Supplemental information for:

The complement receptor C3AR constitutes a novel therapeutic target in *NPM1*-mutated AML

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SUPPLEMENTAL METHODS

Cell surface receptor screen – data analysis

FlowJo (BD Biosciences) was used for data analysis; the median value of the median fluorescent intensity (MFI) for each marker within the $7AAD^-$ $CD3^-$ $CD19^-$ AML cell fraction was divided by the median value of the MFI for each marker within the $7AAD^-$ $CD34^+$ $CD38^{low}$ NBM cell fraction. Subsequently, the quote values were ranked from highest to lowest generating a list of cell surface markers specifically upregulated on the AML blast population (**Figure 1A** and **B**). All antibodies used are listed in **supplemental Table 2**.

Screen validation and cell surface marker evaluation

To validate the results from the screen, nine additional *NPM1*-mutated primary AML MNC samples (bringing the total number of *NPM1*-mutated samples assessed by flow cytometry to 13 cases) were stained with anti-C3AR antibody or corresponding isotype control, together with anti-CD3, anti-CD19 and 7AAD (Biolegend). Three samples were co-stained with anti-GPR56 antibody. For evaluation of C3AR expression in *NPM1* wild type (WT) AML samples (n=30), anti-CD34 and anti-CD38 were also included. Two NBM MNC samples were stained with anti-CD34, anti-CD38, anti-CD90, anti-CD45RA, anti-CD123, anti-lineage cocktail and anti-C3AR to evaluate C3AR-expression in different fractions of the normal MNCs. Anti-CD14 and anti-CD16 was used for detailed analysis of C3AR-expression on monocytes and the dye 7AAD (Biolegend) was used for viability staining in all experiments. The C3AR expression in the leukemia cell lines OCI-AML3 (carrying a *NPM1* mutation), KG-1 (*NPM1* WT) and NALM-6 (*NPM1* WT) (all from German Collection of Microorganisms and Cell

Cultures GmbH, Germany) was also evaluated using an anti-C3AR antibody and a corresponding isotype control. All antibodies used are listed in **supplemental Table 2**.

GPR56 C3AR transplantation experiments

A primary *NPM1*-mutated AML sample was thawed and stained with anti-CD3, anti-CD19, anti-C3AR antibody or corresponding isotype control, anti-GPR56 and 7AAD (Biolegend), as described in the main article, and sorted on a FACS Aria II (BD Bioscience). Animal experiments were approved by the regional animal ethics committee. Irradiated (200-250 cGy) NSGS (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzj) mice were transplanted by tail vain injection with 600'000/mouse of C3AR^{+} GPR56^{+} or C3AR^{+} GPR56^{-} cells. The mice were sacrificed 19 weeks after transplantation and the percentage of human CD33^{+} CD45^{+} in the BM was analyzed on an LSR Fortessa (BD Biosciences). The animal studies were approved by the Swedish Board of Agriculture, Malmö/Lund Animal Ethics committee in Lund, Sweden. All antibodies used are listed in **Supplemental Table 2.**

ADCC in vivo repopulation analysis

The ADCC was executed as described in the main article. 250,000 AML cells/mouse were plated and treated with 10ug/ml of antibody and 2.5 million NK cells. The residual cells from the assay were transplanted into irradiated (200-250 cGy) NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice. Mice were sacrificed 5 weeks after transplantation and the percentage of engrafted human cells in BM was evaluated using viability dye 7AAD (Biolegend) and anti-human CD33 and anti-human CD45 on an LSR Fortessa LSRFortessa (BD Biosciences) instrument. The experiment was performed twice using two different primary AML samples. The animal studies were approved by the Swedish Board of Agriculture, Malmö/Lund Animal Ethics committee in Lund, Sweden. All antibodies used are listed in **Supplemental Table 2**.

