeq (Illumina) with a custom sequencing primer (as shown):

TGAGGACACTGCCGTCTATTATTGTGCTCGC.

The .fastq.gz files were processed using Galaxy (https://usegalaxy.org/). Sequencing artifacts were removed, and adapter sequences were clipped with a custom sequence (as shown):

TACTGGGGTCAAGGAACCCTGGTCAAGATCGGAAGAGCACACGTCTGAACTCCAGTCAC.

A FASTQ masker was applied when the quality score fell below 30. And sequence counts were exported for further analysis. Raw NGS counts for each condition was normalized to counts per million (CPM), and depletion of specific antibody clones was reported as the $\log_2$(fold change) between the library when in the presence or the absence on NK cells.

**IgG and bispecific antibody expression**

IgGs were expressed as previously described[3]. In brief, Expi293 cells were transiently co-transfected with two pFUSE vectors containing the heavy and light chains of interest at a 1:1 ratio. For IgGs, the pFUSE vectors contained a Fab heavy chain was fused to a mouse IgG1 Fc or a Fab light chain. For the bispecific antibodies, the pFUSE vectors contained a Fab heavy chain or a Fab light chain that were fused to the scFv of interest. The ExpiFectamine 293 transfection kit was used for transfections as per manufacturer's instructions. Supernatants were harvested after 5-7 days of expression, and protein was purified by Protein A or Protein L affinity chromatography. Proteins were assessed by SDS-PAGE for purity and quality.

**Calcein release cell cytotoxicity assay**

Calcein release cell cytotoxicity assays were performed as previously described[4]. Target cells were washed and resuspended in to a final concentration of 1-5 $\times 10^6$/mL and labeled in 15

µM calcein-AM for 30 min at 37°C. Cells were washed twice and coincubated with effector cells (purified NK cells, PBMCs, NK92MI, or NCR1+ NKL cells) at the indicated effector to target ratio in the presence of varying antibody concentrations in triplicate. Maximum lysis was induced with 1% Triton X-100. After 2 hours, supernatants were collected and calcein release was measured on an Infinite 200 Pro plate reader (Ex: 485 ± 9 nm; Em: 530 ± 20 nm). Specific lysis was calculated as 100 × (experimental target cell release – target cell spontaneous release)/(maximum release − target cell spontaneous release).

**Fab expression**

Fabs were expressed as previously described[5]. In brief, C43 (DE3) Pro +*E. coli* were transformed with expression plasmids and were grown in TB autoinduction media at 37°C for 6 hours. Incubation temperature was then reduced to 30 °C and were grown for an additional 16–18 hr. Cells were harvested by centrifugation and Fabs were purified by Protein A affinity chromatography. Fab purity and integrity was assessed by SDS-PAGE.

**On cell titration for flow cytometry**

Titrations were performed on primary human NK cells or tetracycline induced overexpression cell lines as indicated. When tetracycline induced overexpression cell lines were used, cells were dosed with 10µg/mL tetracycline for 2 days prior to staining. The starting concentration of each Fab or bispecific antibody was 1 µM, and serial 1:5 dilutions were performed. Antibodies were incubated with cells for 1 hr at 4°C, washed 2 times in 3% BSA in PBS pH 7.4, and stained for 30 min with an Alexafluor-647 conjugated goat anti-human IgG F(ab')$_2$ fragment (Jackson ImmunoResearch Laboratories) at a 1:50 dilution. After an additional 3 washes, cells were fixed and fluorescence was quantified using a CytoFLEX (Beckman Coulter). All flow cytometry data were analyzed using FlowJo software.

**Flow cytometry-based cell cytotoxicity assay**

Flow cytometry-based cell cytotoxicity assays were performed as previously described[6]. In brief, target cells were stained with 1µM CFSE for 20 min at 37°C. Cells were washed and

coincubated with resting NK cells at the indicated effector to target ratio in the presence of varying antibody concentrations in duplicate. Maximum lysis was induced with 0.1% Tween-20. After 2 hours, dead cells were stained with 5nM Sytox Red and fluorescence was quantified using a CytoFLEX (Beckman Coulter). All flow cytometry data were analyzed using FlowJo software.

**IFN-γ secretion assay**

IFN-γ secretion was quantified with the ELISA max deluxe sets (BioLegend) according to the manufacturer's instructions. In brief, NK cells were incubated in the presence or absence of P815 target cells at an effector to target ratio of 1:1 with or without 1µg/mL of each selected antibody for 24 hours. Supernatant was collected and assayed for IFN-γ content.

**Data analysis**

A one-way ANOVA with Dunnett's post hoc test was used for comparison of IFN-γ secretion induced by selected antibodies. Data were analyzed using GraphPad Prism 6.0 software. Dose response curves for the IgGs were fit with a three-parameter logistic model. Dose response curves for the bispecific antibodies were fit with a four-parameter logistic model using a python script.

