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The CD33 Splice Isoform Lacking Exon 2 as Therapeutic Target in Human Acute Myeloid Leukemia

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There is long-standing interest in therapies targeting full-length CD33 ($CD33^{FL}$) for acute myeloid leukemia (AML).[1–3] Longer survival with gemtuzumab ozogamicin (GO) in some patients validates this approach but GO does not benefit many patients with CD33+ leukemias.[4] Numerous efforts to develop more effective CD33-directed therapeutics are therefore ongoing.[5]

The immune-dominant epitopes recognized by existing CD33 therapeutics are located within the membrane-distal V-set domain of $CD33^{FL}$ (Supplementary Figure 1).[5] However, alternative splicing of CD33 results in the transcription of several shorter isoforms in AML cells, with one variant missing exon 2 ($CD33^{\Delta E2}$) being of particular interest.[6] $CD33^{\Delta E2}$ is predicted to contain only the membrane-proximal C2-set domain in its extracellular portion and is, at the mRNA level, present in myeloblasts from all AML patients.[6] A single nucleotide polymorphism (SNP), rs12459419, modulates exon 2 splicing efficiency.[7–9] The minor (T) allele is associated with reduced $CD33^{FL}$ expression on AML cells and, conversely, increased $CD33^{\Delta E2}$ transcription.[9] Data from the pediatric COG-AAML0531 trial, showing improved outcome with GO add-on to intensive chemotherapy was limited to patients homozygous for the major C allele,[9] suggest CD33 splicing events may be clinically important. This observation has prompted interest in targeting $CD33^{\Delta E2}$ with antibodies recognizing the C2-set domain, perhaps particularly for the 50% of patients with rs12459419 CT or TT genotypes. Human AML cells can be generated that display cell surface $CD33^{\Delta E2}$ when expression is forced via lentiviral transduction[6] but whether $CD33^{\Delta E2}$ is a viable

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therapeutic target is unknown. Since commercial antibodies specifically recognizing $CD33^{\Delta E2}$ do not exist, rigorous testing to what degree endogenous *$CD33^{\Delta E2}$* mRNA is translated, post-translationally modified, and transported to the cell surface of AML cells has so far not been possible.

To examine expression and localization of $CD33^{\Delta E2}$ in human AML cell lines and primary AML blasts, we therefore first raised antibodies that bind human $CD33^{\Delta E2}$ but not $CD33^{FL}$ (Supplementary Figure 1). Briefly (see Online Supplement for detailed methods), immunogens consisting of the murine Fc domain and either the entire extracellular domain of human $CD33^{FL}$ or the entire extracellular domain of human $CD33^{\Delta E2}$ were generated, expressed in HEK293 cells, and purified (Supplementary Figure 2). BALB/c, CD1, and F1 mice were injected with a mixture of both immunogens. We identified two hybridomas, 11D5 and 13E11, showing binding to $CD33^{\Delta E2}$ but not $CD33^{FL}$ in early screening assays (data not shown). 11D5 and 13E11 were then sequenced and purified monoclonal antibodies generated (Supplementary Figure 2).[10]

To confirm the specificity of 11D5 and 13E11 as recombinant antibodies, we used parental $CD33^{neg}$ human acute lymphoblastic leukemia (ALL) REH cells and sublines engineered to overexpress either $CD33^{FL}$ or $CD33^{\Delta E2}$.[6] As shown by flow cytometry (Figure 1A) and immunofluorescence microscopy (Supplementary Figure 3), 11D5 and 13E11 only bound cells expressing $CD33^{\Delta E2}$ but neither parental cells nor cells expressing $CD33^{FL}$, indicating the V-set domain (present in $CD33^{FL}$) hinders access of both antibodies to CD33. Testing of both antibodies with additional $CD33^{neg}$ human ALL cell lines (RS4;11, RCH-ACV) and a $CD33^{low}$ human AML cell line (OCI-AML3) engineered to overexpress $CD33^{FL}$ or $CD33^{\Delta E2}$ confirmed their specificity for $CD33^{\Delta E2}$ (Supplementary Figure 4), demonstrating both 11D5 and 13E11 recognize epitopes within the membrane-proximal C2-set domain of CD33 that are only present/accessible in the absence of the V-set domain ("$CD33^{\Delta E2}$-specific antibody").

