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

Running Title: Optimizing CD33-directed NK cell therapy

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Data sharing statement: for original data and reagents, please contact the corresponding author (rwalter@fredhutch.org).

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To the Editor

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 (BiAbs) 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 BiAbs, 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 CD33's 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 ($CD33^{FL}$) *vs*. isogenic cells engineered to express an artificial CD33 molecule lacking the membrane-proximal C2-set domain ($CD33^{\Delta E3-4}$), 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-negative human lymphoblastic leukemia cells were transduced to overexpress either $CD33^{FL}$ or $CD33^{\Delta E3-4}$, and sublines with similar CD33 expression were used (**Supplementary Figure 1A**).

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 BiAbs 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 scFvs 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 flow cytometrically determined in co-culture assays by quantifying numbers and viability (via 4',6-diamidino-2-phenylindole [DAPI] staining) of leukemia cells.

First, we investigated antibody-dependent cellular cytotoxicity (ADCC) of $CD33^{DIST}$ antibodies with NK-92^{CD16a} and primary human NK cells. As shown in **Figure 1B/C** and **Supplementary Figures 1B/C**, all 3 CD33 antibodies (IgG1 formats) elicited dose-dependent ADCC against leukemia cells overexpressing $CD33^{FL}$ or $CD33^{\Delta E3-4}$, whereas they were inactive against CD33- cells. Importantly, the $CD33^{DIST}$ antibodies exhibited greater cytotoxicity against cells expressing $CD33^{\Delta E3-4}$ 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 $CD33^{\Delta E3-4}$ 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 (**Supplementary Figure 1D**). 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 (**Supplementary Figures 1E/F**), consistent with the effect of CD33 antibodies (IgG1) being CD33-specific. Affinity measurements showed that $CD33^{DIST}$ antibodies had slightly lower affinity for $CD33^{\Delta E3-4}$ compared to $CD33^{FL}$, indicating that affinity differences could not account for enhanced efficacy observed with $CD33^{\Delta E3-4}$ (footnote; **Supplementary Figure 1**). Together, these data support the hypothesis that binding CD33 closer to the cell membrane enhances ADCC.

Second, we tested the effect of membrane proximity on the efficacy of $CD33^{DIST}$/CD16a BiAbs. As shown in **Figure 1D/E**, the $CD33^{DIST}$/CD16a BiAb elicited dose-dependent cytolytic activity against human acute leukemia cells expressing $CD33^{FL}$ or $CD33^{\Delta E3-4}$ but not isogenic CD33-negative cells, demonstrating CD33-specificity of the BiAb activity (**Supplementary Figure 2A** depicts results with a non-binding control BiAb). The minimal cytotoxic effects of lintuzumab-IgG4 at the same molar concentrations and effector-to-target (E:T) ratio (**Supplementary Figure 2B**) 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 BiAbs.

Third, we investigated human NK cells (KHYG-1 cells) transduced with 2nd generation $CD33^{DIST}$ CARs (**Supplementary Figure 2C**) using a 3rd generation lentivirus (pSLCAR). Both lintuzumab- and hP67.6-based $CD33^{DIST}$ CAR-NK cells demonstrated dose-dependent, CD33-specific cytotoxicity (**Figure 1F** and **Supplementary Figure 2D**). 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 overnight co-culture with $CD33^{\Delta E3-4}$-expressing ML-1 cells compared to $CD33^{FL}$-expressing counterparts (**Supplementary Figure 2E/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 $CD33^{FL}$ (**Supplementary Figure 2G**), supporting the hypothesis that the distance of the binding epitope from the cell membrane influences CAR-NK efficacy.

The findings summarized above demonstrate membrane-proximal CD33 binding enhances cytotoxic effects of NK cell-engaging therapies, providing rationale to explore 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 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. As depicted in **Supplementary Figure 3A**, 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. 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 **Supplementary Figure 3B**). No ADCC was observed against isogenic cells lacking CD33, confirming CD33-specificity of the cytotoxicity (**Supplementary Figure 3C**).