## 3.11 Supplemental References

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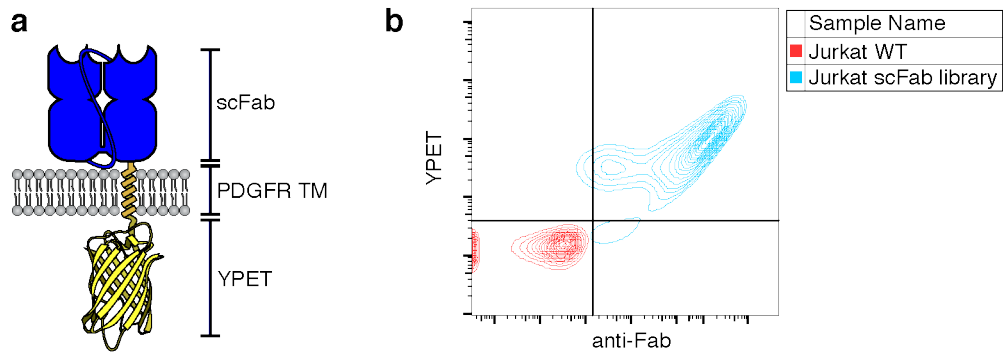
## 3.12 Supplemental Figures
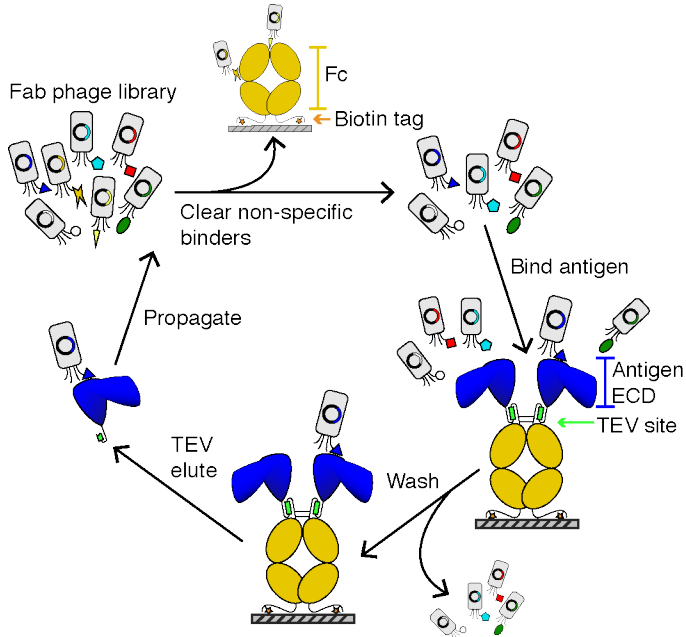


**Supplemental Figure 3.1 Schematic of phage display selections and Fc-fusion construct used to enrich for Fab-phage that selectively bind to NK cell antigens.**
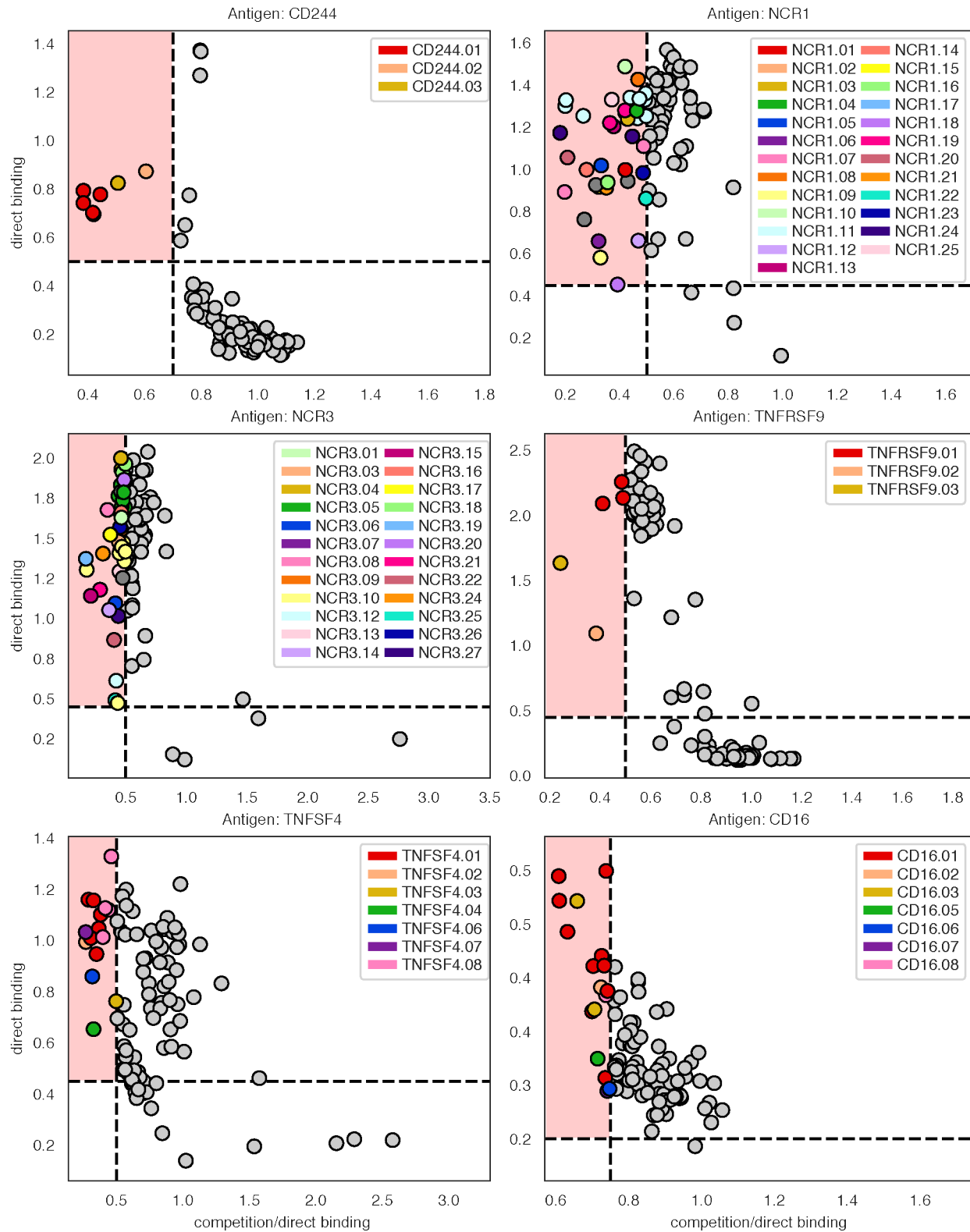
**Supplemental Figure 3.2 Fab-phage ELISAs from selections against NK cell antigens to identify high affinity binders.** On the y-axis, direct binding of Fab-phage to the antigen of interest is plotted. On the x-axis, the competition to direct ratio is shown. For competitive

