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Supplementary Information for

Gene-edited stem cells enable CD33-directed Immune therapy for myeloid malignancies

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Supplementary Text
Materials and Methods

Cell line and primary human cells

The Immortalized human acute myeloid cell line, HL-60 was obtained from ATCC and cultured in IMDMEM with 20% Fetal Bovine Serum and 1% Penicillin Streptomycin. In order to follow leukemia engraftment and progression over time, the HL-60 cells were transduced with lentiviral particles expressing a dTomato fluorescent protein under an EF1α promoter. The lentivirus vector and particles were produced by Vectalys (Toulouse, France).

Human Bone Marrow or Cord Blood $CD34^+$ stem cells were purchased from StemExpress (Folsom, CA, USA) and maintained in StemSpan SFEM II (STEMCELL technologies inc) containing 1% Penicillin Streptomycin, 100ng/mL TPO, 100ng/mL SCF, 100 ng/mL IL6 and 100ng/mL FLT3L and UM171 0.35 nM (Xcessbio, San Diego, CA, USA). All human cytokines were purchased from Biolegend (San Diego, CA, USA).

Human T cells were purified from fresh peripheral blood normal donor leukopaks purchased from the New York Blood Center. Briefly, the leukopak was diluted with 2-4 volumes of Phosphate Buffered Saline (1X) supplemented with 2 mM EDTA, store at 4C. Then 35 mL of diluted leukopak was carefully layered on 15 mL of Ficoll-PaqueTM Premium (GE) and centrifuged at 400g, 30 mins at 25°C, in swinging rotor buckets. The layer of mono nuclear cells was then transferred to a new tube, diluted 1:1 with PBS (1X) containing 2mM EDTA and centrifuged 400g, 15 mins at 25°C. The red blood cells of the pellet were then removed with 1X ACK lysis buffer (Gibco), incubated 5-8 mins at RT, washed with PBS (1X) containing 2mM EDTA and centrifuged again 400g, 10 mins at 25°C. The $CD4^+$ and $CD8^+$ T cells were then positively selected from the mono nuclear cells pellet with Miltenyi Biotec $CD4^+$ and $CD8^+$ microbeads, following manufacturer protocols. The $CD4^+$ and $CD8^+$ T cells were then activated the same day using CD3/CD28 dynabeads 1:1 bead to cell ratio (Gibco) and expanded separately in the Gibco OpTmizer™ CTS™ T-Cell Expansion SFM medium containing IL7 10ng/mL and IL15 5ng/mL.

CAR constructs, lentiviral production, and transduction

The anti-CD33 Chimeric Antigen Receptor was generated by cloning the light and heavy chain of the humanized anti-human CD33 scFv (clone My96) fused in frame, to the CD8 alpha hinge domain, the CD8 transmembrane domain, the 4-1BB signaling domain and the CD3zeta intracellular domain into the lentiviral plasmid pHIV-Zsgreen, a gift from Bryan Welm & Zena Werb (Addgene plasmid # 18121). All cDNA fragments were codon optimized and synthesized by GeneArt (Regensburg, Germany). The lentiviral particles were produced by Vectalys (Toulouse, France). 24 hours after activation, $CD4^+$ T cells were transduced with lentiviral particles at an MOI=30 and in parallel, $CD8^+$ T cells at an MOI=40.

In vitro cytotoxicity assays

Effector sorted CAR-T cells stably expressing zsGreen were mixed at a different ratio with the following target cells HL-60 stably expressing dTomato and or $CD34^{+}CD33^{WT}$ cells stained with Celltrace blue and or $CD34^{+}CD33^{Del}$ stained with Celltrace Violet (Invitrogen). 16 to 24 hours after incubation, using 7AAD or Sytox Red as a viability dye, data were acquired with the BioRad ZE5 flow cytometry analyzer in high-throughput mode in order to assess cytotoxicity. After subtracting the spontaneous lysis in negative control, CART33 cells specific cytotoxicity (%) was calculated as cells positive for both CFSE and 7-AAD or Sytox Red with the following formula: ((% positive cells with CART33)- (% positive cells with control T cells))/(100– ((% positive cells with control T cells)) x 100.

