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A functional mammalian display screen identifies rare antibodies that stimulate NK cell–mediated cytotoxicity

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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.

antibody | natural killer cells | immunotherapy | bispecific antibodies

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

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 desig immunotherapies.

Author contributions: E.K. and J.A.W. designed research; E.K. performed research; C.K., J.L.R., and L.L.L. contributed new reagents/analytic tools; E.K. analyzed data; L.L.L. advised on research; and E.K. and J.A.W. wrote the paper.

Competing interest statement: E.K., L.L.L., and J.A.W. have filed a patent application through the University of California, San Francisco and the Parker Institute for Cancer Immunotherapy to cover some of the antibody sequences described herein. C.K. is the Scientific Founder, fiduciary Board of Directors member, Scientific Advisory Board member, shareholder, and consultant for Foghorn Therapeutics, Inc. (Cambridge, MA).

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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.

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 (*SI Appendix*, Fig. S1), 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 (*SI Appendix*, Fig. S2). Multiple antibodies were generated against each receptor, with a total of 69 antibodies isolated against six NK cell receptors (*SI Appendix*, Table S1).

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 (Fig. 1*A*) to generate a small mammalian display library. The scFabs were robustly expressed at the cell surface, as determined by staining for human-specific Fab (*SI Appendix*, Fig. S3). 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 (Fig. 1*B*).

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 (*SI Appendix*, Fig. S4). 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 (Fig. 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.

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 (Fig. 3*A* 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

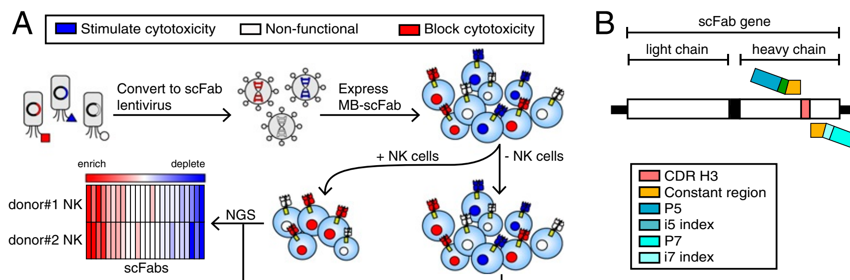

Fig. 1. Schematic of the functional screen. (*A*) The phage from NK cell antigen selections were screened via ELISA, and Fabs with unique CDRs were converted into scFabs. Jurkat cells were transduced with membrane-bound (MB) scFabs to generate the mammalian display library. The library was incubated 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.

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Fig. 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.

nonfunctional, NCR3.12, appeared to stimulate NK cell–mediated cytotoxicity.

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 (Fig. 3*B* and Dataset S3). Although NCR3.12 was able to stimulate NK cell activity, it does not

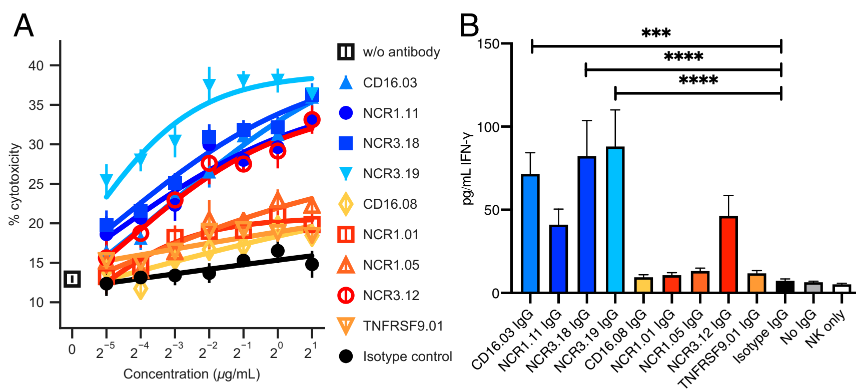

Fig. 3. In vitro activity of antibodies identified from a functional screen against FcγR+ cell lines. (*A*) A redirected lysis assay using peripheral blood NK cells against FcγR+ THP-1 cells in the presence of varying concentrations of antibodies was used to assay the ability of antibodies to stimulate NK cell-mediated cytotoxicity. Data are representative of five independent experiments. (*B*) An IFN-γ 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-γ secretion. Values represent mean $\pm$ SEM of eight different donors. $^{***}P < 0.001$; $^{****}P < 0.0001$.