binding, Fab-phage is preincubated with 20 nM of soluble antigen and then allowed to bind to the antigen coated plates.

**Supplemental Figure 3.3 Expression of membrane bound (MB) scFab library on mammalian target cells.** (A) Format of construct used to display scFabs on mammalian target cells. (B) Expression of MB scFab library on Jurkat target cells. WT Jurkats are in red. MB-scFAB expressing Jurkats are in blue. Expression of scFab at the cell surface was detected with an anti-human Fab antibody.
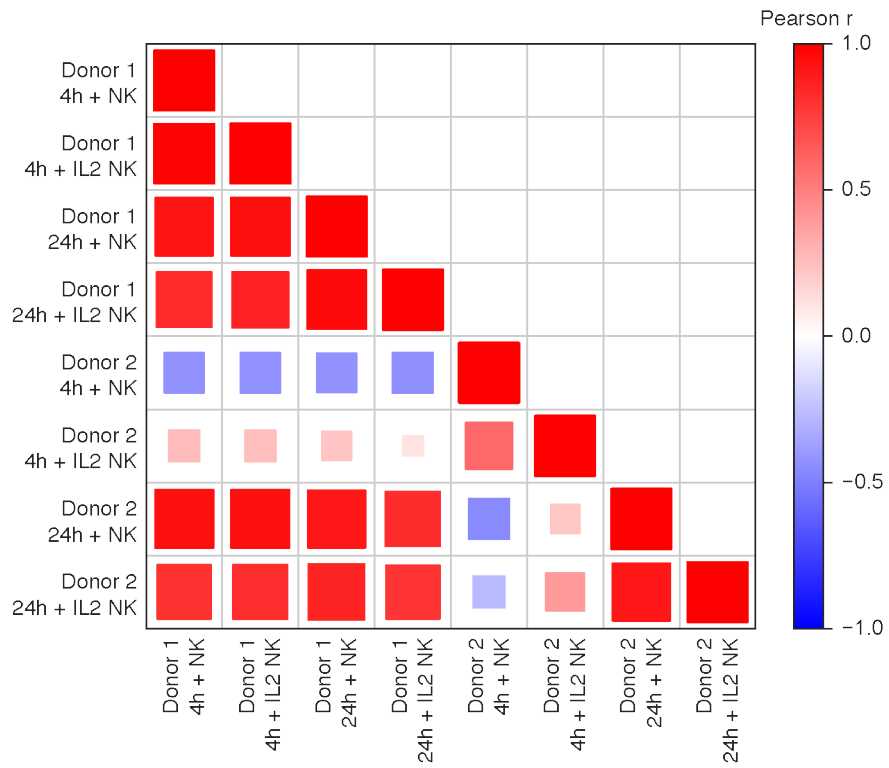
**Supplemental Figure 3.4 Comparisons of biological replicates from two different blood donors correlate well.**
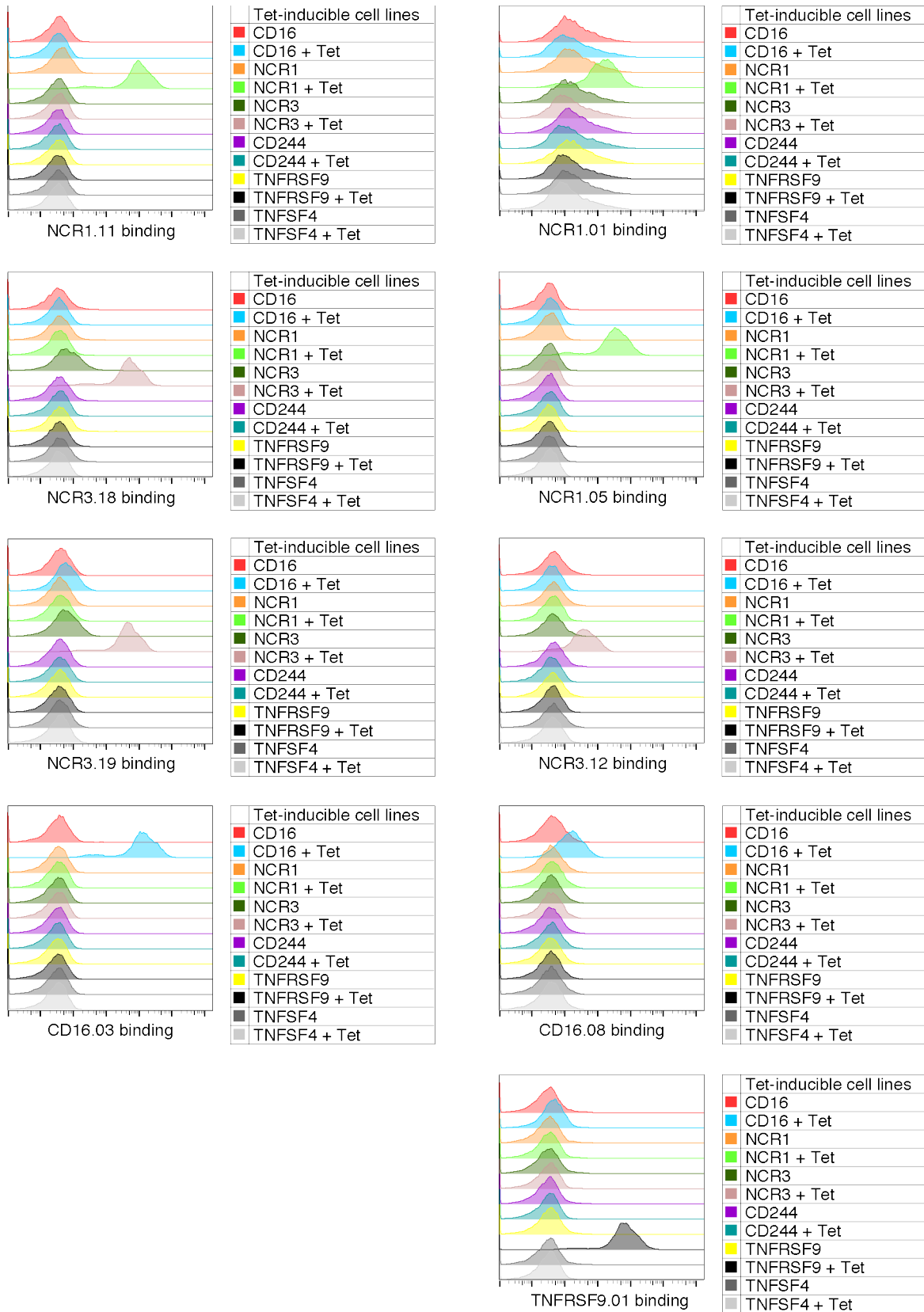
**Supplemental Figure 3.5 Representative flow cytometry histograms demonstrate the**