The predicted canonical signal peptide of CD33 consists of 16 amino acids, with 12 amino acids encoded by exon 1 and 4 amino acids encoded by exon 2, and cleavage predicted to occur after the 16^{th} residue (Supplementary Figure 5). Since $CD33^{\Delta E2}$ retains exon 1 but lacks exon 2, the predicted signal peptide cleavage site present in $CD33^{FL}$ is lost. Computational models identified only 2 low-probability cleavage sites in the 5' portion of exon 3 (Supplementary Figure 5). Thus, we considered the possibility that the amino acids encoded by exon 1 (which are cleaved in $CD33^{FL}$) are retained in the mature $CD33^{\Delta E2}$ protein. Consistent with this possibility, mass spectrometry detected the signal peptide as part of the $CD33^{\Delta E2}$ immunogen (Supplementary Figure 6). To test whether the signal peptide served as binding epitope for 11D5 and/or 13E11, we generated two variant CD33 constructs with modified signal peptide sequences. Specifically, we generated a $CD33^{\Delta E2}$ variant in which the N-terminal amino acids of exon 2 that comprise the signal peptide for $CD33^{FL}$ were included (Supplementary Figure 5, "$CD33^{\Delta E2}$ + SP^{FL}"). Additionally, we generated a $CD33^{FL}$ variant in which the N-terminal amino acids encoded by exon 2 were replaced with the N-terminal amino acids of exon 3 to mimic the structure of the signal peptide in $CD33^{\Delta E2}$ (Supplementary Figure 5, "$CD33^{FL}$ + $SP^{\Delta E2}$"). As depicted in Supplementary Figure 7, 13E11 but not 11D5 bound $CD33^{FL}$ + $SP^{\Delta E2}$, whereas 11D5 but

not 13E11 bound $CD33^{\Delta E2}$ + SP^{FL}. Together, these findings suggest 13E11 and 11D5 bind non-overlapping epitopes on $CD33^{\Delta E2}$, with 13E11 but not 11D5 recognizing the signal peptide retained in $CD33^{\Delta E2}$.

We previously reported universal expression of *CD33*$^{\Delta E2}$ at the mRNA transcript level in human AML cell lines and primary bone marrow and peripheral blood samples from patients with AML.[6] With 13E11 and 11D5 now available, we tested whether $CD33^{\Delta E2}$ could be detected as protein on the cell surface of human AML cells. In a panel of human AML cell lines shown to express *CD33*$^{\Delta E2}$ mRNA (Supplementary Figure 8), neither 13E11 nor 11D5 detected cell surface $CD33^{\Delta E2}$ (Figure 1B). Moreover, in 2 primary human AML specimens (both of which expressed *CD33*$^{\Delta E2}$ mRNA; Supplementary Figure 8), flow cytometric analyses with 11D5 similarly did not demonstrate any $CD33^{\Delta E2}$ protein on AML blasts (Figure 1C). We also found no evidence of $CD33^{\Delta E2}$ in 4 healthy donor bone marrow specimens which all expressed $CD33^{FL}$ (Supplementary Figure 9A). Of note, all three rs12459419 genotypes were represented in our panel of cell lines, primary AML cells, and healthy donor samples. To study the potential expression of $CD33^{\Delta E2}$ on primary AML cells in more detail, we performed multiparameter flow cytometric analyses of 21 residual clinical samples positive for AML or myelodysplastic syndrome in which directly-labeled 11D5 was integrated in a multi-tube reagent panel. In all 21 samples, no convincing evidence of expression of $CD33^{\Delta E2}$ was identified above background.

