Targeting the membrane-proximal domain of CD33 to maximize the efficacy of natural killer cell-based immunotherapies

Improved survival of some patients with the antibody-drug conjugate gemtuzumab ozogamicin (GO) validates CD33 as therapeutic target for acute myeloid leukemia (AML),[1] but GO is often ineffective. As one limitation, GO and most other CD33 antibodies recognize the membrane-distal V-set domain.[2] For T cell-engaging therapeutics, this may be suboptimal: we recently showed CD33/CD3 bispecific antibodies (BiAb) and chimeric antigen receptor (CAR)-modified T cells binding CD33 closer to the cell membrane exert greater T cell-mediated cytotoxicity than those binding distally.[3,4] Here, we investigated whether this principle applies to therapies harnessing natural killer (NK) cells, which are of growing interest. For unconjugated antibodies, CD16-directed BiAb, and CAR-NK cells, we here demonstrate improved NK cell-mediated cytotoxicity with membrane proximal CD33 targeting. We also show antibodies binding the C2-set domain yield effective therapeutics, supporting further development of NK cell-based therapies directed at the CD33 membrane-proximal domain.

We followed a strategy previously employed for T-cell therapies,[3] namely to compare cytotoxicity against human acute leukemia cells expressing full-length CD33 ($CD33^{FL}$) *versus* isogenic cells engineered to express an artificial CD33 molecule lacking the membrane-proximal C2-set domain ($CD33^{\Delta E3\text{-}4}$), bringing the V-set domain into immediate cell membrane proximity (~4 nm closer) (Figure 1A); for comparison, the cleft at activating human NK cell immune synapses is ~10-30 nm.[5] Human AML cells with CRISPR/Cas9-mediated deletion of CD33 and endogenously $CD33^{-}$ human lymphoblastic leukemia cells were transduced to overexpress either $CD33^{FL}$ or $CD33^{\Delta E3\text{-}4}$, and sublines with similar CD33 expression were used (*Online Supplementary Figure S1A)*.

In research approved by the Fred Hutchinson Cancer Center's Institutional Review Board, we examined 3 therapeutic modalities: 1) unconjugated V-set-directed CD33 IgG1 antibodies (lintuzumab, 2D3 [developed in-house], and "A33" [obtained from publicly available sequences]); 2) $CD33^{DIST}$/CD16a BiAb in the IgG-single chain variable fragment (IgG4-scFv) format,[6] using published sequences for CD33 (lintuzumab) and CD16a (4-LS21); and 3) $CD33^{DIST}$-directed CAR-NK cells, generated by transducing the human KHYG-1 NK-cell line with a P2A/EGFP-containing lentivirus encoding a 4-1BB co-stimulatory domain-based second-generation CAR with scFv from lintuzumab and hP67.6 (used in GO).[1,7] Human NK cell lines (KHYG-1 and NK-92 and/or sublines transduced with high-affinity CD16a [$CD16a^{158V}$]) and primary human NK cells from healthy volunteers served as effector cells. Primary cells were thawed and rested overnight before use in cytotoxicity assays. Cytotoxicity was determined by flow cytometry in co-culture assays by quantifying numbers and viability (via 4',6-diamidino-2-phenylindole [DAPI] staining) of leukemia cells.