In vitro phagocytosis assays

$CD34^{+}CD33^{WT}$ cells or $CD34^{+}CD33^{Del}$ were differentiated into monocytes for 14 days, with StemSpan SFEM II containing 1% Penicillin Streptomycin and the StemSpan Myeloid Expansion Supplement II (100X). At day 15, differentiated monocytes were preincubated for 30 mins with PBS or 25uM Cytochalasin D and then incubated with pHrodo red E coli bioparticles (thermofisher) with or without 25uM cytochalasin D for 1h30 mins. Cells were then transferred to FACS tubes, washed with PBS-FBS and incubated 10 mins at RT with Human TruStain FcX and True-Stain Monocyte blocker (Biolegend). Antibodies mix containing CD14-APC/Cy7, CD13-BV786, CD33-APC and ebioscience fixable viability dye e450 was then added directly. After 25 mins of incubation, cells were washed and phagocytosis was assessed by FACS.

LPS induced cytokine assay

For LPS induced innate immune response assessment, 8 weeks after injection of $CD34^{+}CD33^{WT}$ or $CD34^{+}CD33^{Del}$ cells, NSGS mice received 15ugr of LPS (from E coli O26:B6, Sigma) intraperitoneally. Plasma was collected in EDTA tube at t=0 and 4h30 after injection. Cytokines level was measured using Legendplex human cytokine panel from Biolegend, following manufacturer instructions.

In vivo Phagocytosis assay

For *in vivo* phagocytosis, mice received 150uL of pHrodo red E coli bioparticles (thermofisher) via i.v. and were sacrified 2 hours after for peritoneal cavity analysis.

Statistics

All statistics were performed using Graphpad Prism 7. For continuous variables, an unpaired two tailed t-test was performed. Differences between means were considered significant when the p value is <0.05, else not significant (ns; $p>0.05$).