Finally, we generated $CD33^{PROX}$-directed CAR-NK cells using sequences from $CD33^{PROX}$ antibodies (9G2, 6H9, 1A9, 1B9 and 1E6; **Supplementary Figure 3D**). As expected, $CD33^{PROX}$ CAR-NK cells induced cytotoxicity against REH cells overexpressing either $CD33^{FL}$ or $CD33^{\Delta E2}$ (**Supplementary Figure 3E**). $CD33^{PROX}$ 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-negative parental REH cells, and minimal cytotoxicity was observed against isogenic cells lacking CD33, further highlighting the CD33-specificity of $CD33^{PROX}$ CAR-NK cells (**Supplementary Figures 3E/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 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 membrane proximity matters 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. Like for CAR-T cells, our data suggest it is advantageous to target 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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FIGURE LEGENDS

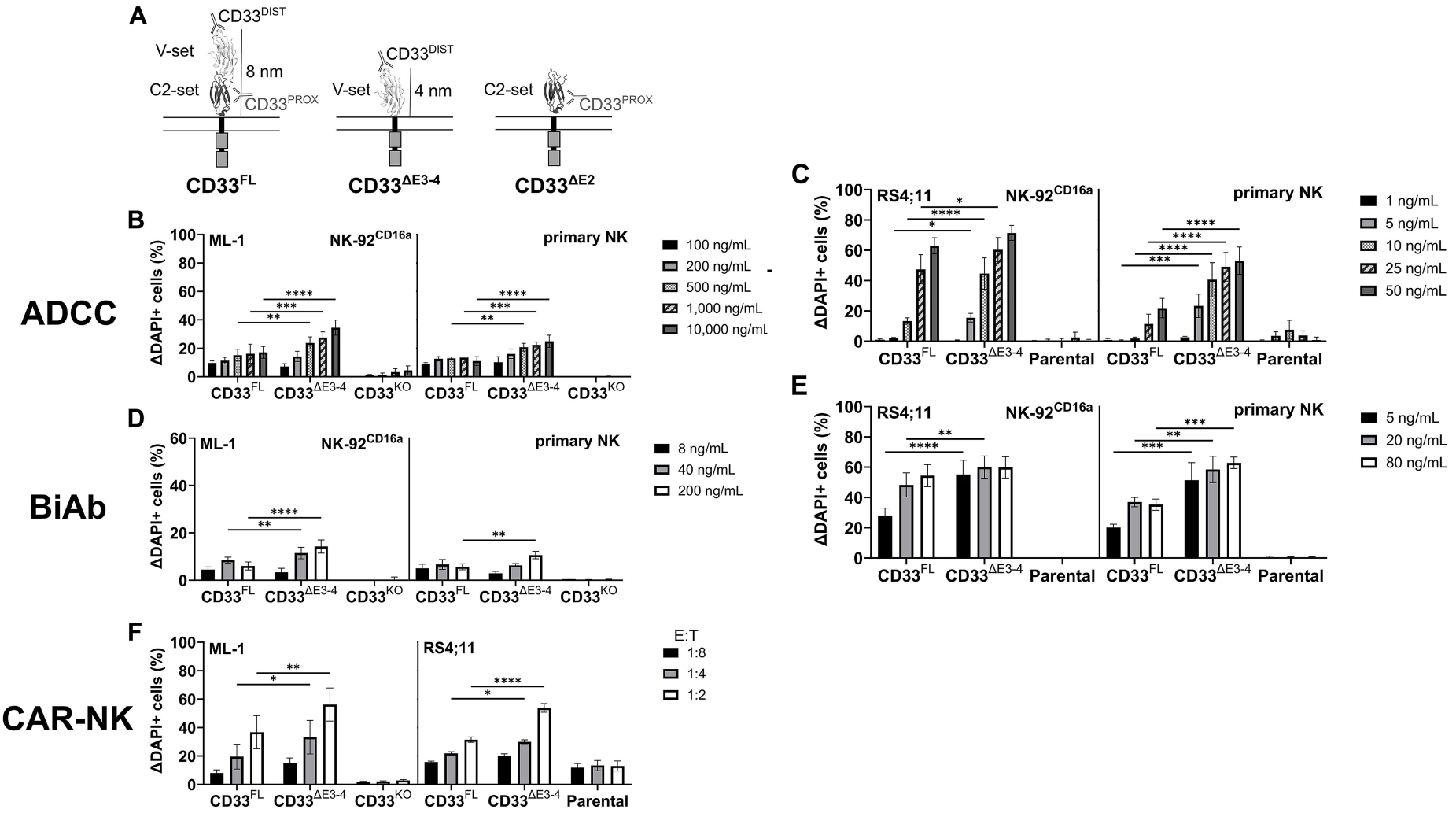
Figure 1

Effect of membrane proximity on the anti-leukemia efficacy of NK 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 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 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 DAPI to detect non-viable cells, was determined flow cytometrically. Results are presented as percentage change in non-viable cells compared to control cells treated with NK effector cells but without **(B-C)** antibody or **(D-E)** $CD33^{DIST}$/CD16a BiAb, or **(F)** control cells left untreated with NK cells. For all experiments, mean±SEM 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.