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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 (*SI Appendix*, Fig. S5), 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 (*SI Appendix*, Fig. S6 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 (Fig. 4*A*). 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 (*SI Appendix*, Fig. S7) and promote the lysis of Daudi cells (Fig. 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 (Fig. 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.

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 (*SI Appendix*, Fig. S8 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 (Fig. 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 (*SI Appendix*, Fig. S9). 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.

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.

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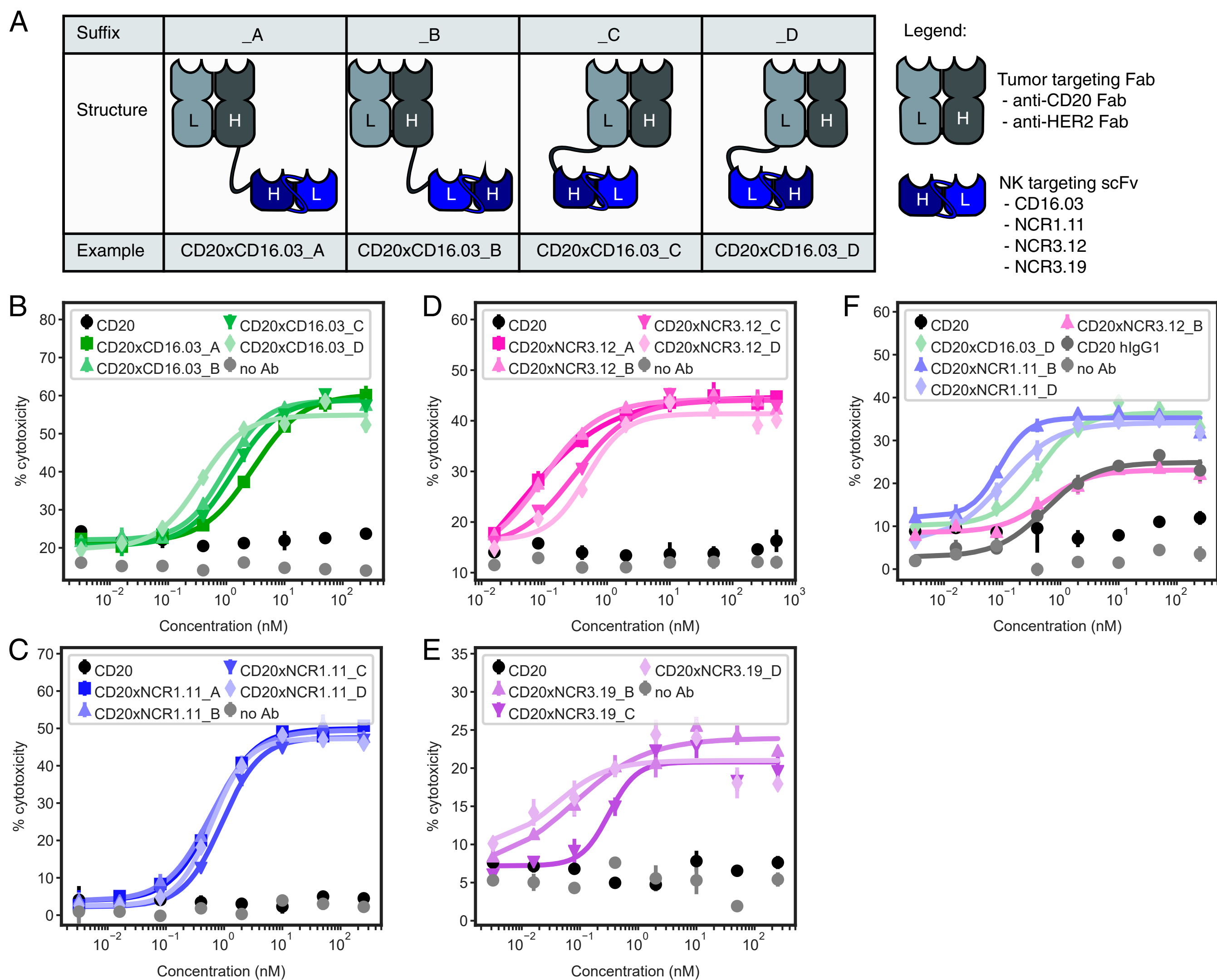

Fig. 4. Bispecific constructs generated and cytotoxicity induced by bispecific constructs against CD20+ Daudi. (*A*) Each NK-targeting antibody was converted into an scFv (in blue) and attached to either the light chain (L) or heavy chain (H) of the tumor-targeting Fab (in gray). The tumor antigen was 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.