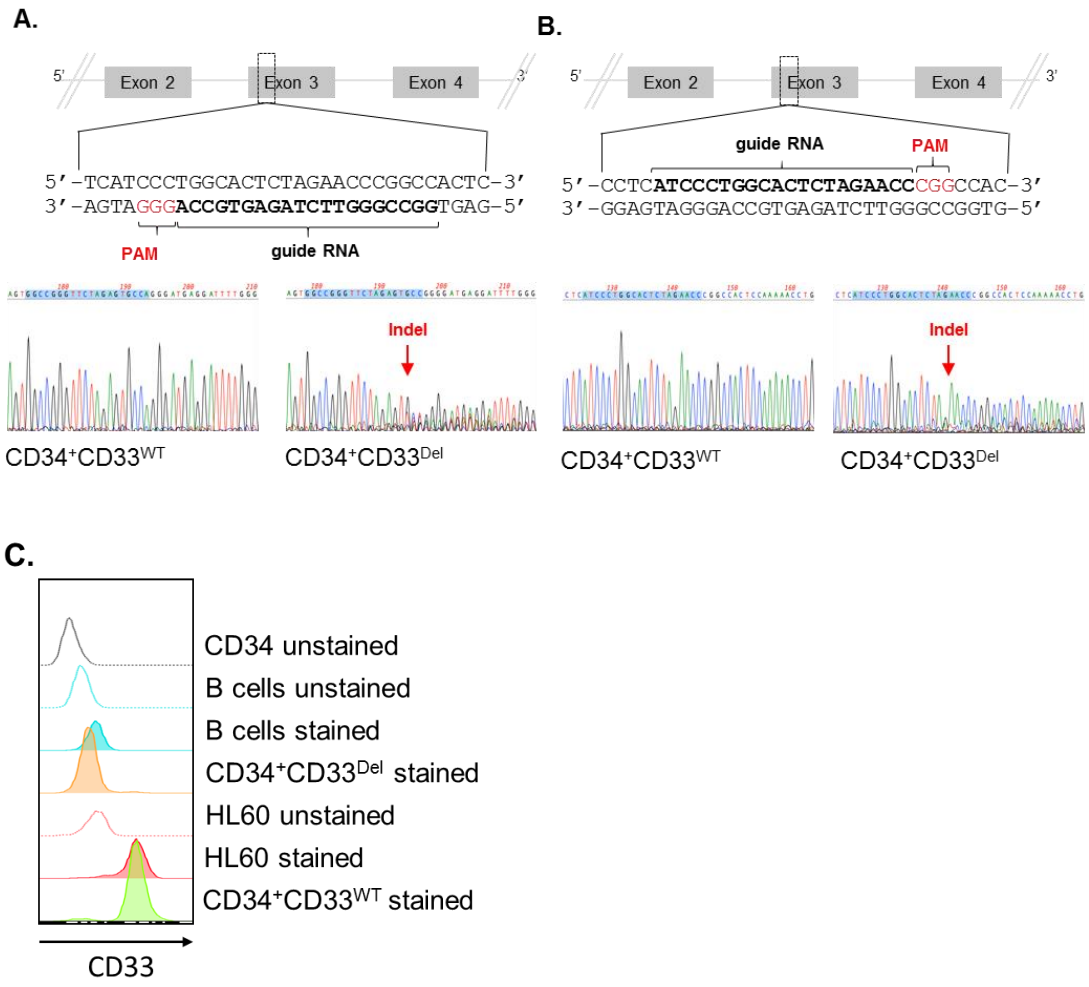

Fig. S1. Additional sgRNA tested to ablate CD33 expression show high level of indels. (*A & B*) and expression of CD33 on gene-edited and unedited cells (C). A & B Schematic representation of CD33 genomic locus showing exon 2-4 and location and sequence of two additional sgRNA targeting CD33 locus. Chromatogram at the bottom are screenshot of Sanger sequencing showing a region surrounding the DNA double strand break site, left: $CD34^{+}CD33^{WT}$ cells and right: $CD34^{+}CD33^{Del}$. Guide sequences are highlighted in blue on the chromatogram and appearance of indels is indicated by red downward arrow.

C. CD33 expression on CD34+ cells, HL60 AML cells and B cells and $CD34^{+}CD33^{Del}$cells. CD33 expression was determined by flow cytometry. Dotted lines indicate unstained cells, filled histogram show cells stained with CD33 antibody. $CD34^{+}CD33^{Del}$ cells show overlap with the B cells (negative control for CD33 expression) and virtually no overlap with the $CD34^{+}CD33^{WT}$ and HL-60 cells positive control) suggesting efficient CD33 deletion.