**selectivity of resultant Fabs towards the antigen they were selected against.** Six tetracycline-inducible FlpIn cell lines were generated to over-express each NK cell antigens upon tetracycline addition.
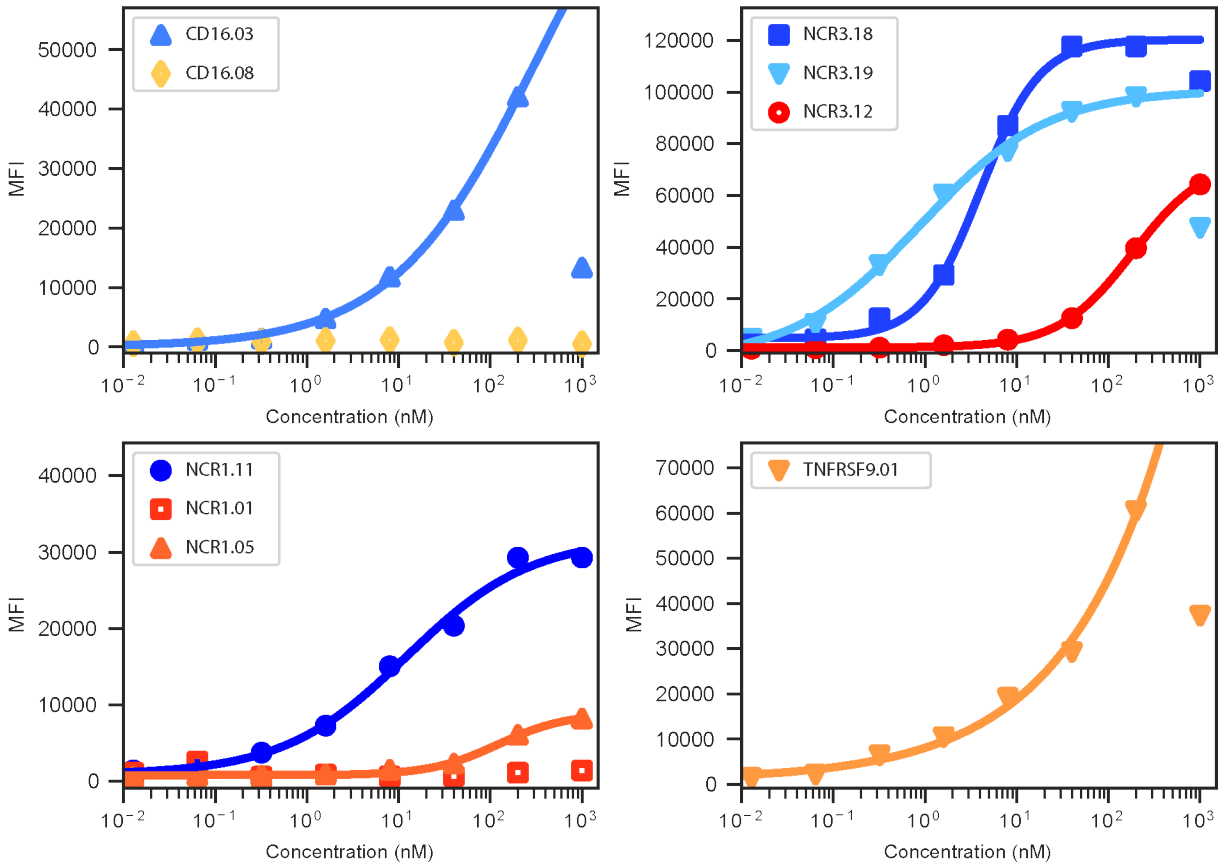
**Supplemental Figure 3.6 Titration of antibodies identified from functional screen against peripheral blood NK cells.** Median fluorescence intensities of NK cells were plotted over a range of antibody concentrations. Antibodies that were identified as activating from the functional screen bound at lower concentrations than almost all of the antibodies that were identified as non-functional.
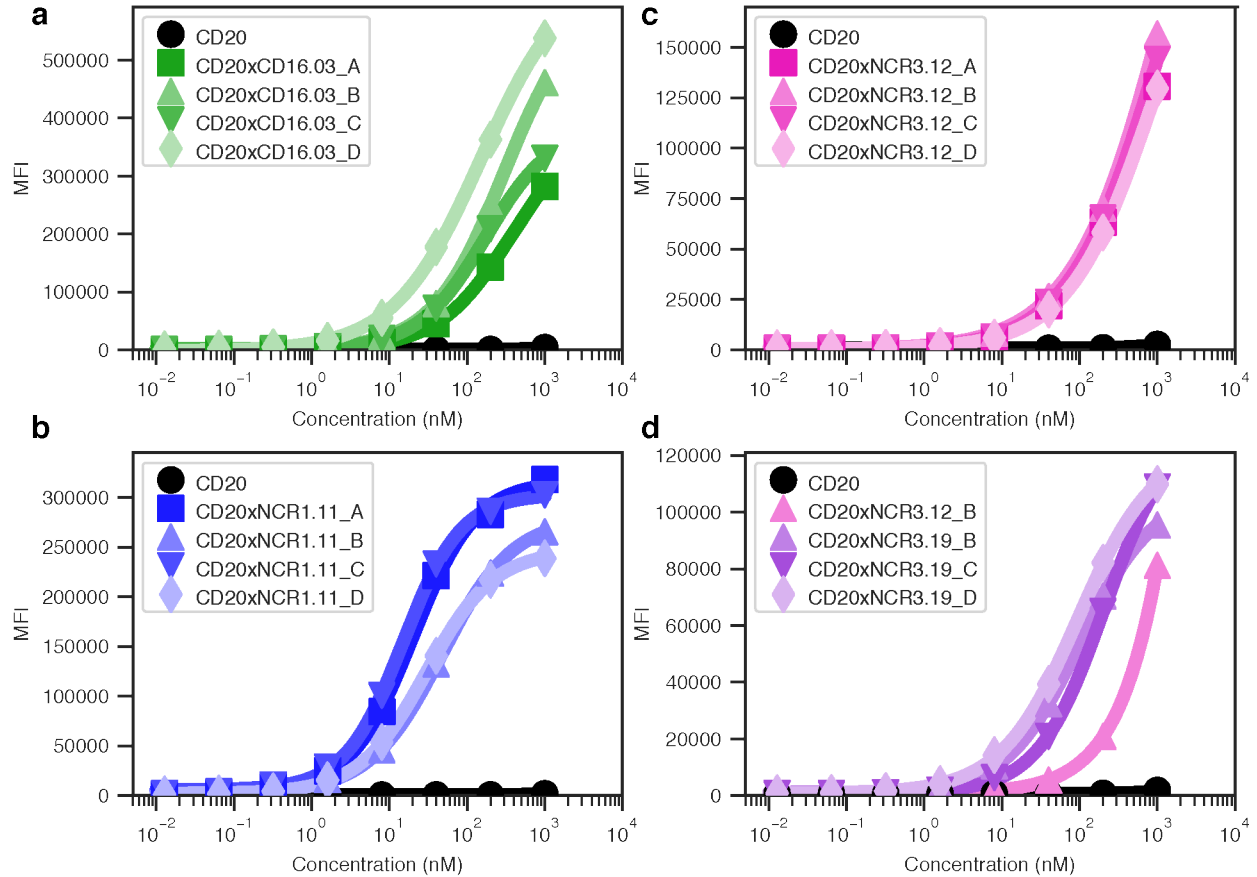