Firstly, we investigated antibody-dependent cellular cytotoxicity (ADCC) of $CD33^{DIST}$ antibodies with NK-92^{CD16a} and primary human NK cells. All 3 CD33 antibodies (IgG1 formats) elicited dose-dependent ADCC against leukemia cells overexpressing $CD33^{FL}$ or $CD33^{\Delta E3\text{-}4}$, whereas they were inactive against $CD33^{-}$ cells (Figure 1B, C and *Online Supplementary Figure S1B)*. Importantly, the $CD33^{DIST}$ antibodies exhibited greater cytotoxicity against cells expressing $CD33^{\Delta E3\text{-}4}$ than cells expressing similar levels of $CD33^{FL}$ with either NK-92^{CD16a} or primary NK cells. While speculative, the larger difference seen in ML-1 relative to RS4;11 cells may be due to lower CD33 expression on the former. Differences in cytotoxicity against cells expressing $CD33^{FL}$ or $CD33^{\Delta E3\text{-}4}$ were unlikely due to differences in expression of NK activating/inhibitory ligands as NK cells were similarly effective against individual sublines in the absence of antibodies (*Online Supplementary Figure S1D*). Unlike IgG1 versions, no significant cytotoxicity was observed with IgG4 versions of CD33 antibodies (IgG4 has significantly reduced affinity for CD16a than IgG1[8]) or a non-targeting human IgG1 control antibody (13R4) in the presence of NK cells (*Online Supplementary Figure S1E, F*), consistent with the effect of CD33 antibodies (IgG1) being CD33-specific. Affinity measurements showed that $CD33^{DIST}$ antibodies had slightly lower affinity for $CD33^{\Delta E3\text{-}4}$ compared to $CD33^{FL}$, indicating that affinity differences could not account for the enhanced efficacy observed with $CD33^{\Delta E3\text{-}4}$ (*Online Supplementary Figure S1*). Together, these data support the hypothesis that binding CD33 closer to the cell membrane enhances ADCC.

Secondly, we tested the effect of membrane proximity on the efficacy of $CD33^{DIST}$/CD16a BiAb. The $CD33^{DIST}$/CD16a BiAb elicited dose-dependent cytolytic activity against human acute leukemia cells expressing $CD33^{FL}$ or $CD33^{\Delta E3\text{-}4}$ but not isogenic $CD33^{-}$ cells (Figure 1D, E), demonstrating CD33-specificity of the BiAb activity. (See results with a non-binding control BiAb in *Online Supplementary Figure S2A*.) The minimal cytotoxic effects of lintuzumab-IgG4 at the same molar concentrations and effector-to-target (E:T) ratio (*Online Supplementary Figure S2B*) suggest the activity of the $CD33^{DIST}$/CD16a BiAb is via CD16a scFv engagement

rather than the Fc portion. As seen for CD33 antibodies, the CD33^{DIST}/CD16a BiAb exhibited greater cytotoxicity against cells expressing CD33$^{\Delta E3-4}$ than isogenic cells expressing CD33^{FL}, supporting the hypothesis that a tighter synapse between the NK and leukemia cell via membrane-proximal binding of CD33 enhances cytotoxicity of NK cell-engaging BiAb.

Thirdly, we investigated human NK cells (KHYG-1 cells) transduced with second-generation CD33^{DIST} CAR (*Online Supplementary Figure S2C*) using a third-generation lentivirus (pSLCAR). Both lintuzumab- and hP67.6-based CD33^{DIST} CAR-NK cells demonstrated dose-dependent, CD33-specific cytotoxicity (Figure 1F and *Online Supplementary Figure S2D*). Both CD33^{DIST}-directed CAR-NK cell products exhibited greater *in vitro* cytotoxicity against ML-1 and RS4;11 cells expressing CD33$^{\Delta E3-4}$ than cells expressing CD33^{FL} at similar levels, supporting the concept that binding of the CD33-directed CAR-NK cells closer to the leukemia cell membrane enhances cytotoxicity. Consistent with this, CD33^{DIST} CAR-NK cells showed higher intracellular TNFα and IFNγ levels after