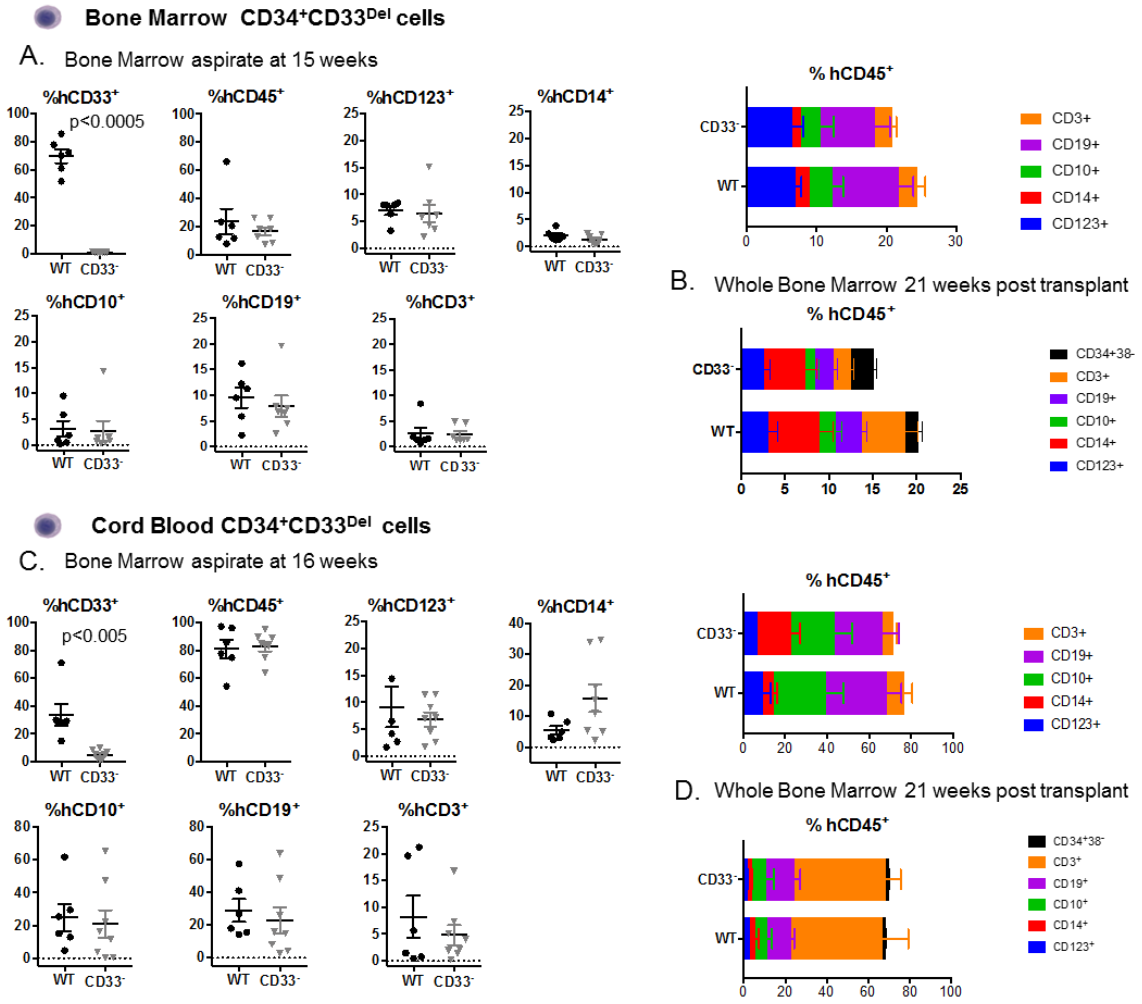

Fig. S2. Deletion of CD33 does not impair engraftment and hematopoietic repopulation in NSGM3 mice. (*A-B*) Bone marrow derived CD34$^+$ cells engraftment and repopulation: (*A*) Bone marrow aspirate (15 weeks) post-transplant analyzed for cells of various lineages, as indicated. CD34$^+$CD33^{Del} cells show same engraftment (CD45$^+$) as control cells as well as comparable percentage of mature myeloid and lymphoid cells. The bar diagram represents summary of individual panels. **(*B*)** Summary of data from main Fig. 2C. **(*C* to *D*) Cord blood derived CD34$^+$ cells engraftment and repopulation: (*C*)** Bone marrow aspirate (16 weeks) post-transplant analyzed for cells of various lineages, as indicated. CD34$^+$CD33^{Del} cells show same engraftment (CD45$^+$) as control cells as well as comparable percentage of mature myeloid and lymphoid cells. The bar diagram represents summary of individual panels. **(*D*)** Summary of data from main Fig 2E. No significant differences were observed between both groups in all cell types analyzed ($p>0.05$), unpaired t test. All data are represented as mean ± SEM.