**Supplemental Figure 3.7 Titration of bispecific antibodies towards HEK293 FlpIn cell lines over expressing NK antigens of interest.** (a) Binding of anti-CD20-scFv CD16.03 bispecifics towards CD16 over expression cell line. (b) Binding of anti-CD20-scFv NCR1.11 bispecifics towards NCR1 over expression cell line. (c) Binding of anti-CD20-scFv NCR3.12 bispecifics towards NCR3 over expression cell line. (d) Binding of anti-CD20-scFv NCR3.19 bispecifics towards NCR3 over expression cell line.
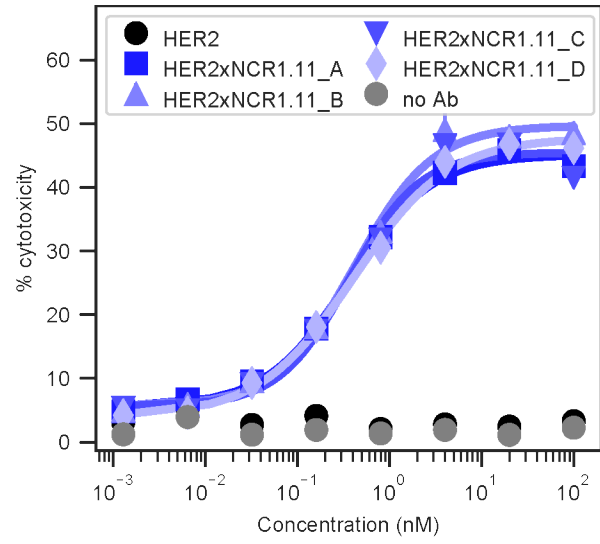
**Supplemental Figure 3.8 Cytotoxicity induced by HER2 targeting bispecific constructs**