Bulk RNA-sequencing and external data

Gene expression was determined as fragments per kilobase of transcript per million reads (FPKM) using RSEM 1.2.30. STAR 2.5.2b was used as alignment tool and hg19 as human reference genome. External RNA-sequencing gene expression data from the Cancer Genome Atlas program (TCGA) AML dataset[1] was downloaded from cbioportal (https://cbioportal-datahub.s3.amazonaws.com/laml_tcga_pub.tar.gz). The expression data were annotated with *NPM1*, *DNMT3A* and *FLT3*-ITD mutations from supplemental data in the original TCGA

AML publication.[1] External RNA-sequencing gene expression data from the Beat AML program AML dataset[2] (dbGaP Study Accession: phs001657.v1.p1) was downloaded from The National Cancer Institute's (NCI's) Genomic Data Commons (GDC) Data Portal with The GDC Data Transfer Tool, and aligned to reference genome hg19 using STAR version 2.7.8a.[3] Gene expression was summarized as expected counts using RSEM version 1.2.30[4] and converted to count per million (CPM) using voom[5] from limma version 4.44.1.[6]

Single cell RNA-sequencing

The reads were aligned to the hg19 reference genome and converted into single cell gene expression data using cellranger count (10x Genomics, v.3.1.0). Further analysis was performed using the R package Seurat (v.4.0.0).[7] Low quality cells were excluded, (<200 informative genes or >15 % expressed transcripts representing mitochondrial genes). Normalization and variance stabilization was performed using the R package sctransform (v0.3.2).[8] The data were visualized using uniform manifold approximation and projection (UMAP)[9] based on a principal component analysis (PCA) with 80 components. A graph-based clustering approach was applied with 80 nearest neighbors and a resolution of 0.5-0.8 was used to portion the dataset into clusters. For lineage inference of the cells within the NBM samples, a carefully annotated dataset,[10] processed by Weighted Nearest Neighbor analysis, was used as a reference.[10,11] The cells in the full dataset (including both NBM and AML cells) were classified by projection on to the PCA structure from the annotated NBM dataset using Seurats "TransferData" function. Cell classifications were modified based on the origin of the cells within each cluster: clusters with only a negligible contribution from NBM samples (less than 20% when normalized for differences in total cell contribution) were re-classified as “AML-specific cells”. The cells from AML samples were evaluated by single cell mutation identification of the known *NPM1*-variant in that sample using VarTrix (https://github.com/10xgenomics/vartrix , v1.1.14).

REFERECES SUPPLEMENTAL METHODS

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. CD22 and CD127 show inconsistent expression in *NPM1*-mutated AML. Histograms showing expression of CD22 and CD127 in the $CD3^-$ $CD19^-$ cell population in the primary *NPM1*-mutated samples used in the screen. Marker stain in red and isotype control in gray.

Supplemental Figure 2. C3AR is not expressed in normal hematopoietic stem and progenitor cells (A) and (B) Flow cytometric analysis of C3AR expression in different fractions of NBM MNCs from two healthy donors shown as contour plots. The same samples as in Figure 2 are presented. C3AR stain in red and isotype control in black. (C), (D), (E) and (F) Flow cytometric analysis of C3AR expression in $Lin^-CD34^+CD38^-$ (including HSC, MPP and LMPP) and $Lin^-CD34^+CD38^+$ (including CMP, MEP and MEP) from four healthy donors shown as histograms. C3AR stain in red and isotype control in black. NBM, normal bone marrow; HSC, hematopoietic stem cell (Lin^- $CD34^+$ $CD38^-$ $CD90^+$ $CD45RA^-$) ; MPP, multipotent progenitor (Lin^- $CD34^+$ $CD38^-$ $CD90^-$ $CD45RA^-$); LMPP, lymphoid-primed multipotent progenitor (Lin^- $CD34^+$ $CD38^-$ $CD45RA^+$); CMP, common myeloid progenitor (Lin^- $CD34^+$ $CD38^+$ $CD123^+$ $CD45RA^-$); GMP, granulocyte-macrophage progenitor (Lin^- $CD34^+$ $CD38^+$ $CD123^+$ $CD45RA^+$); MEP, megakaryocyte-erythroid progenitor (Lin^- $CD34^+$ $CD38^+$ $CD123^-$ $CD45RA^-$); MNC, mononuclear cell.

Supplemental Figure 3. CD16 positive monocytes in MNCs from NBM express C3AR (A) and (B) C3AR-expression in different monocytes subsets based on CD14 and CD16 expression in NBM MNCs from two donors. C3AR stain in red and isotype in filled gray. NBM, normal bone marrow; MNC, mononuclear cells.