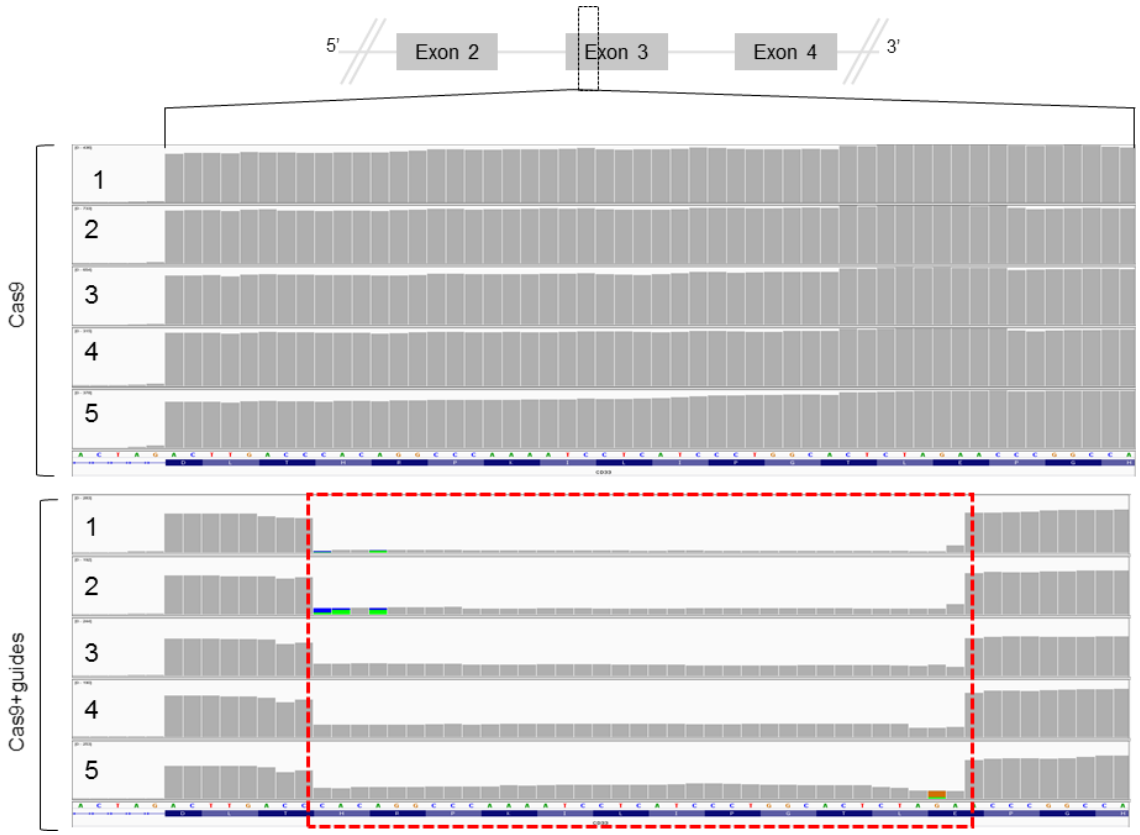

Fig. S3. Coverage of reads in RNAseq data. Integrated genomic viewer (IGV) screenshot of coverage track showing CD33 genomic regions surrounding the guides in Cas9+sgRNA (n=5; bottom) and Cas9 only (n=5; top) cells as indicated in the left. The grey bars in the coverage track (indicated on right) show the depth of the reads displayed at each locus. Generally, the coverage should be uniform and hence the bar height should be same but deletions results in dip in the height of the bars, marked by a red dotted rectangle in lower panel.