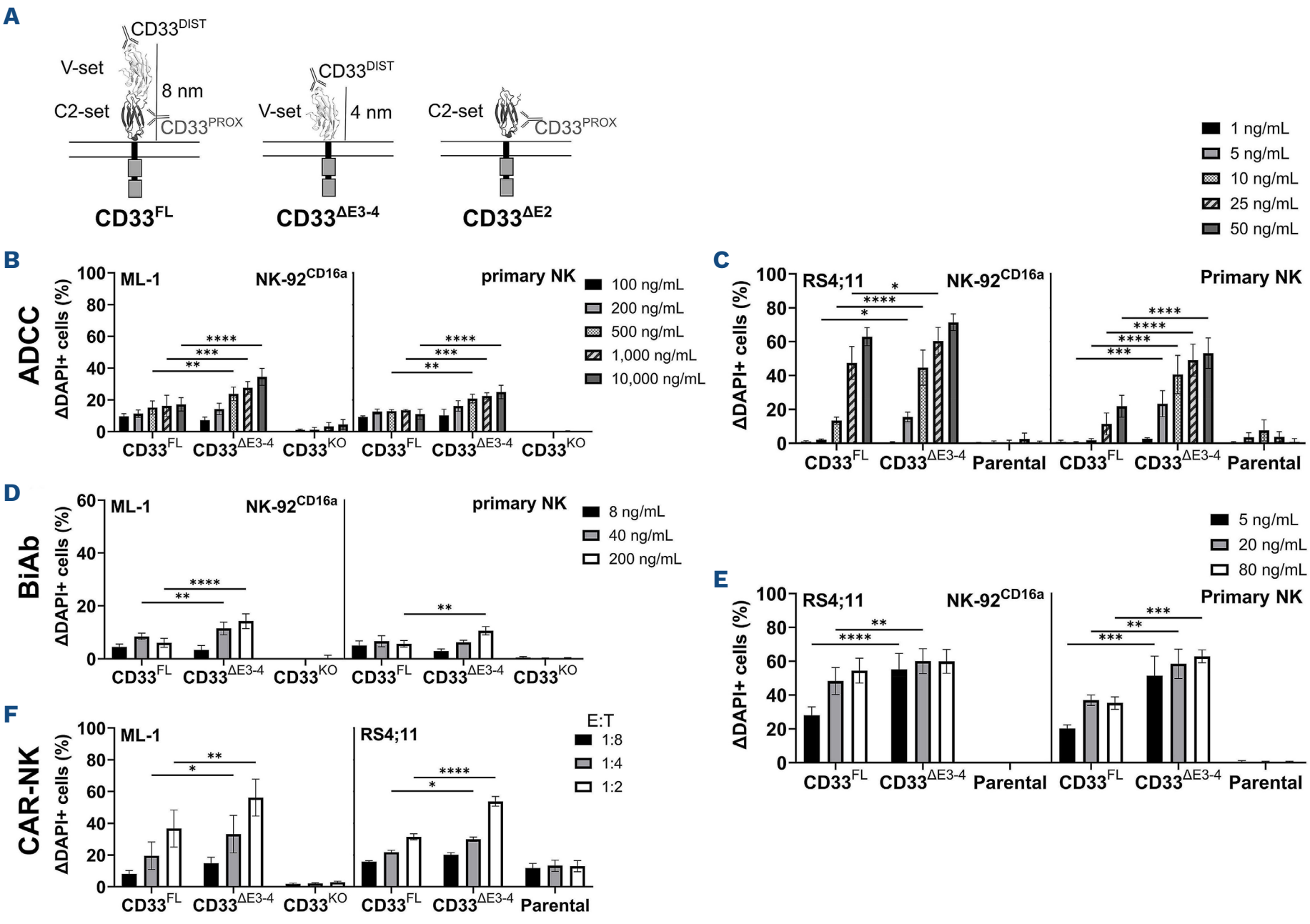

Figure 1. Effect of membrane proximity on the anti-leukemia efficacy of natural killer cell-engaging therapeutics. (A) Schematic of full-length CD33 and CD33 variants as well as binding of CD33^{DIST} and CD33^{PROX} antibodies. (B) ML-1 cells with CRISPR/Cas9-mediated deletion of endogenous CD33 and (C) CD33-negative parental RS4;11 cells and corresponding sublines expressing CD33^{FL} or CD33$^{\Delta E3-4}$ were incubated with NK-92^{CD16a} or primary human natural killer (NK) cells at an effector:target (E:T) cell ratio of 3:1 and increasing concentrations of a CD33^{DIST} IgG1 antibody (lintuzumab) as indicated. (D) ML-1 and (E) RS4;11 target cells were incubated with NK effector cells at an E:T cell ratio of 1:1 and increasing concentrations of a CD33^{DIST}/CD16a bispecific antibody (BiAb) (built with scFv sequence from anti-CD16a 4-LS21 fused to light chain of lintuzumab IgG4) as indicated. (F) ML-1 and RS4;11 target cells were incubated with CD33^{DIST} CAR-modified KHYG-1 cells (built with scFv sequences from lintuzumab) at various E:T cell ratios as indicated. After 48 hours, drug-induced cytotoxicity, using 4',6-diamidino-2-phenylindole (DAPI) to detect non-viable cells, was determined by flow cytometry. Results are presented as percentage change in non-viable cells compared to control (CTRL) cells treated with NK effector cells but without (B and C) antibody or (D and E) CD33^{DIST}/CD16a BiAb, or (F) CTRL cells left untreated with NK cells. For all experiments, mean±standard error of mean from at least 3 separate experiments is shown. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-way ANOVA with Tukey's test. ADCC: antibody-dependent cellular cytotoxicity.

overnight co-culture with CD33$^{\Delta E3-4}$-expressing ML-1 cells compared to CD33^{FL}-expressing counterparts (*Online Supplementary Figure S2E, F*). We also compared cytotoxicity of CD33-directed CAR-NK cells against ML-1 cells expressing CD33^{FL} or similar levels of an artificial CD33 molecule with both domains positioned further from the transmembrane domain via addition of CD22 C2-set domains as spacers[3] (CD33^{FL} + CD22 4D). As expected, CAR-NK cells showed decreased killing against cells expressing the elongated version compared to CD33^{FL} (*Online Supplementary Figure S2G*), supporting the hypothesis that the distance of the binding epitope from the cell membrane influences CAR-NK efficacy.

These findings demonstrate membrane-proximal CD33 binding enhances cytotoxic effects of NK cell-engaging therapies, providing a rationale for exploring therapeutics recognizing the C2-set domain. As a potential additional benefit, such therapeutics would recognize a shorter isoform of CD33 lacking the exon 2-encoded V-set domain (CD33$^{\Delta E2}$).[1,9] Whether such an isoform exists naturally as a protein is currently unclear, but transcription of CD33$^{\Delta E2}$ via alternative splicing is well established.[1,9,10] We previously