Supplemental Figure 4. C3AR is specifically expressed on *NPM1*-mutated AML cells (A) Fold change of the C3AR MFI in the myeloid enriched ($CD3^-$ $CD19^-$) cells of different AML subtypes compared to isotype control MFI (n=43), as assessed by flow cytometry. (B) Fold change of C3AR MFI in AML subgroups with *NPM1* WT, in the stem cell enriched ($CD34^+$ $CD38^{low}$) compartment, compared to isotype control MFI (n=43), as assessed by flow cytometry. MFI, median fluorescent intensity; WT, wild type; chrom-splice, chromatin-spliceosome. Bars show median with interquartile range, *≤0.05, **≤0.01 and ***≤0.001 using a nonparametric Mann-Whitney test.

Supplemental Figure 5. Single cell RNA sequencing confirms *C3AR1* expression in *NPM1*-mutated AML blasts and in normal monocytes. UMAPs showing the expression of *C3AR1* in 12 *NPM1*-mutated AMLs, five NBM MNCs and three NBM $CD34^+$, in individual plots. NBM, normal bone marrow; UMAP; Uniform Manifold Approximation and Projection for Dimension Reduction

Supplemental figure 6. Bulk RNA sequencing confirms low *C3AR1* expression is normal $CD34^+$ cells. CPM values of *C3AR1* in MNCs (n=20) and normal $CD34^+$ (n=13) cells from NBM, in the publicly available Beat AML dataset, represented as box plots. CPM, counts per million; NBM, normal bone marrow. ****≤0.0001 using a nonparametric Mann-Whitney test.

Supplemental Figure 7. Single cell RNA sequencing shows *GPR56* to be expressed in HSCs, NK cells, some T-cells and *NPM1*-mutated AML cells. UMAPs showing the expression of *GPR56* in 12 *NPM1*-mutated AMLs, five NBM MNCs and three NBM $CD34^+$ in individual plots. NBM, normal bone marrow; UMAP; Uniform Manifold Approximation and Projection for Dimension Reduction

Supplemental Figure 8. Co-expression of GPR56 and C3AR marks *NPM1*-mutated AML cells with leukemia initiating capacity. Contour plots showing the percentage of $CD45^+$

$CD33^+$ cells in the BM of immunodeficient NSGS mice 19 weeks after transplantation with $C3AR^+GPR56^+$ and $C3AR^+GPR56^-$ cells from a primary *NPM1*-mutated patient sample, as shown in Figure 5B and C.

Supplemental Figure 9. C3a-stimulation of primary AML cells and C3AR expression on KG-1, OCI-AML3 and NALM-6 cells. (A) Expression of pERK1/2 after 5-minute stimulation with 100 ng/ml of human C3a in five primary AML sample. Same samples as in Figure 6A. Unstimulated cells in filled gray and C3a-stumlated in red. (B) Number of primary *NPM1*-mutated AML cells after a 3-day culture with increasing concentrations of human C3a. 100,000 primary AML cells were seeded/well in serum free medium. The experiments were performed in triplicates. (C) Expression of C3AR in OCI-AML3, KG-1 and NALM-6 leukemia cell lines. C3AR stain in red and isotype in gray. (D) Number of NALM-6 cells after a 3-day culture with increasing concentrations of human C3a. 20,000 NALM6 cells were seeded/well in serum free medium. The experiment was performed in triplicates. Bars in B and D show mean cell numbers with standard deviation.