Consistent with our findings, recent studies have indicated $CD33^{\Delta E2}$ protein is not expressed on the cell surface of healthy donor neutrophils and monocytes.[11] Rather, some evidence suggested $CD33^{\Delta E2}$ was retained intracellularly, accumulating in peroxisomes.[11] To address this possibility in AML, we conducted flow cytometric studies with 11D5 on permeabilized AML cells that we found to express *CD33*$^{\Delta E2}$ mRNA. In REH cells lentivirally-transduced to overexpress $CD33^{\Delta E2}$, staining was retained following permeabilization, demonstrating that the target antigen can still be detected after the permeabilization process. Likewise, 11D5 bound ML-1 cells in which endogenous CD33 was deleted via CRISPR/Cas9 and $CD33^{\Delta E2}$ introduced via lentiviral mediated gene transfer (ML-$1^{del33+CD33\Delta E2}$) (Figure 2A). Neither $REH^{CD33\Delta E2}$ nor ML-$1^{del33+CD33\Delta E2}$ cells showed increased 11D5 staining following permeabilization. Similarly, neither HL-60 or U937 cells nor primary AML patient cells stained with 11D5 after permeabilization. An ML-1 cell subline with CRISPR/Cas9-mediated deletion of exon 2 with resulting exclusive expression of *CD33*$^{\Delta E2}$ mRNA[12] likewise did not show cell surface or intracellular $CD33^{\Delta E2}$ protein expression (Figure 2A), arguing against the possibility that heterodimer formation between $CD33^{FL}$ and $CD33^{\Delta E2}$ could prevent antibody access to endogenously-expressed $CD33^{\Delta E2}$. These cells, however, stained positive with an antibody against myeloperoxidase used as a positive control for detection of an intracellular antigen. These findings suggested the lack of $CD33^{\Delta E2}$ cell surface expression in AML is not due to sequestration of $CD33^{\Delta E2}$ protein in intracellular compartments. To test further whether $CD33^{\Delta E2}$ protein might be retained in intracellular compartments, we performed immunoprecipitations using commercial antibodies raised against C-terminal CD33 peptides to enrich for both $CD33^{FL}$ and $CD33^{\Delta E2}$. As shown in Figure 2B, we found CD33 protein with the predicted size of $CD33^{\Delta E2}$ in human ALL cell lines forced to overexpress $CD33^{\Delta E2}$. In contrast, a similar protein band was not found in any of the parental human AML cell lines or 4 primary AML patient samples studied, again

suggesting that CD33$^{\Delta E2}$ protein is not present at a level detectable by antibody staining in human AML cells. It is plausible but speculative that retention of a non-cleaved signal peptide could alter intracellular trafficking and target CD33$^{\Delta E2}$ for degradation, as has been demonstrated for other proteins (e.g. references[13,14]).

Together, in our studies conducted with newly-developed CD33$^{\Delta E2}$-specific antibodies, we were unable to detect CD33$^{\Delta E2}$ on human AML cell lines or primary blast cells from a smaller cohort of AML patients, which contained cells with CC, CT, and TT rs12459419 genotypes. Different from what has been reported previously in normal neutrophils and monocytes,[11] we could not find CD33$^{\Delta E2}$ protein accumulations in intracellular compartments of AML cells or maturing healthy donor myeloid cells. While we cannot exclude that CD33$^{\Delta E2}$ might be expressed on the cell surface of myeloblasts in a smaller subset of AML patients, or might be expressed at earlier differentiation stages in AML, our data do not provide evidence for the value of CD33$^{\Delta E2}$ as a therapeutic target in AML regardless of the CD33 rs12459419 genotype of the patient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of interest: H.P.K. is a consultant to and has ownership interests with Rocket Pharma and Homology Medicines and is a consultant to CSL Behring and Magenta Therapeutics. R.B.W. received laboratory research grants and/or clinical trial support from Agios, Amgen, Aptevo Therapeutics, Arog, BioLineRx, Jazz, Pfizer, Seattle Genetics, and Selvita; has ownership interests with Amphivena Therapeutics; and is (or has been) a consultant to Agios, Amphivena Therapeutics, Astellas, BiVictrix, Boehringer Ingelheim, Covagen, Emergent Biosolutions/ Aptevo Therapeutics, Jazz, Kite, Pfizer, and Seattle Genetics. The other authors declare no competing financial interests.

