LETTER TO THE EDITOR

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

Authors

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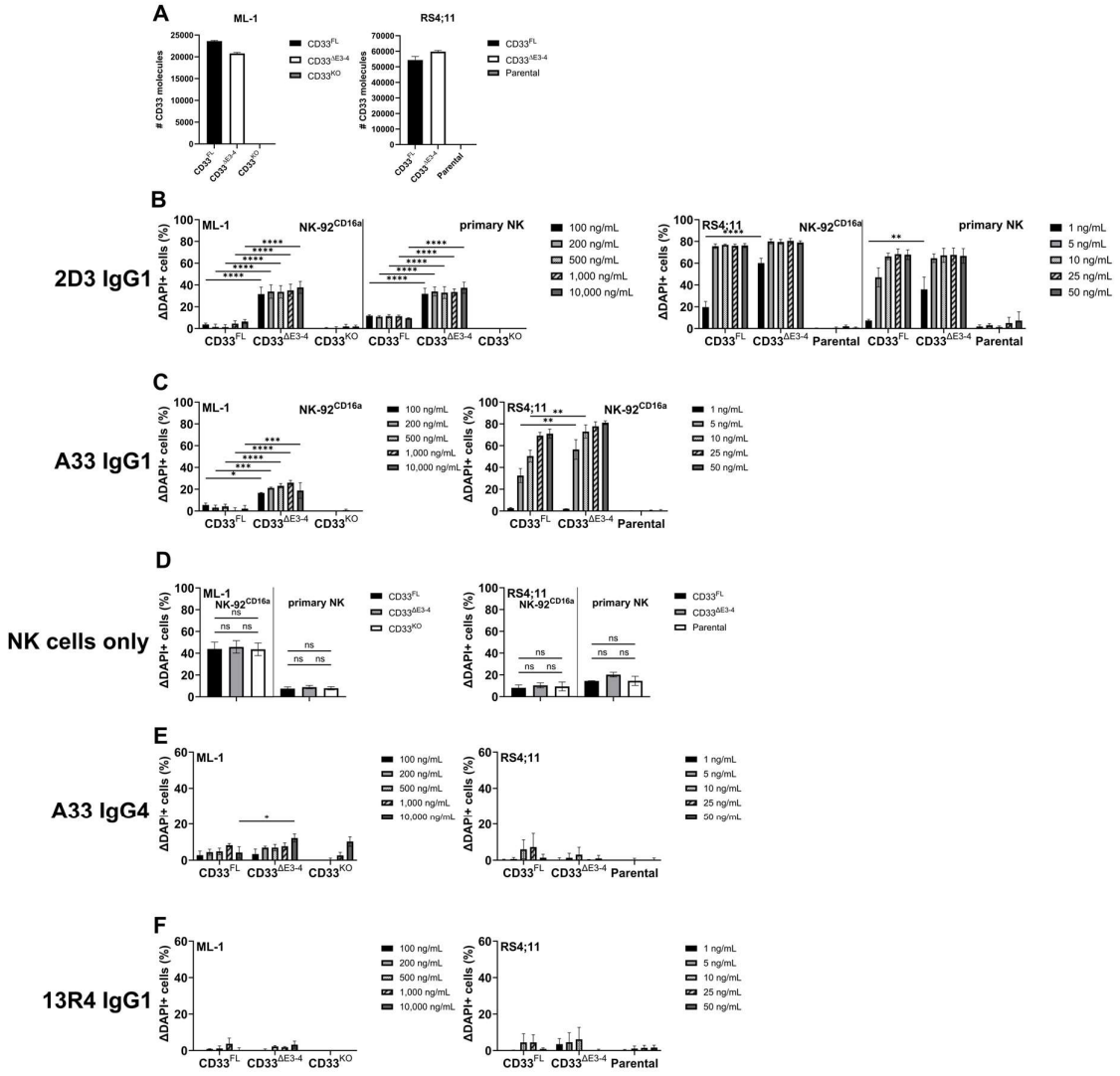
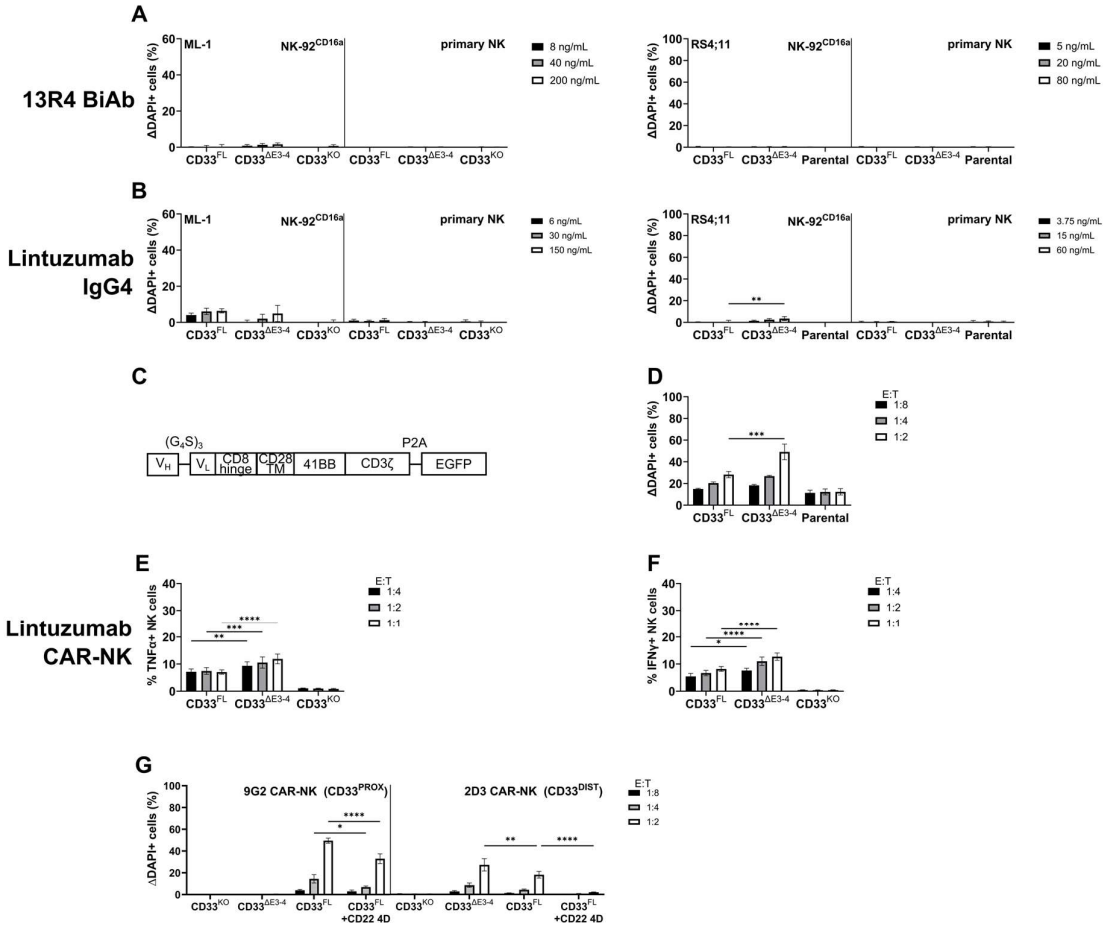
SUPPLEMENTARY FIGURE 1