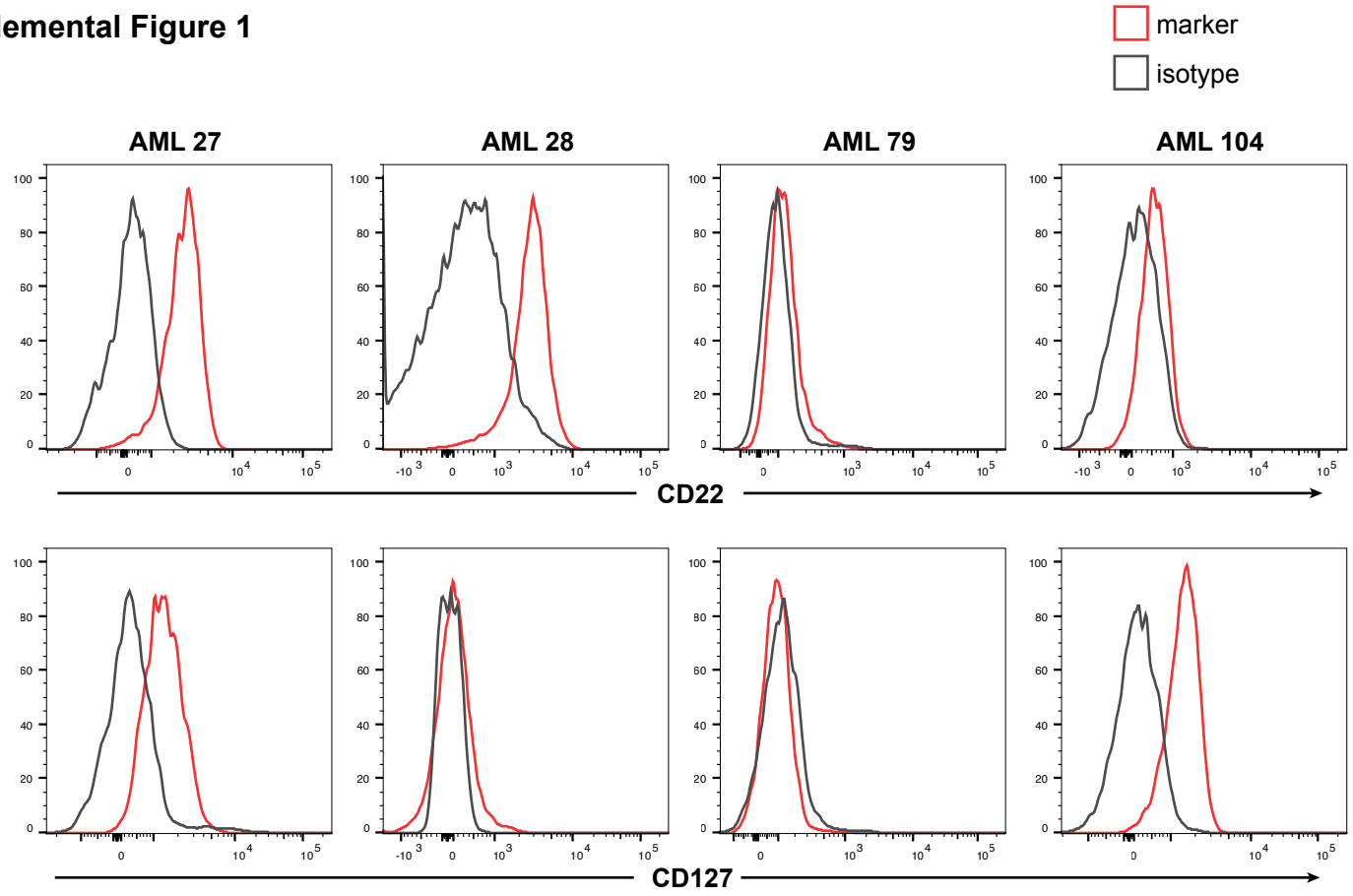
Supplemental Figure 10. Single cell RNA sequencing shows no expression of *C3* in NBM mononuclear cells or *NPM1*-mutated AML cells. UMAPs showing the expression of *C3* in 12 *NPM1*-mutated AMLs, five NBM MNCs and three NBM $CD34^+$ in individual plots. NBM, normal bone marrow; UMAP; Uniform Manifold Approximation and Projection for Dimension Reduction

Supplemental Figure 11. Bulk RNA-seq reveals expression of complement genes associated with the alternative complement pathway in AMLs of different subtypes. FPKM values for *C3*-related complement genes in the AML Lund cohort, represented as box plots. Same data as in Figure 6D, but including AML subgroups with less than 3 patients (n=112).