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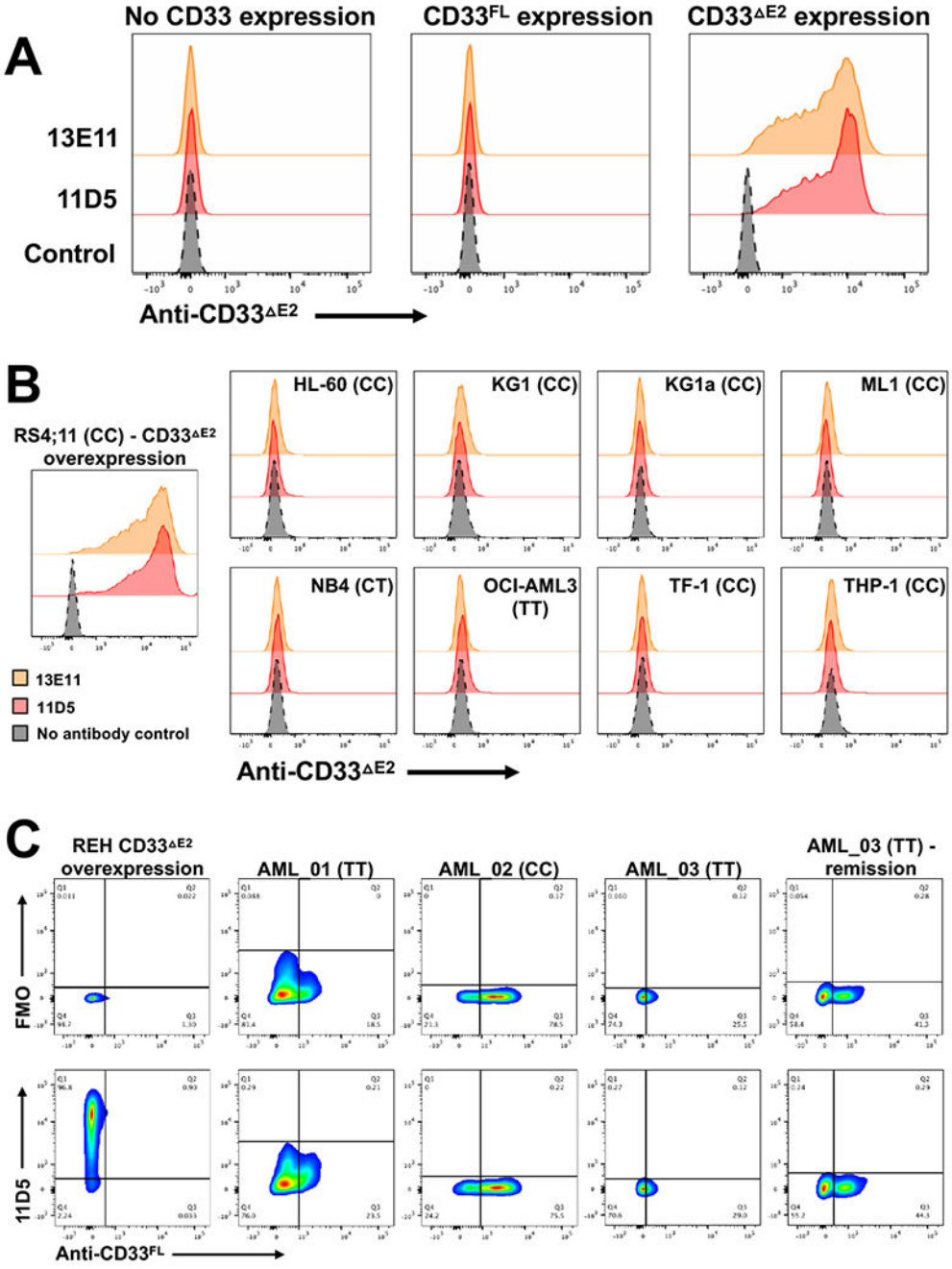