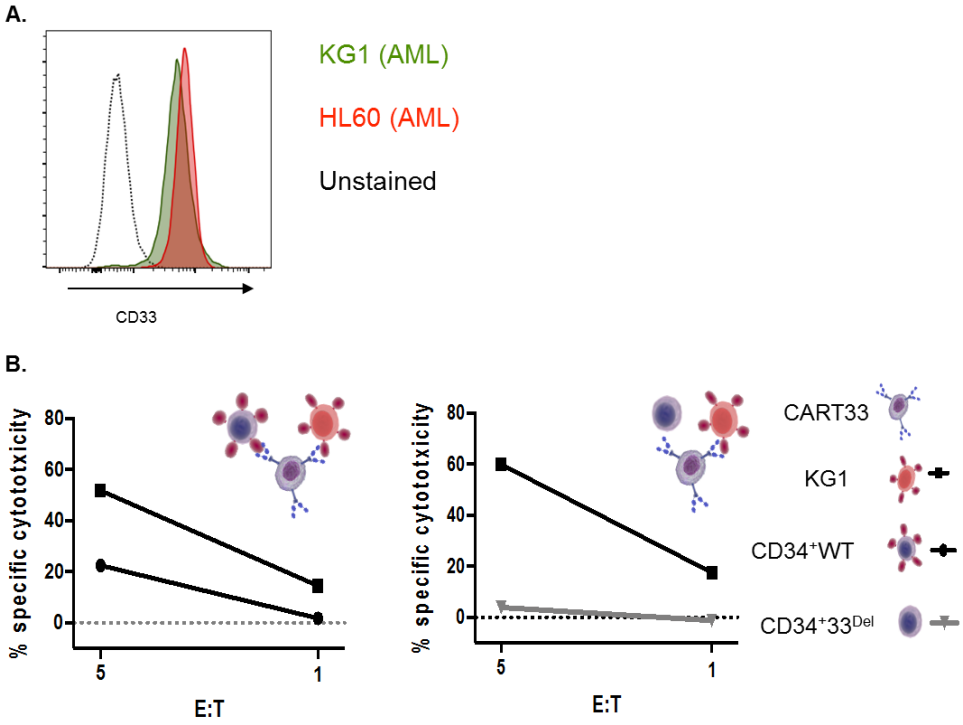

Fig. S4. Cytotoxicity of CART33 over AML derived KG1 cells (**A**) CD33 expression was measured and found to be relatively less on KG1 cells compared to HL-60 cells. (**B**) CART33 cells or control T were incubated with KG1 and CD34+CD33WT or CD34+CD33Del at two different effector (E) to target (T) ratio (E:T). Cytotoxicity was assessed by flow cytometry and percent specific cytotoxicity was measured. We saw a CD33-antigen dependent cytotoxicity of KG1 cells.