Supplemental Figure 12. C3AR can be specifically targeted with anti-C3AR antibodies. ADCC assays on primary *NPM1*-mutated AML samples from three individual patients, showing the percentage of AML cells after overnight incubation with a polyclonal anti-C3AR antibody and NK cells or corresponding isotype and NK cells, compared to control with only NK cells. 10ug/ml of antibody was used and NK cells from two different donors. Bars show mean cell numbers with standard deviation. (B) Leukemic engraftment 5 weeks after ADCC,

as the percentage of $CD45^{+}CD33^{+}$ human cells in the BM of mice transplanted with isotype treated or anti-C3AR treated AML cells, as in Figure 7B. 10ug/ml of antibody was used. Line indicates median engraftment. *≤0.05, **≤0.01 and ****≤0.0001 using a student's t-test in A. ADCC; antibody-dependent cellular cytotoxicity assay.

Supplemental Figure 1



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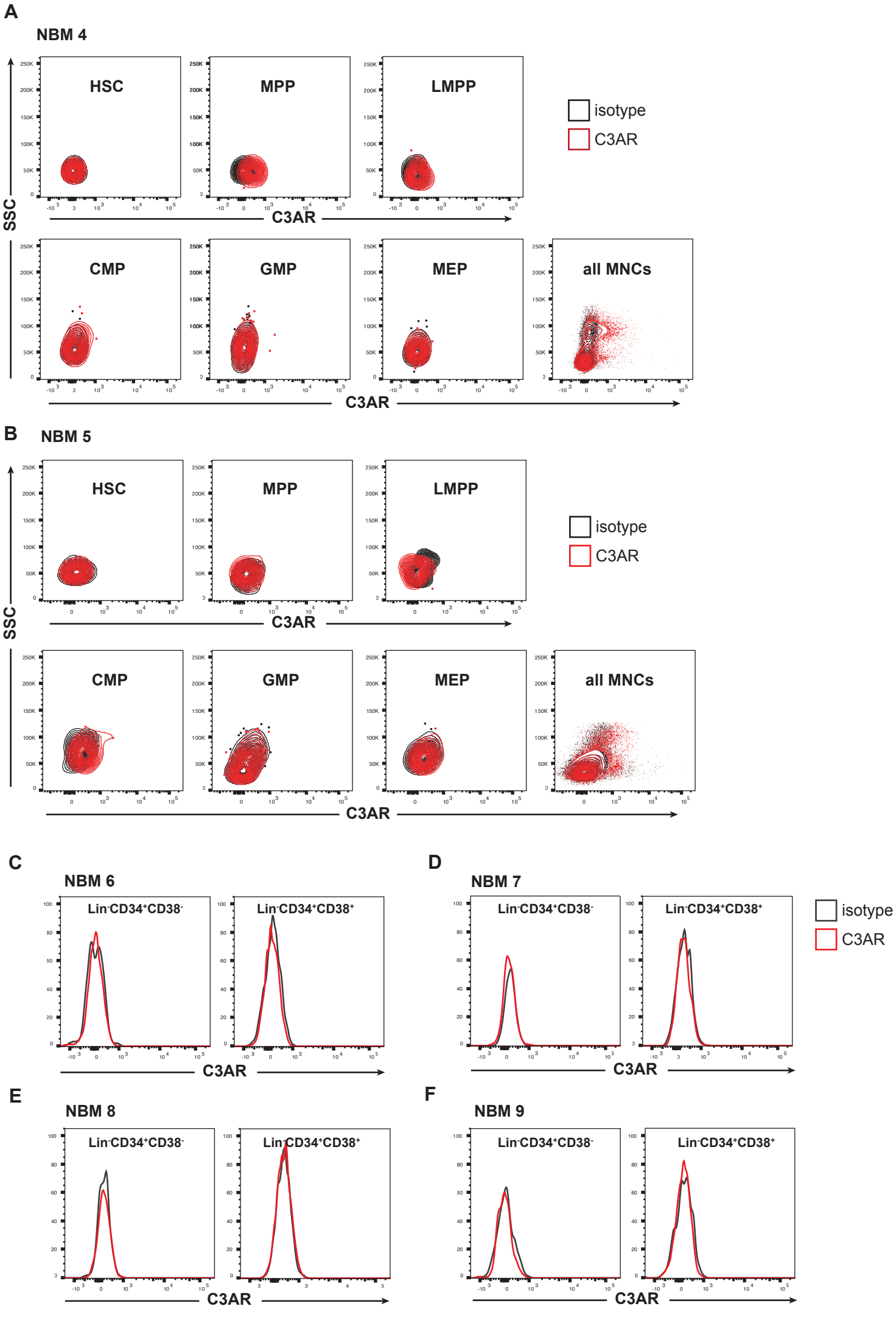