## 3.13 Supplemental Tables

**Supplemental Table 3.1 Antibodies and their target antigen and CDRs.**

| Name | Antigen | LC3 | HC1 | HC2 | HC3 |
|---|---|---|---|---|---|
| CD16.01 | CD16 | YRGGRLI | VYSYYI | YISPSSGSTY | GYYSAWRYSMGL |
| CD16.02 | CD16 | AMYWELL | VSSSYI | SIYSYYGYTS | SYSYYSTWYMHRYFYAAF |
| CD16.03 | CD16 | SSAELI | FSSYSI | SIYSSSGSTS | WSYDQYYDQHGYYFYYWGF |
| CD16.05 | CD16 | WPGSLV | FYYYSI | SIYPSSGSTS | WSYHGM |
| CD16.06 | CD16 | SSSSLI | FSSSSI | SISSSSGSTS | FPYRWSYYWYSYYKWYAM |
| CD16.07 | CD16 | YGQRNPI | FYSSYI | SISPYSGYTY | SWYYFSAM |
| CD16.08 | CD16 | SSHHLI | FSSSSI | SIYPSYGYTS | TYSYYAM |
| CD244.01 | CD244 | SVGVPV | LSYSSI | SIYSYYGYTS | YYEYYGYGYAAM |
| CD244.02 | CD244 | SYNYSPL | ISYYSI | SIYPYYGYTS | NYYLYYGM |
| CD244.03 | CD244 | SVGVPV | ISYYSI | SIYSYYGYTS | YYEYYGYGYAAM |
| NCR1.01 | NCR1 | SYYYDLV | FSSSSI | SIYSYYGYTS | YKFKNAYWWSYYNTQGVGM |
| NCR1.02 | NCR1 | SMYHQLI | VSYSSI | SIYSYYGSTS | YSFEPSLYSYYWDSRSAF |
| NCR1.03 | NCR1 | WWSSPI | VSSYSI | SIYSSYGSTY | KMGWYYYWYDGL |
| NCR1.04 | NCR1 | SYYYYLV | FSYSYI | SISSSSGYTS | HYYRDFYQWTGGM |
| NCR1.05 | NCR1 | SWAWPV | FSYSSI | SIYSSYGSTY | FLGGAM |
| NCR1.06 | NCR1 | SYFPPI | LYSSSI | SIYPYYSSTS | SDGYWYWYGWYWGM |
| NCR1.07 | NCR1 | STYEPI | VYYSSI | SISSYSSYTS | EVHWYRYSYWYYYLTQAM |
| NCR1.08 | NCR1 | NWYYPLI | VYYSSI | SISPYSGYTS | MYNYQMYWLFGGGYGM |
| NCR1.09 | NCR1 | SSGWYPL | FSSYSI | SIYSYYGYTS | SESYWPYGWYGF |
| NCR1.10 | NCR1 | SSSSLI | LSSYSI | SISSYYSSTS | SWPYSSQQWYYYYYWYAM |
| NCR1.11 | NCR1 | SSSSLI | VYYSYI | SISSYYGSTY | SRYLQDYWSSWWVSWYGL |
| NCR1.12 | NCR1 | SSSSLI | FSSSSI | SISSSSGSTS | SSMWSFPGGWQHYSFGM |
| NCR1.13 | NCR1 | SSYSLV | FYSSSI | SIYPYYGYTS | PWQPAYYGYAYGM |
| NCR1.14 | NCR1 | SSSSLI | VYSSSI | SISPYYGYTS | TGQYYYSYVYGL |
| NCR1.15 | NCR1 | GYENPL | VYSSYI | SIYSSYGYTS | YAFSLPSMWWYSYSGM |
| NCR1.16 | NCR1 | SWYYPI | FSSSSI | SISSSSGSTS | SYVGRYSYYYYPGMGI |
| NCR1.17 | NCR1 | SAHYPV | FSYSYI | SIYSSSSSTY | EGLGWTWGAWLYWYGGI |
| NCR1.18 | NCR1 | SGWSSPI | VYSSSI | SISPSYGYTS | DILFFSYWYSAL |
| NCR1.19 | NCR1 | SSYHPL | FSYSYI | SISSSSGSTS | WNTMYYYWYRPWGSWAM |
| NCR1.20 | NCR1 | SWHSSSLI | VSYSSI | SIYSYYGYTS | EYQQSYWYKWAL |
| NCR1.21 | NCR1 | GWYFTLI | VYYSSI | SISSSYGSTS | YPRSYFWGMVSRTQGL |
| NCR1.22 | NCR1 | VRFGYPV | VYSSSI | SISSSSGSTS | SDLQQYWWWPSYYGYGGF |
| NCR1.23 | NCR1 | SYVSPI | LYSSSI | YISSSYGYTY | DMYKPGWYYYWGSGDYGF |
| NCR1.24 | NCR1 | SYAWPV | VYSYSI | SISSYSGYTY | NSWLRGTMSAYRYQGL |
| NCR1.25 | NCR1 | YYFKPI | VYSSSI | SISSYSSSTY | NHYYAQSYGIYQYFSGF |