Figure 2

Anti-leukemia efficacy of $CD33^{PROX}$ NK cell-engaging therapeutics. (A) Parental AML cell lines (EOL-1 or TF-1 cells) were incubated with NK-92^{CD16a} effector cells at an 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) CAR-NK cells or control 13R4 CAR-NK cells at various E:T cell ratios as indicated. After 48 hours, drug-induced cytotoxicity, using DAPI to detect non-viable cells, was determined flow cytometrically. Results are presented as percentage change in non-viable cells compared to **(A)** control cells treated with NK effector cells but without antibody, or **(B)** control cells left untreated with NK cells. For all experiments, mean±SEM 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 via flow cytometry, and changes in the number of viable cells with CAR-NK cells added compared to cells without NK cells is shown. Mean±SEM from 7 different patient specimens with three replicates each is shown. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-way ANOVA with Dunnett's test.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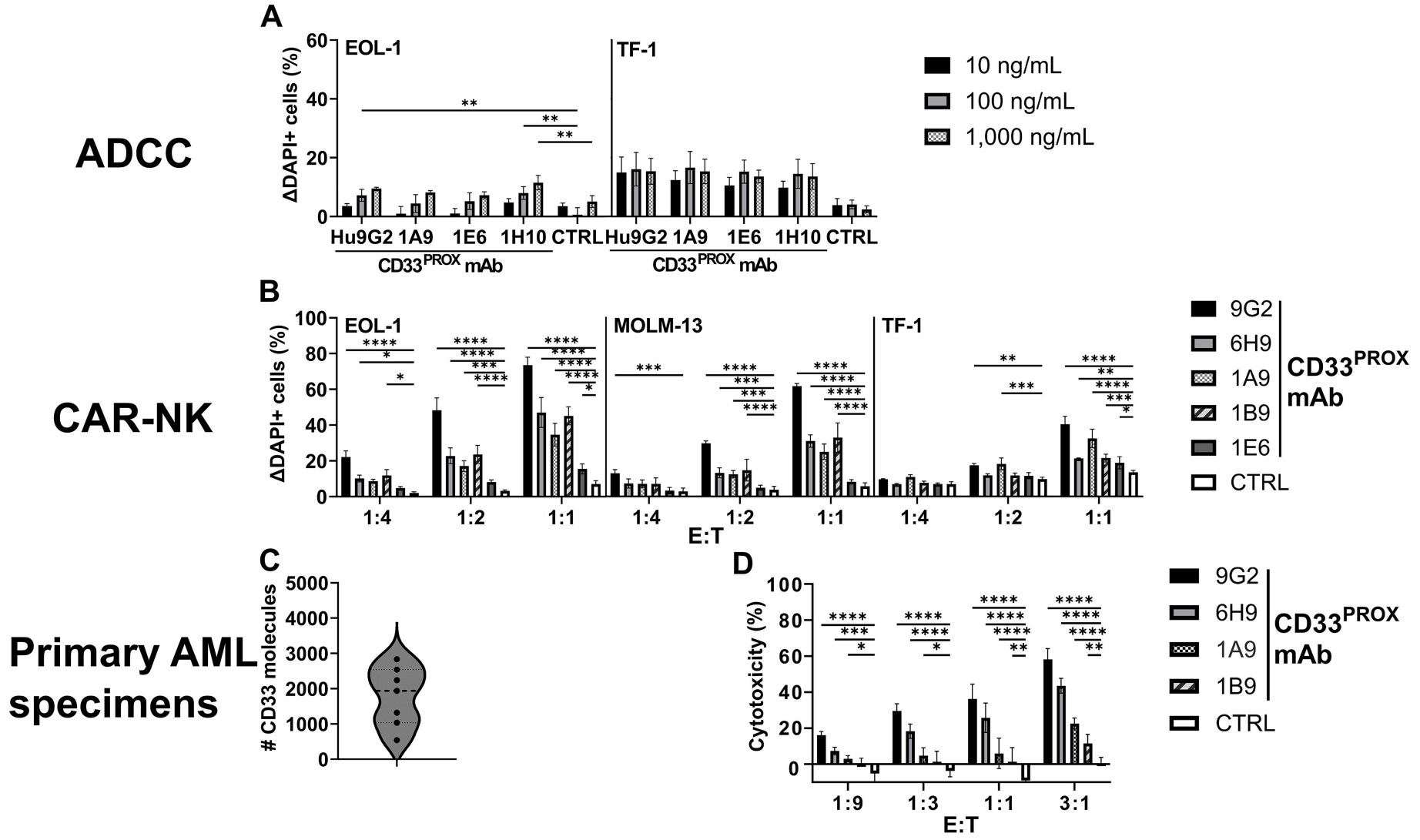
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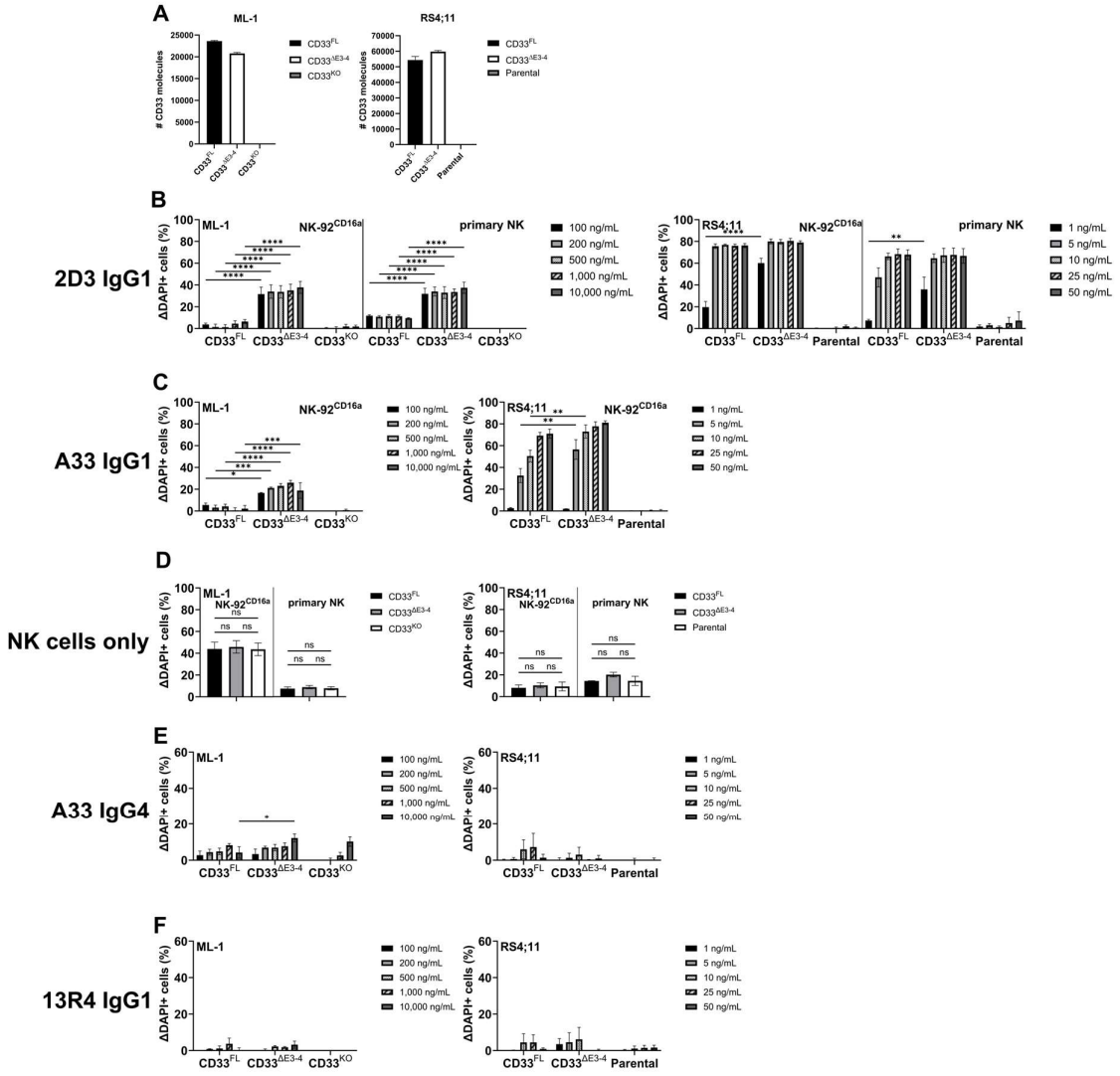
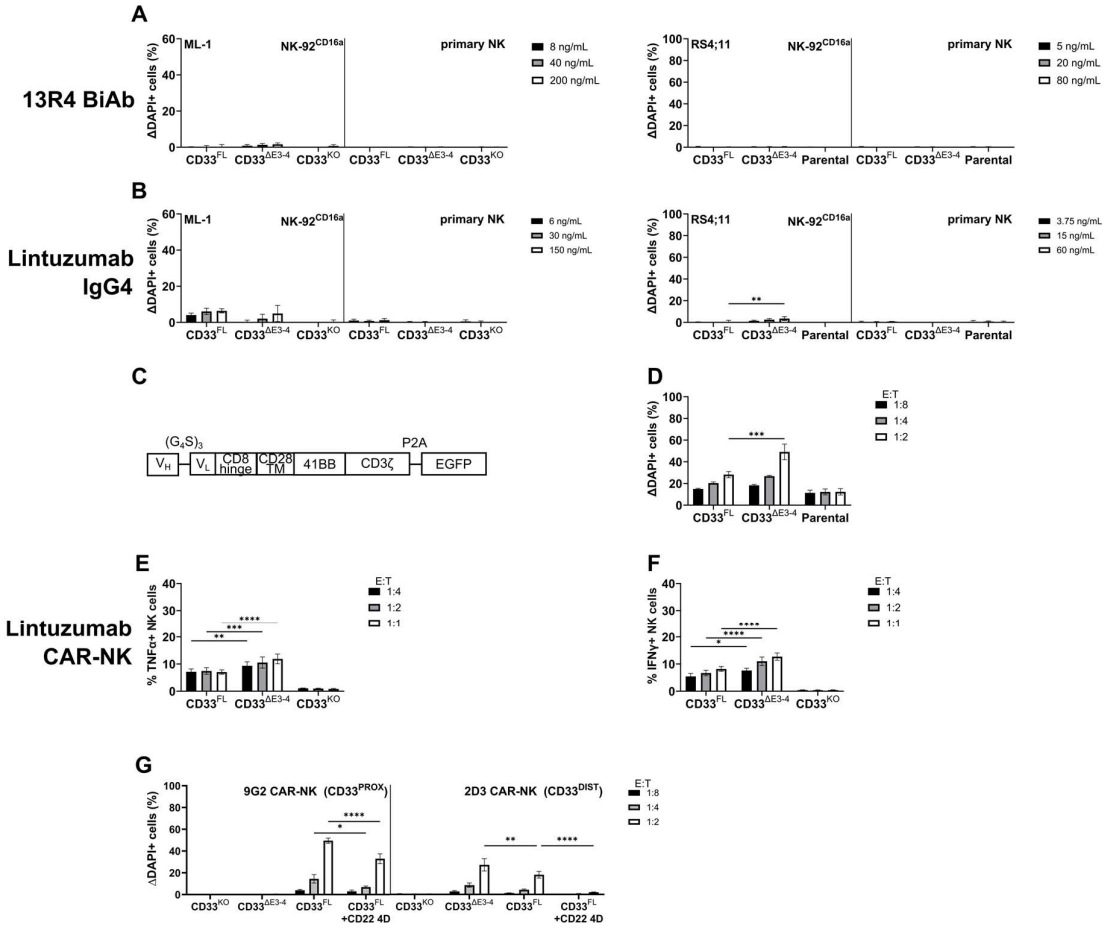
SUPPLEMENTARY FIGURE 1