Figure 1. Cell surface characterization of human acute leukemia cells with anti-CD33$^{\Delta E2}$ antibodies.

Anti-CD33$^{\Delta E2}$ antibody clones 11D5 and 13E11 were tested by flow cytometry against **(A)** parental REH cells (human acute lymphoblastic cell line, endogenously CD33^{neg}) and sublines engineered to overexpress CD33^{FL} and CD33$^{\Delta E2}$ as well as **(B)** a panel of human AML cell lines and, as a positive control, RS4;11 (human acute lymphoblastic cell line, endogenously CD33^{neg}) cells engineered to overexpress CD33$^{\Delta E2}$. A no-primary-antibody control was included. Data are representative of three separate experiments. CD33

rs12459419 genotypes are shown in parentheses. **(C)** The anti-CD33$^{\Delta E2}$ antibody 11D5 and standard anti-CD33^{FL} antibody clone p67.6 were used to co-stain three frozen/thawed samples from AML patients with active disease (AML_01, AML_02, AML_03) as well as a corresponding remission sample from AML_03. As a positive control, engineered REH cells overexpressing CD33$^{\Delta E2}$ were included. Both full stain and fluorescence minus one (FMO, −11D5) are shown. CD33 rs12459419 genotypes are shown in parentheses. Abbreviations: CD33^{FL}, full length CD33 isoform; CD33$^{\Delta E2}$, CD33 isoform arising from transcript missing exon 2.