| Name | Antigen | LC3 | HC1 | HC2 | HC3 |
|---|---|---|---|---|---|
| NCR3.01 | NCR3 | SSYEYPV | VYYSSI | SIYPSSGSTY | WYIKWDTWYYSDAM |
| NCR3.02 | NCR3 | SYGSLI | VSSYSI | SIYPYYGSTS | WYQYYYQYSLGF |
| NCR3.03 | NCR3 | SYGFLI | VYSYSI | SIYPYSGYTS | WYNYHHWWSRAF |
| NCR3.04 | NCR3 | SGRQLV | VSSYSI | SIYSYYGSTY | WYQYYPSQGGMAM |
| NCR3.05 | NCR3 | SSWYYPF | LYSYSI | SIYPYYGSTS | WYQYWDWSYSRGAM |
| NCR3.06 | NCR3 | FSLSLL | VYYSSI | SISPSYGYTS | SYSYGFAM |
| NCR3.07 | NCR3 | SFGFYYPF | VYYSSI | SIYPYSGYTS | DYTSNSYGDYYYYGGYGF |
| NCR3.08 | NCR3 | SGYYWPI | FSSSSI | SISSSSGSTS | WLMWFSYAHGAYHMPYGL |
| NCR3.09 | NCR3 | SSKYLV | VYSYSI | YIYPYSGSTS | WFTYHWPGSIAF |
| NCR3.10 | NCR3 | SSYEYPV | VYYSSI | SISPYYGYTS | WYQYSDSIAM |
| NCR3.12 | NCR3 | SSYWPF | ISSSSI | YISSSSGYTS | YSYFYGGYFYWTSWGAF |
| NCR3.13 | NCR3 | YYGYLI | VYSYSI | SISPYYGYTS | WYQYSDSIAM |
| NCR3.14 | NCR3 | SSSSLI | FYSYSI | SISSSSGSTS | YFYDSPYYTYFSNYPSAL |
| NCR3.15 | NCR3 | SSSSLI | VYSSSI | SISSSSGSTS | YVGPSYHSVWYYYSWYYAI |
| NCR3.16 | NCR3 | SWESLV | VSYSSI | SISPYYGYTS | YHDRYTYYFGYYGWSYGM |
| NCR3.17 | NCR3 | HWRVSLI | VSSYSI | SIYSYSGSTS | WYMYYNSDYFGL |
| NCR3.18 | NCR3 | SSSSLI | VSSSSI | SISSSSGSTS | RISSYYMSYYDSFYYAGM |
| NCR3.19 | NCR3 | QWYPLI | VYSYSI | SIYSYYGSTS | WYQYYYIGTAAM |
| NCR3.20 | NCR3 | SSSSLI | LSYSSI | SISSSSGSTS | RYKGIVWWSYWSNWYYMGL |
| NCR3.21 | NCR3 | KGNRLI | ISYSSI | SIYSYYSSTS | YSGGYFLFYTYDYYVYGF |
| NCR3.22 | NCR3 | SSSSLI | VYSYSI | SISSSSGSTS | WYGYYYYGEFAF |
| NCR3.23 | NCR3 | SSYGLF | VSSYSI | SISPYYGYTS | WYQYSDSIAM |
| NCR3.24 | NCR3 | TSSLPI | VYSSSI | SISPYSGYTY | WYMYYYKIAM |
| NCR3.25 | NCR3 | SSSSLI | VSSYSI | SISSYYGSTS | QWEYSSYHYSTWYYAWAM |
| NCR3.26 | NCR3 | SSSSLI | ISYSSI | SISSSSGSTS | RYDSYYWYYYSSYWYYGM |
| NCR3.27 | NCR3 | LYSRLV | VSYSSI | SIYPYSGYTS | PYDYYGWYWGAF |
| TNFRSF9.01 | TNFRSF9 | GSWYSGLI | LYSSSI | SIYCSYGSTY | NWYWYAL |
| TNFRSF9.02 | TNFRSF9 | SYYYDPV | IYSSSI | SIYSYSGSTY | EFVQWHYGYFYYDDWYAF |
| TNFRSF9.03 | TNFRSF9 | SSLGYLI | VSSYSI | SISSYYGYTS | EWWEGWAL |
| TNFSF4.01 | TNFSF4 | TSSSYLI | VYSYSI | SIYPYSGSTS | WYQYSRPRDWGL |
| TNFSF4.02 | TNFSF4 | GYSSSSLI | IYSYSI | SIYPYYSYTS | WYMYAEADAM |
| TNFSF4.04 | TNFSF4 | SWVSGPF | LSSYSI | SIYSYSGYTS | WYHHFYHMAM |
| TNFSF4.06 | TNFSF4 | YYGYLI | VYSYSI | SISPLLWLYF | WYQYSDSIAM |
| TNFSF4.08 | TNFSF4 | SYSDSSLF | VYSYSI | SIYPSYGSTY | WYGYSYYSHEAM |
| GFP.01 | GFP | SWGLI | ISYYSI | SIYPYYSSTS | AGWVASSGM |