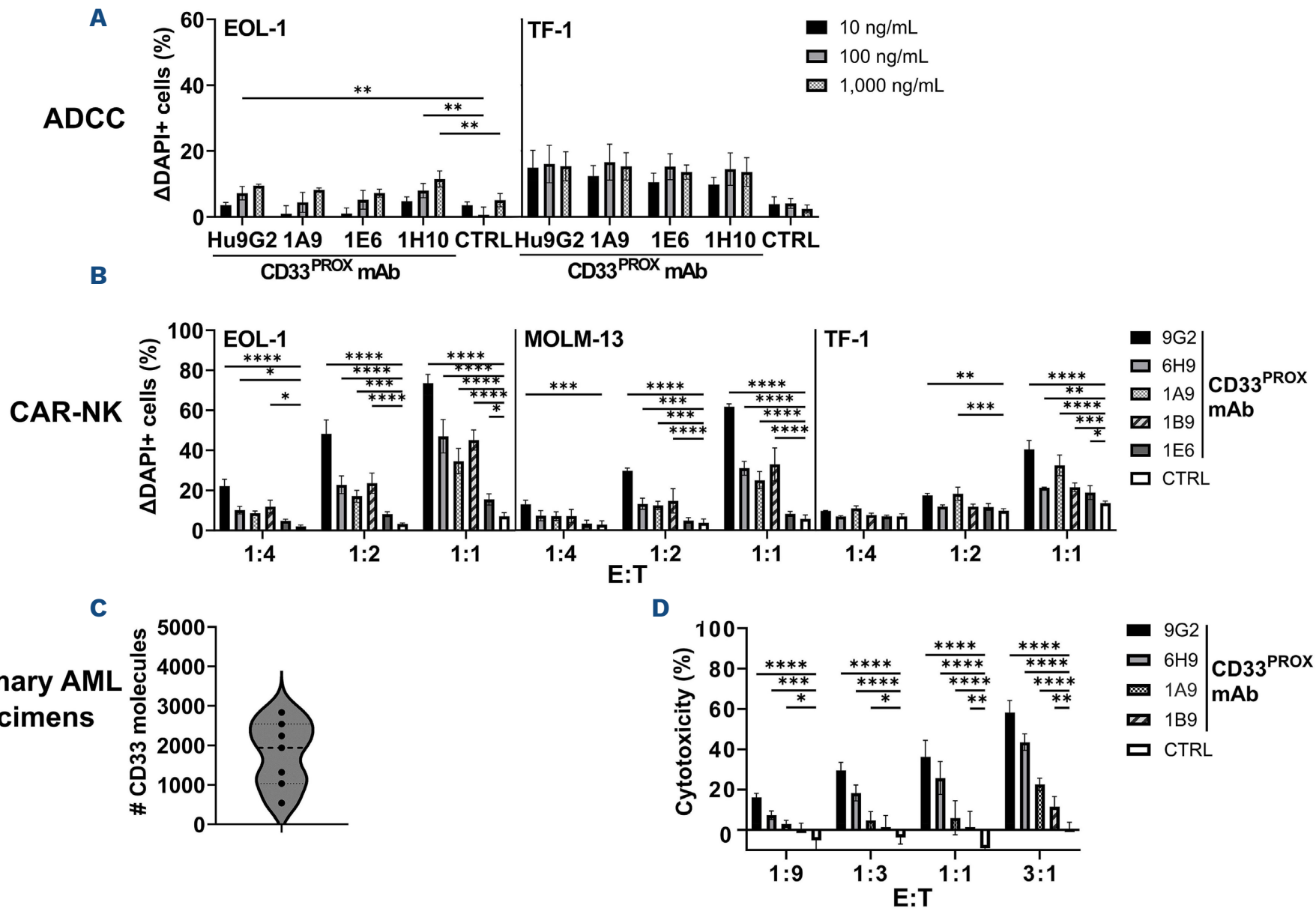

Figure 2. Anti-leukemia efficacy of CD33^{PROX} natural killer cell-engaging therapeutics. (A) Parental acute myeloid leukemia (AML) cell lines (EOL-1 or TF-1 cells) were incubated with NK-92^{CD16a} effector cells at an effector:target (E:T) cell ratio of 3:1 and increasing concentrations of CD33^{PROX} antibodies (Hu9G2, 1A9, 1E6, 1H10) or a negative isotype control antibody (13R4) in human IgG1 format as indicated. For TF-1 cells, all comparisons P<0.001 relative to 13R4. (B) Parental AML cell lines (EOL-1, MOLM-13, or TF-1 cells) were incubated with CD33^{PROX} (9G2, 6H9, 1A9, 1B9, 1E6) chimeric antigen receptor (CAR) natural killer (NK) cells or control (CTRL) 13R4 CAR-NK cells at various E:T cell ratios as indicated. After 48 hours, drug-induced cytotoxicity, using 4',6-diamidino-2-phenylindole (DAPI) to detect non-viable cells, was determined by flow cytometry. Results are presented as percentage change in non-viable cells compared to (A) CTRL cells treated with NK effector cells but without antibody, or (B) CTRL cells left untreated with NK cells. For all experiments, mean±standard error of mean from 3 separate experiments is shown. (C) Cell surface expression of the CD33 molecules on AML patient specimens was assessed by flow cytometry using a CD33^{DIST} antibody, P67.6, in conjunction with Quantibrite beads. (D) Primary AML cells were incubated overnight with CD33^{PROX} (9G2, 6H9, 1A9, 1B9) CAR-NK cells or non-targeting control (13R4) CAR-NK cells at various E:T cell ratios as indicated. Viable target cells were enumerated by flow cytometry, and changes in the number of viable cells with CAR-NK cells added compared to cells without NK cells is shown. Mean±standard error of mean from 7 different patient specimens with 3 replicates each is shown. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-way ANOVA with Dunnett's test. ADCC: antibody-dependent cellular cytotoxicity; mAb: monoclonal antibodies.