(A) Relative cell surface expression of the CD33 molecules was assessed by flow cytometry using a $CD33^{DIST}$ antibody, P67.6, in conjunction with Quantibrite beads. Mean+SD values from 3 replicates are shown. **(B-C)** ML-1 cells with CRISPR/Cas9-mediated deletion of endogenous CD33 loci and 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 NK cells at an effector:target (E:T) cell ratio of 3:1 and increasing concentrations of a $CD33^{DIST}$ IgG1 antibody [**(B)** 2D3 or **(C)** A33] for 48 hours. Summary of binding affinities of $CD33^{DIST}$ human IgG1 antibodies (lintuzumab or 2D3) for ectodomain of $CD33^{FL}$: K_d = 4.75 nM, R^2 = 0.9942 and 4.09 nM, R^2 = 0.9989, respectively. K_d for ectodomain of $CD33^{\Delta E3-4}$ = 54.6 nM, R^2= 0.9952 and 19.3 nM R^2 = 0.953, respectively. Binding affinities were measured by Octet Biolayer Interferometry (BLI) assays. The non-binding isotype control antibody (13R4) used as negative control, showed no binding to either $CD33^{FL}$ or $CD33^{\Delta E3-4}$ analyte. **(D)** ML-1 and RS4;11 target cells were co-cultured with NK-92^{CD16a} or primary human NK cells at an E:T cell ratio of 3:1 without any antibodies present for 48 hours. **(E-F)** ML-1 and RS4;11 target cells were incubated with NK-92^{CD16a} or primary human NK cells at an E:T cell ratio of 3:1 and increasing concentrations of **(E)** a $CD33^{DIST}$ IgG4 antibody (A33), or **(F)** a