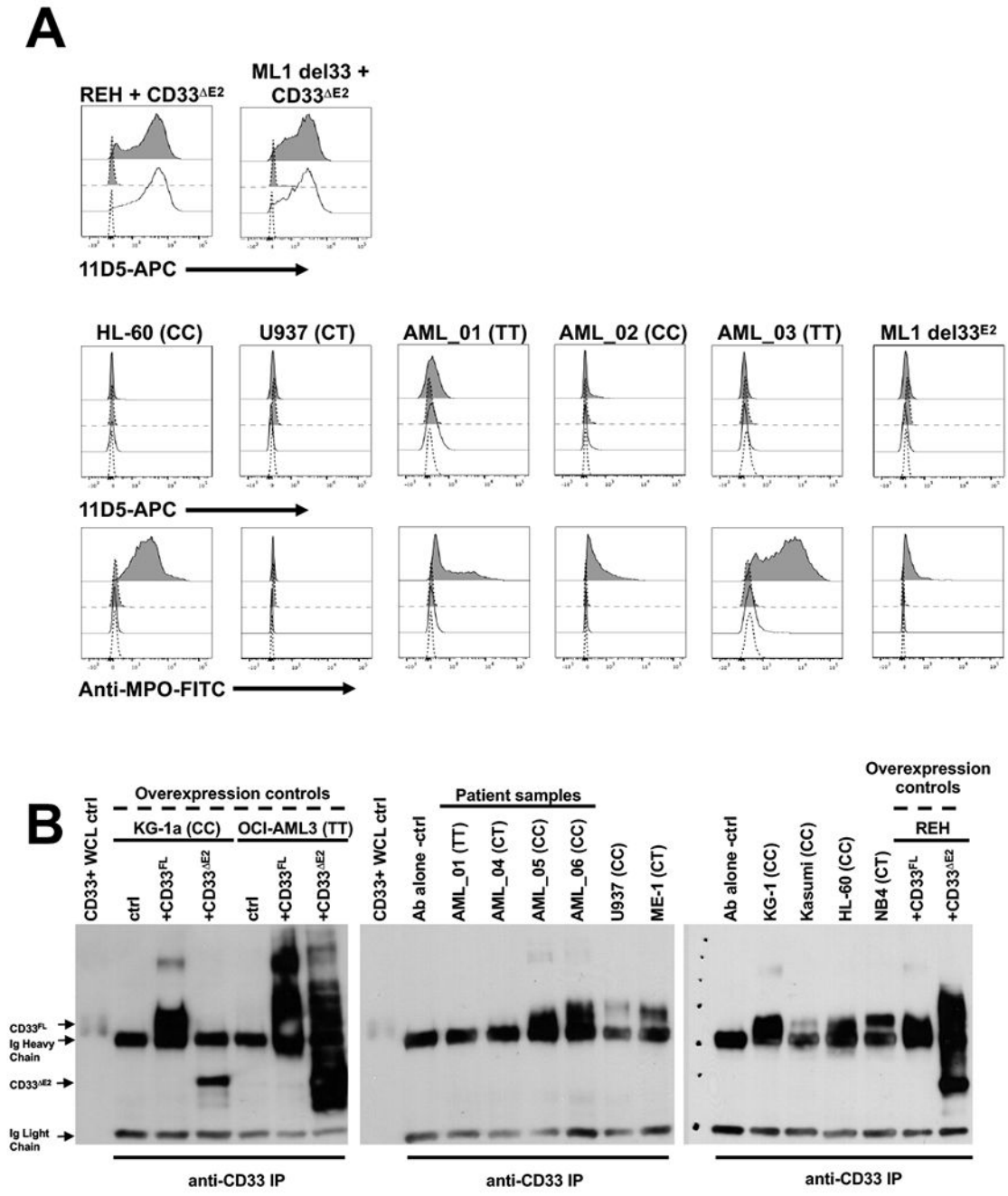

Figure 2. Whole cell characterization of human acute leukemia cells with anti-CD33$^{\Delta E2}$ antibodies.

(A) Engineered REH cells overexpressing CD33$^{\Delta E2}$, ML-1 cells with deletion of CD33 (del CD33) via CRISPR/Cas9 and subsequent overexpression of CD33$^{\Delta E2}$, HL-60 cells, U937 cells, three primary AML patient specimens (AML_01, AML_02, AML_03) and an ML-1 cell subline with genomic deletion of CD33 exon 2 via CRISPR/Cas9 (del33^{E2}) with resulting exclusive expression of *CD33$^{\Delta E2}$* transcript[12] were included. All cell lines and patient specimens were stained with 11D5-APC with (gray histograms) or without (white histograms) permeabilization to detect intracellular antigens. Anti-myeloperoxidase (MPO)

antibody was used as a positive control for intracellular antigen detection. Dotted lines represent isotype control antibody. Results are representative of N=3 separate experiments. CD33 rs12459419 genotypes are shown for non-engineered cell lines and patient specimens in parentheses. **(B)** Antibodies against the C-terminal portion of CD33 (present in both $CD33^{FL}$ and $CD33^{\Delta E2}$) were used for immunoprecipitation and subsequent immunoblotting to enrich for, and detect, CD33 isoforms. KG-1a, OCI-AML3, and REH cells engineered to overexpress either $CD33^{FL}$ or $CD33^{\Delta E2}$ are shown as positive/negative controls together with a panel of parental AML cells lines and thawed AML patient samples. CD33 rs12459419 genotypes are shown in parentheses. Ab, antibody; ctrl, control; IP, immunoprecipitation; WCL, whole cell lysate.