generated a panel of murine and human CD33$^{C2\text{-}set}$ antibodies that bind both CD33^{FL} and CD33$^{\Delta E2}$, i.e., are CD33^{PROX} antibodies with properties of "CD33^{PAN}" antibodies;[3] some of the murine CD33^{PROX} antibodies (e.g., 9G2 [Hu9G2]), were subsequently humanized by CDR grafting. Only CD33^{PROX} antibodies (Hu9G2, 1A9, 1E6 and 1H10) but not CD33^{DIST} antibodies (lintuzumab, 2D3, A33) showed binding to REH cells overexpressing CD33$^{\Delta E2}$, confirming CD33^{PROX} antibodies bind the C2-set domain even when the membrane-distal V-set domain is not present (*Online Supplementary Figure S3A*). By comparison, both CD33^{DIST} and CD33^{PROX} antibodies bound REH cells overexpressing CD33^{FL}. In the presence of NK-92^{CD16a} cells, all CD33^{PROX} antibodies (Hu9G2, 1A9, 1E6, and 1H10) in IgG1 format but not the non-targeting isotype control antibody (13R4) induced ADCC against two different human AML cell lines with varying CD33 levels (Figure 2A and *Online Supplementary Figure S3B*). No ADCC was observed against isogenic cells lacking CD33, confirming CD33-specificity of the cytotoxicity (*Online Supplementary Figure S3C*).

Finally, we generated CD33^{PROX}-directed CAR-NK cells using sequences from CD33^{PROX} antibodies (9G2, 6H9, 1A9, 1B9 and 1E6) (*Online Supplementary Figure S3D*). As expected, CD33^{PROX} CAR-NK cells induced cytotoxicity against REH cells overexpressing either CD33^{FL} or CD33$^{\Delta E2}$ (*Online Supplementary Figure S3E*). CD33^{PROX} CAR-NK cells induced greater cytotoxicity against AML cell lines (EOL-1, MOLM-13 and TF-1) than non-targeting CAR-NK cells (13R4) (Figure 2B). There was no clear correlation between the magnitude of cytotoxic effects of individual CAR-NK cells and their CAR expression levels. No cytotoxicity was observed against CD33^{-} parental REH cells, and minimal cytotoxicity was observed against isogenic cells lacking CD33, further highlighting the CD33-specificity of CD33^{PROX} CAR-NK cells (*Online Supplementary Figure S3E, F*). We then assessed the anti-tumor efficacy of CD33^{PROX} CAR-NK cells against primary human AML cells. In a set of 7 samples from AML patients with a range of CD33 molecules expressed on the surface of leukemic cells (Figure 2C), we found all four CD33^{PROX} CAR-NK products induced CD33-specific cytotoxicity in an E:T cell ratio-dependent manner compared to the non-targeting CAR-NK cells (13R4) (Figure 2D).