non-targeting IgG1 antibody (13R4) for 48 hours. For all experiments, drug-induced cytotoxicity, using DAPI to detect non-viable cells, was determined flow cytometrically. Results are presented as percentage change in non-viable cells compared to **(B-C, E-F)** control cells treated with NK effector cells but without antibody or **(D)** control cells left untreated with NK effector cells. For all experiments, mean+SEM values from 3 separate experiments are shown. **(B-C, E-F)** **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 by two-way ANOVA with Tukey's test and **(D)** ns (not significant) by one-way ANOVA with Tukey's test.

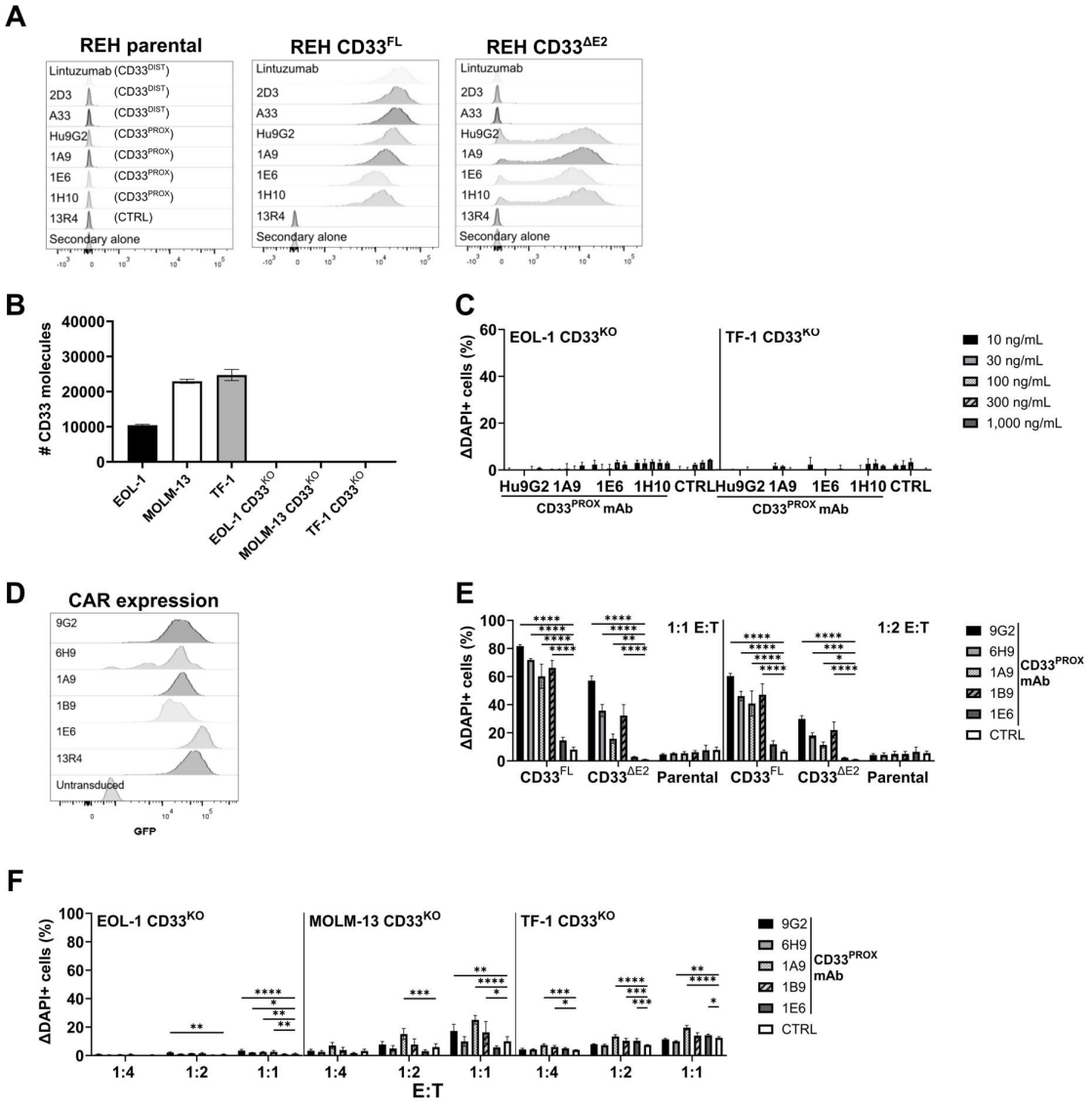
SUPPLEMENTARY FIGURE 2



(A-B) ML-1 cells with CRISPR/Cas9-mediated deletion of endogenous CD33 loci and CD33-negative parental RS4;11 cells and corresponding sublines overexpressing $CD33^{FL}$ or $CD33^{\Delta E3-4}$ were incubated with NK-92^{CD16a} or primary human NK cells at an effector:target (E:T) cell ratio of 1:1 and increasing concentrations of **(A)** a non-targeting control BiAb 13R4/CD16a or **(B)** $CD33^{DIST}$ antibody (lintuzumab) in human IgG4 format. After 48 hours, drug-induced cytotoxicity, using DAPI to detect non-viable cells, was determined flow cytometrically. Results are presented as percentage change in non-viable cells compared to control cells treated with NK effector cells but without BiAb/antibody. Given a molecular weight of 200,000 g/mol for an IgG4-scFv BiAb and a molecular weight of 150,000 g/mol for an IgG4 antibody (without the additional CD16a scFv fused to the light chains), the molar concentrations of the BiAb used in **Figure 1C/D** are identical to those of the IgG4 antibody used in **(B)** here. The minimal cytotoxic effects of lintuzumab in IgG4 format at the same molar concentrations and E:T ratio suggest the anti-leukemia activity of the $CD33^{DIST}$/CD16a BiAb required engagement of the CD16a scFv rather than the Fc portion of the BiAb. **(C)** Schematic of the anti-CD33 second-generation CAR construct used for lentiviral