(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.

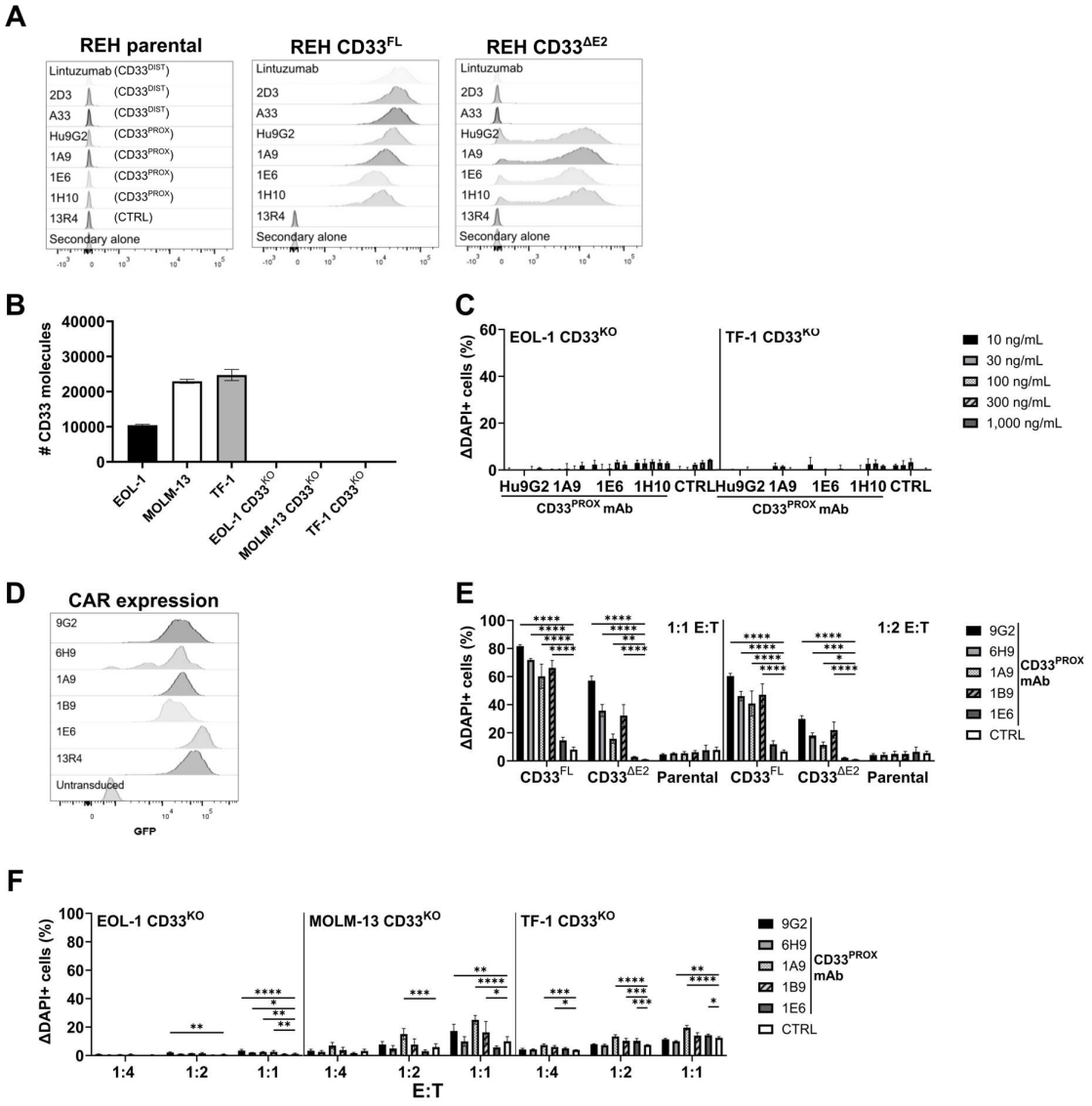
SUPPLEMENTARY FIGURE 2



(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.