Together, our data indicate that decreasing the distance between CD33 binding epitope and leukemia cell membrane enhances the efficacy of CD33-directed NK-cell therapies. While relevance for membrane-proximal targeting has been shown for other antigens,[11-15] these findings are the first to show the importance of membrane proximity for ADCC efficacy, even for a relatively small target antigen like CD33, with a change in epitope position by ~4 nm yielding ADCC benefit. We believe our studies are the first to investigate how membrane proximity affects CAR-NK-cell efficacy. As for CAR-T cells, our data suggest it is advantageous to target the CD33 membrane proximally for enhanced activation/efficacy even though the exact mechanisms in doing so for CAR-NK cells may differ from CAR-T cells. While almost all existing CD33-targeting therapeutics bind CD33 distally, our findings support the further development of therapies directed at the membrane-proximal domain for optimization of the anti-tumor efficacy of NK-cell-based treatments for AML and other CD33-expressing neoplasms.

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Contributions

SYTL, FMC and GSL performed research, analyzed and interpreted data, and wrote the manuscript. MCL-H, JH, JL and ARK performed research, and analyzed and interpreted data. RBW conceptualized and designed this study, participated in data analysis and interpretation, and wrote the manuscript. All authors revised the manuscript critically and gave final approval to submit for publication.

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Data-sharing statement

For original data and reagents, please contact the corresponding author.

References

1. Godwin CD, Gale RP, Walter RB. Gemtuzumab ozogamicin in acute myeloid leukemia. Leukemia. 2017;31(9):1855-1868.
2. Walter RB. Investigational CD33-targeted therapeutics for acute myeloid leukemia. Expert Opin Investig Drugs. 2018;27(4):339-348.
3. Godwin CD, Laszlo GS, Fiorenza S, et al. Targeting the membrane-proximal C2-set domain of CD33 for improved CD33-directed immunotherapy. Leukemia. 2021;35(9):2496-2507.
4. Fiorenza S, Lim SYT, Laszlo GS, et al. Targeting the membrane-proximal C2-set domain of CD33 for improved CAR T cell therapy. Mol Ther Oncol. 2024;32(3):200854.
5. McCann FE, Vanherberghen B, Eleme K, et al. The size of the synaptic cleft and distinct distributions of filamentous actin, ezrin, CD43, and CD45 at activating and inhibitory human NK cell immune synapses. J Immunol. 2003;170(6):2862-2870.
6. Orcutt KD, Ackerman ME, Cieslewicz M, et al. A modular IgG-scFv bispecific antibody topology. Protein Eng Des Sel. 2010;23(4):221-228.
7. Laszlo GS, Estey EH, Walter RB. The past and future of CD33 as therapeutic target in acute myeloid leukemia. Blood Rev. 2014;28(4):143-153.
8. Bruhns P, Iannascoli B, England P, et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood. 2009;113(16):3716-3725.
9. Laszlo GS, Harrington KH, Gudgeon CJ, et al. Expression and functional characterization of CD33 transcript variants in human acute myeloid leukemia. Oncotarget. 2016;7(28):43281-43294.
10. Godwin CD, Laszlo GS, Wood BL, et al. The CD33 splice isoform lacking exon 2 as therapeutic target in human acute myeloid leukemia. Leukemia. 2020;34(9):2479-2483.
11. Bluemel C, Hausmann S, Fluhr P, et al. Epitope distance to the target cell membrane and antigen size determine the potency of T cell-mediated lysis by BiTE antibodies specific for a large melanoma surface antigen. Cancer Immunol Immunother. 2010;59(8):1197-1209.
12. Lin TS. Ofatumumab: a novel monoclonal anti-CD20 antibody. Pharmgenomics Pers Med. 2010;3:51-59.
13. Haso W, Lee DW, Shah NN, et al. Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. Blood. 2013;121(7):1165-1174.
14. Cleary KLS, Chan HTC, James S, Glennie MJ, Cragg MS. Antibody distance from the cell membrane regulates antibody effector mechanisms. J Immunol. 2017;198(10):3999-4011.
15. Xiao Q, Zhang X, Tu L, Cao J, Hinrichs CS, Su X. Size-dependent activation of CAR-T cells. Sci Immunol. 2022;7(74):eabl3995.