**3.14 Supplemental Files**

**Supplemental Datasets S1-S9 can be found at https://www.pnas.org/lookup/suppl/**

**doi:10.1073/pnas.2104099118/-/DCSupplemental**

**Dataset S1:** Dataset_S01.csv

> Description: Raw sequencing count information obtained from NGS. Sequencing counts
>
>> for each donor and experimental condition tested are listed. Total counts for
>>
>> each CDR-H3 and each respective antibody is recorded. This data was used
>>
>> for Figure 3.2.
>
> Published link:
>
> https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/1/pnas.21
>
> 04099118.sd01.csv

**Dataset S2:** Dataset_S02. xlsx

> Description: Raw data from each donor used in the calcein release assay where mIgG1
>
>> were used to redirect NK cell-mediated cytotoxicity towards THP-1 cells (as
>>
>> shown in Figure 3.3A). The work up for each donor is also displayed.
>
> Published link:
>
> https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/2/pnas.21
>
> 04099118.sd02.xlsx

**Dataset S3:** Dataset_S03. xlsx

> Description: Data from each donor used in the IFN-$\gamma$ release assay (as shown in Figure
>
>> 3.3B)
>
> Published link:

https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/4/pnas.21

04099118.sd04.xlsx

**Dataset S4:** Dataset_S04.xlsx

Description: Raw MFI values from the on cell titrations to determine binding affinities of

each Fab towards primary NK cells (as shown in Supplemental Figure 3.6).

Data from two different donors is displayed.

Published link:

https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/5/pnas.21

04099118.sd05.xlsx

**Dataset S5:** Dataset_S05. xlsx

Description: Raw data from each donor used in the calcein release assay where

bispecific antibodies made from the CD16.03 antibody were used to redirect

NK cell-mediated cytotoxicity towards Daudi cells (as shown in Figure 3.4B).

The work up for each donor is also displayed.

Published link:

https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/6/pnas.21

04099118.sd06.xlsx

**Dataset S6:** Dataset_S06.csv

Description: Raw data from each replicate used in the calcein release assay where bispecific antibodies made from the NCR1.11 antibody were used to redirect NK cell-mediated cytotoxicity towards Daudi cells (as shown in Figure 3.4C). The work up for each replicate is also displayed.

Published link:

https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/7/pnas.21 04099118.sd07.xlsx


**Dataset S7:** Dataset_S07.csv

Description: Raw data from each replicate used in the calcein release assay where bispecific antibodies made from the NCR3.12 antibody were used to redirect NK cell-mediated cytotoxicity towards Daudi cells (as shown in Figure 3.4D). The work up for each replicate is also displayed.

Published link:

https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/8/pnas.21 04099118.sd08.xlsx


**Dataset S8:** Dataset_S08.csv

Description: Raw data from each replicate used in the calcein release assay where bispecific antibodies made from the NCR3.19 antibody were used to redirect NK cell-mediated cytotoxicity towards Daudi cells (as shown in Figure 3.4E). The work up for each replicate is also displayed.

Published link:

https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/9/pnas.21 04099118.sd09.xlsx

**Dataset S9:** Dataset_S09.csv

Description: Raw data from each donor used in the calcein release assay where the activity of different bispecifics were compared against a CD20 targeting hIgG1 antibody (as shown in Figure 3.4E). The work up for each donor is also displayed.
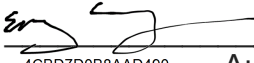
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https://www.pnas.org/highwire/filestream/993695/field_highwire_adjunct_files/10/pnas.2104099118.sd10.xlsx

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8/31/2021

Date