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

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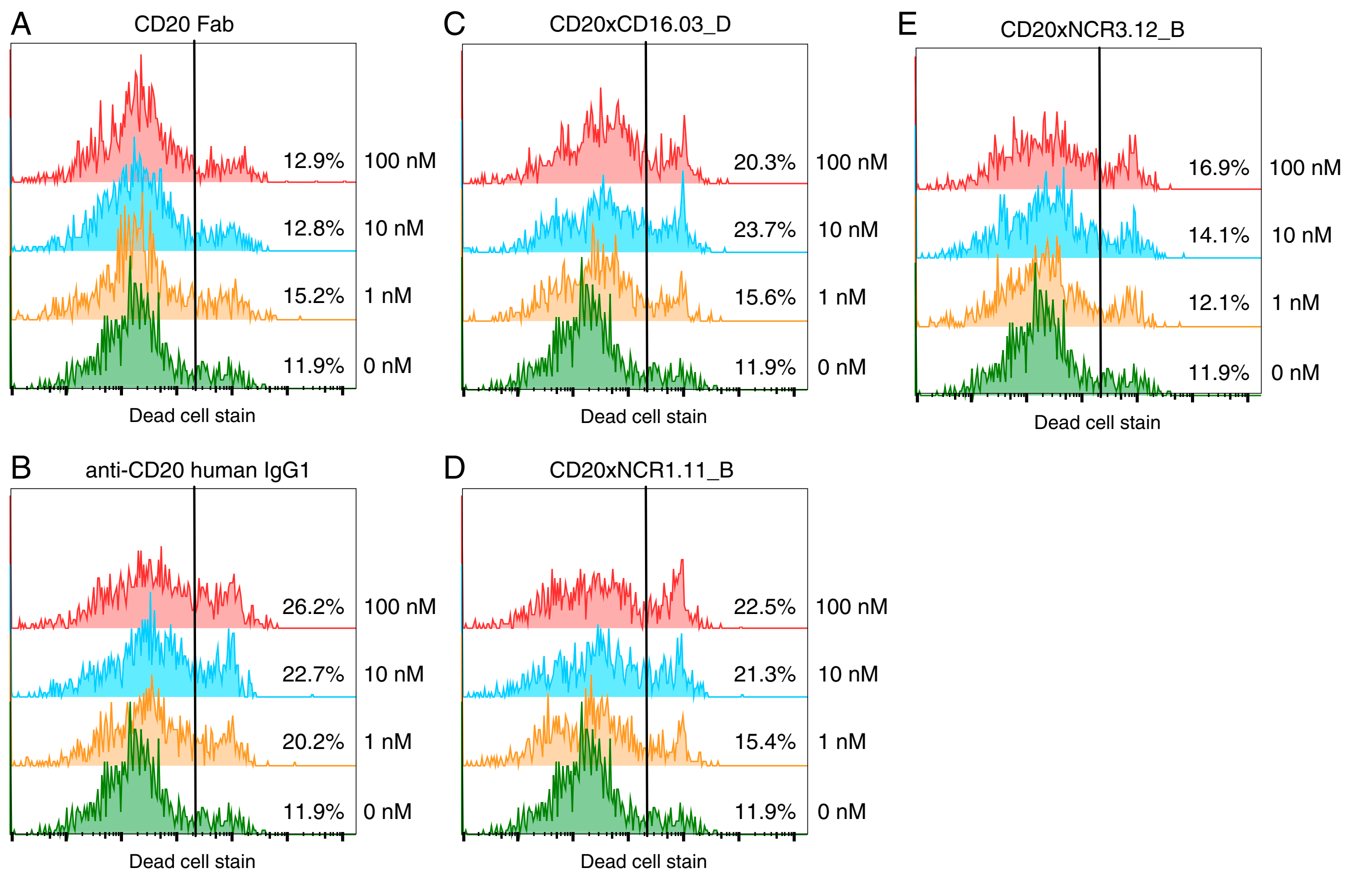

Fig. 5. Cytotoxicity of SC1 lymphoma cells by the following antibodies: (*A*) Anti-CD20 Fab. (*B*) Anti-CD20 human IgG1 mAb. (*C*) Anti-CD20–scFv CD16.03 bispecifics. (*D*) Anti-CD20–scFv NCR1.11 bispecifics. (*E*) Anti-CD20–scFv NCR3.12 bispecifics.

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 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

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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.

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 *SI Appendix*. Additional questions pertaining to methods, protocols, and reagents are available upon request.

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.

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