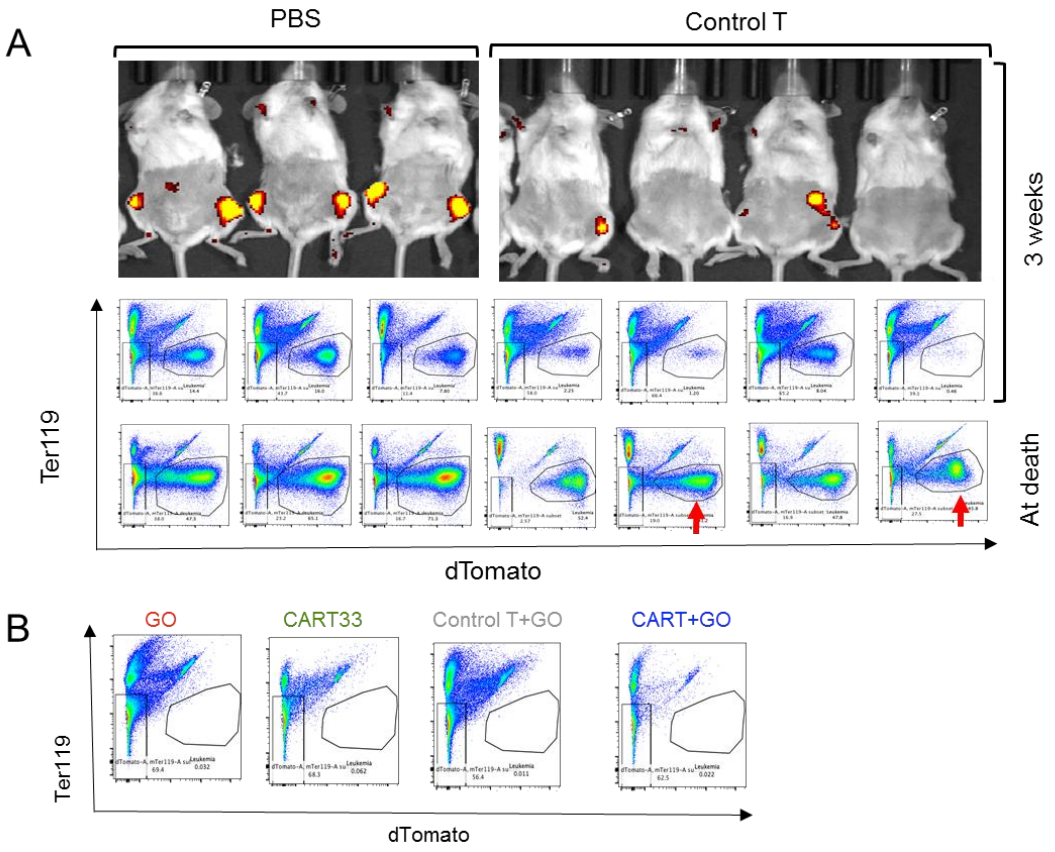

Fig. S5. Therapy model: CD34^{+}CD33^{Del} cells resist CD33-targeted immunotherapy. **(*A*)** Leukemia burden in control groups of mice at 3 weeks (Top panel: imaging and bottom panel: flow cytometry of bone marrow aspirate) and 8 weeks (Flow cytometry dot plots) Leukemia cells were gated on Ter119^{-}dtomato^{+}. **(*B*)** Representative flow cytometry dot plot from one mice showing absence of leukemia burden in treated groups.

Table S1. Summary of reads and variants found in whole genome sequencing.

	Cas9			Cas9+sgRNA		
Total PF Reads	629,724,054			659,744,702		
Percent Q30 Bases	93.46%			93.43%		
	SNVs	Insertions	Deletions	SNVs	Insertions	Deletions
Small Variants Summary						
Total Passing	3,743,126	401,796	418,883	3,744,331	401,386	418,187
Percent Found in dbSNP	94.24%	85.75%	86.70%	94.25%	85.82%	86.89%
Het/Hom Ratio	1.613	2.008	2.358	1.614	2	2.343
Ts/Tv Ratio	2.063	-	-	2.063	-	-
Variants by Consequence						
Frameshifts	-	58	67	-	57	67
Non-synonymous	10,248	144	192	10,300	157	191
Synonymous	11,638	-	-	11,625	-	-
Stop Gained	77	6	0	77	6	0
Stop Lost	19	0	1	19	0	1

Table S2. Off-target sites for sgRNA 846 (sgRNA: **AUCCCUGGCACUCUAGAACC;** PAM: **CGG)**. No indels were found within 100 bp of the expected off-target cut site in whole genome sequencing analysis. Mismatch with guide sequence is denoted in red.

OFF TARGET SITE	MISMATCHES	CHROMOSOME	CUT SITE	PAM	GENE	Indels
ATCCCTGGCACTCAGGAGCC	3	chr9	129,640,094	AGG	ASB6	None
AACCCTGGCTCTCTAAAACC	3	chr12	112,995,250	AGG	RP1-71H24.1	None
ATCCCTGGCACCCTGGCACC	3	chr14	99,627,517	TGG		None
ATCCCTGGCACTCCAGGGCC	3	chr17	9,253,589	TGG	STX8	None
AGCCCTGGCAGTCTGGAACC	3	chr8	52,941,254	TGG		None
ATCCCAGGCCCTCTATAACC	3	chr1	236,691,946	TGG	ACTN2	None
ATCCATGGTACTCTGGAACC	3	chr22	35,609,583	AGG	MB	None
AACCCAGGCACTCTAGCACC	3	chr2	203,480,837	AGG	RAPH1	None
ACCCCTAGCACTCTAGAGCC	3	chr1	26,560,691	AGG	RPS6KA1	None
ACCCCTGGCAGTCTAGAGCC	3	chr4	25,435,756	AGG		None
ATCCCTGACACTCTGGGACC	3	chr12	3,595,172	TGG		None
AGCCCTCGCACTCTGGAACC	3	chr14	74,598,895	AGG	LTBP2	None
ATCTCTGGCAGACTAGAACC	3	chr5	94,207,405	AGG	KIAA0825	None
ACCCCTGACACTCTAGAAAG	4	chr16	57,399,318	AGG		None
ATCCCTGGCACACTGGGCCC	4	chr2	106,853,582	AGG	ST6GAL2	None
ATCACTGTCTCCCTAGAACC	4	chr9	125,259,035	TGG		None
ATCCCTGACCCTCCAGAACA	4	chr9	131,545,888	TGG	RP11-40A7.2	None
ACCCCTGGCTCCCTAGAGCC	4	chr10	125,810,087	TGG	UROS	None
CTGCCTGGCTCTCTAGCACC	4	chr11	205,054	TGG	BET1L	None
GTCTCTGGCACTCTAGGCCC	4	chr11	2,689,716	AGG	KCNQ1	None

Table S3. Off-target sites for sgRNA 811 (sgRNA: **CCUCACUAGACUUGACCCAC**; PAM: **AGG**). No indels were found within 100 bp of the expected off-target cut site in whole genome sequencing analysis. Mismatch with guide sequence is denoted in red.