transduction of KHYG-1 cells. **(D)** RS4;11 and **(E-F)** ML-1 target cells were incubated with $CD33^{DIST}$ CAR-modified KHYG-1 cells (built with scFv sequences from **(D)** hP67.6 or **(E-F)** lintuzumab) at various effector:target (E:T) cell ratios as indicated. **(D)** After 48 hours, CAR-NK-induced cytotoxicity, using DAPI to detect non-viable cells, was determined flow cytometrically. Results are presented as percentage change in non-viable cells compared to control cells left untreated with NK cells. **(E-F)** After overnight incubation, intracellular cytokine staining was carried out to determine the percentage of CAR-NK cells positive for **(E)** TNFα and **(F)** IFNγ by flow cytometry. **(G)** ML-1 target cells expressing truncated, full-length or elongated CD33 variants were incubated with CD33-directed CAR-modified KHYG-1 cells (9G2 or 2D3) at various E:T cell ratios as indicated. After 48 hours, CAR-NK-induced cytotoxicity was determined flow cytometrically like in **(D)**. For all experiments, mean+SEM values from 3 separate experiments are shown. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 by two-way ANOVA with Tukey's test.

SUPPLEMENTARY FIGURE 3



(A) Binding of CD33 antibodies to CD33-negative REH parental cells or sublines overexpressing CD33^{FL} or CD33$^{\Delta E2}$ that lacks the membrane-distal V-set domain. **(B)** Relative cell surface expression of CD33 molecules on parental AML cell lines and sublines with CRISPR/Cas9-mediated deletion of endogenous CD33 loci were assessed by flow cytometry using a CD33^{DIST} antibody, P67.6, in conjunction with Quantibrite beads. Mean±SD values from 3 replicates are shown. **(C)** AML cell lines with CRISPR/Cas9-mediated deletion of endogenous CD33 loci (EOL-1 CD33^{KO} or TF-1 CD33^{KO}) were incubated with NK-92^{CD16a} effector cells at effector:target (E:T) cell ratio of 3:1 and increasing concentrations of CD33^{PROX} antibodies (Hu9G2, 1A9, 1E6, 1H10) or isotype control antibody (13R4) in human IgG1 format. After 48 hours, drug-induced cytotoxicity, using DAPI to detect non-viable cells, was determined flow cytometrically. Results are presented as

percentage change in non-viable cells compared to control cells treated with NK effector cells but without antibody. **(D)** Expression of the $CD33^{PROX}$ CARs in transduced and EGFP-sorted CAR-NK cells was assessed by flow cytometry via quantification of EGFP levels. **(E)** CD33-negative REH parental cells or sublines overexpressing $CD33^{FL}$ or $CD33^{\Delta E2}$ or **(F)** AML cell lines with CRISPR/Cas9-mediated deletion of endogenous CD33 loci (EOL-1 $CD33^{KO}$, MOLM-13 $CD33^{KO}$ or TF-1 $CD33^{KO}$) were incubated for 48 hours with $CD33^{PROX}$ (9G2, 6H9, 1A9, 1B9, 1E6) CAR-NK cells at various effector:target (E:T) cell ratios. CAR-NK-induced cytotoxicity, using DAPI to detect non-viable cells, was determined flow cytometrically. Results are presented as percentage change in non-viable cells compared to control cells untreated with NK cells. For all experiments, mean±SEM values from 3 separate experiments are shown. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-way ANOVA with Dunnett's test.