OFF TARGET SITE	MISMATCHES	CHROMOSOME	CUT SITE	PAM	GENE	Indels
CCTCACAAGATTAGACCCAC	3	chr12	86,341,556	TGG	MGAT4C	None
TCTCACTGGCCTTGACCCAC	3	chr9	134,687,890	TGG	COL5A1	None
CCTCACTGGACTTGACTCAG	3	chr1	209,621,235	GGG	LAMB3	None
CCTCACTGGACAAGACCCAC	3	chr12	19,574,394	TGG	AEBP2	None
CCTCACTAGACTAGATCCAT	3	chr9	973,903	TGG		None
CCTCGCTAGCCTTTACCCAC	3	chr7	157,876,963	AGG	PTPRN2	None
CCTCACAAGACAAGACCCAC	3	chr8	62,858,829	TGG	NKAIN3	None
CTTCACCAGCCTTGACCCAC	3	chr19	51,125,605	AGG	SIGLEC9	None
CCTCACCAGCCCTGACCCAC	3	chr19	51,143,004	AGG	SIGLEC7	None
CTTCACCAGCCTTGACCCAC	3	chr19	51,144,414	AGG	SIGLEC7	None
CCTCACCAGCCCTGACCCAC	3	chr19	51,185,448	AGG	SIGLEC20P	None
CCTCACCAGCCCTGACCCAC	3	chr19	51,211,800	AGG	SIGLEC22P	None
CCTCACCAGCCCTGACCCAC	3	chr19	51,501,106	AGG	SIGLEC12	None
CCTCACCAGCCCTGACCCAC	3	chr19	51,509,057	AGG	SIGLEC27P	None
CCTCACCAGCCCTGACCCAC	3	chr19	51,530,952	AGG	SIGLEC6	None
TCACACTGGCCTTGACCCAC	4	chr9	130,997,236	TGG		None
CCTTACTAGCCTCCACCCAC	4	chr5	127,098,351	GGG		None
CCTCACAAGATAAGACCCAC	4	chr2	223,246,492	TGG		None
CCTAACTAGAATTGCCACAC	4	chr2	228,144,164	AGG	SPHKAP	None
CCTCTATATACTTGTCCCAC	4	chr1	46,408,719	AGG	FAAH	None

Table S4. List of genes differentially expressed in CD33 deleted cells. Genes whose expression is low in CD33 deleted cells compared to CD33 wildtype cells are indicated by negative sign.

Gene	log2 Fold Change	pvalue	padj	Description
CD33	-0.837	0.000455048	0.999	Myeloid cell surface antigen CD33
CDON	1.033	0.009579405	0.999	Cell adhesion molecule-related
AC027279.1	0.824	0.01015653	0.999	Non-coding RNA
LDLR	-0.563	0.012597131	0.999	Low-density lipoprotein receptor
PRAME	-0.87	0.016800466	0.999	Melanoma antigen preferentially expressed in tumors
INSIG1	-0.494	0.017206864	0.999	Insulin-induced gene 1 protein
HMGB1P31	-0.797	0.022454929	0.999	Non-coding RNA
AP005019.1	-1.261	0.032232654	0.999	Non-coding RNA
PCLAF	0.382	0.035486149	0.999	PCNA-associated factor;PCLAF
CD22	-0.76	0.03640001	0.999	B-cell receptor CD22
TPTE2	0.712	0.037739962	0.999	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase TPTE2
ZNF469	0.811	0.039222723	0.999	Zinc finger protein 469
SVOPL	-0.867	0.04599215	0.999	Putative transporter SVOPL
FFAR4	0.678	0.046205968	0.999	Free fatty acid receptor 4