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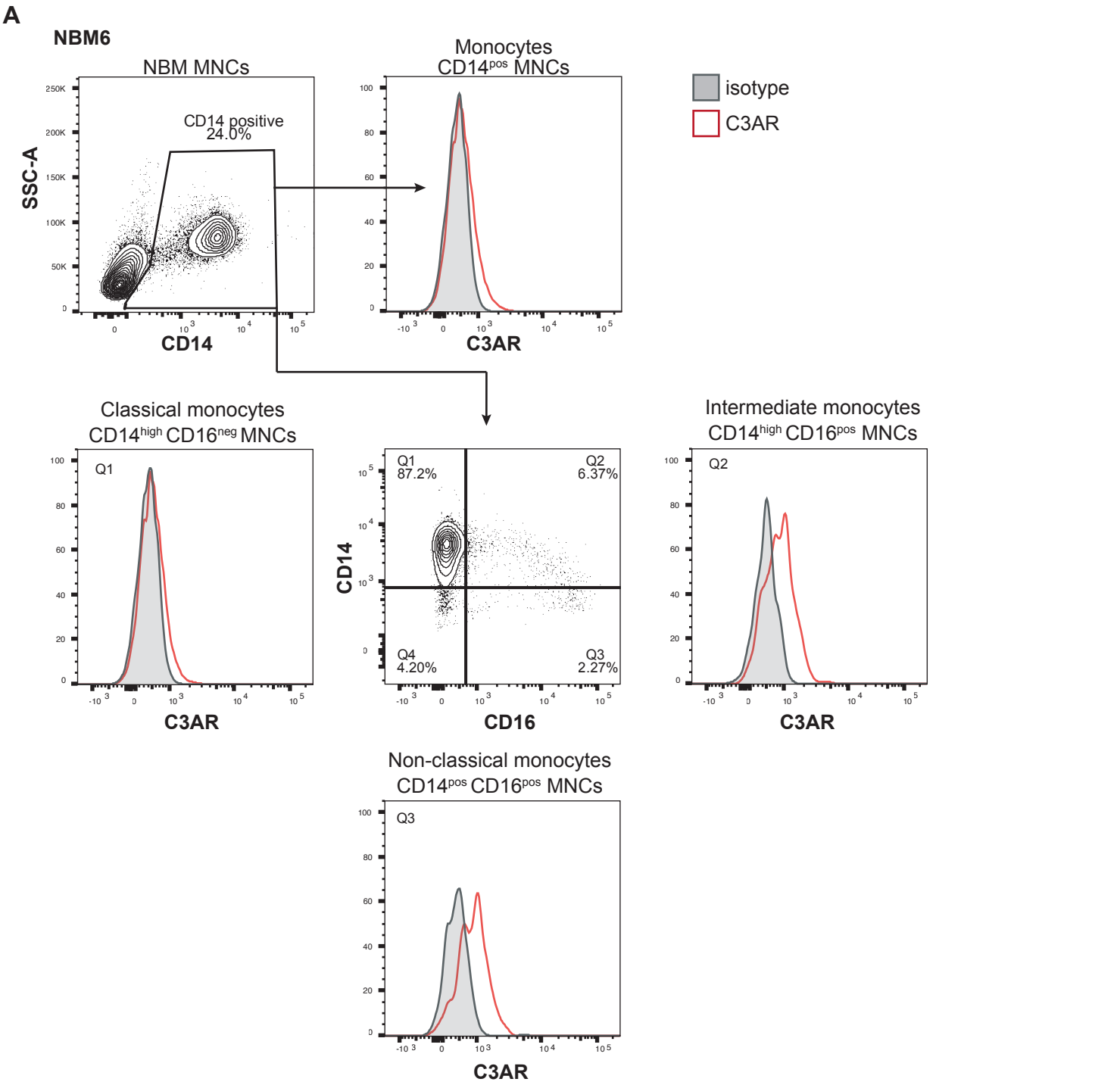

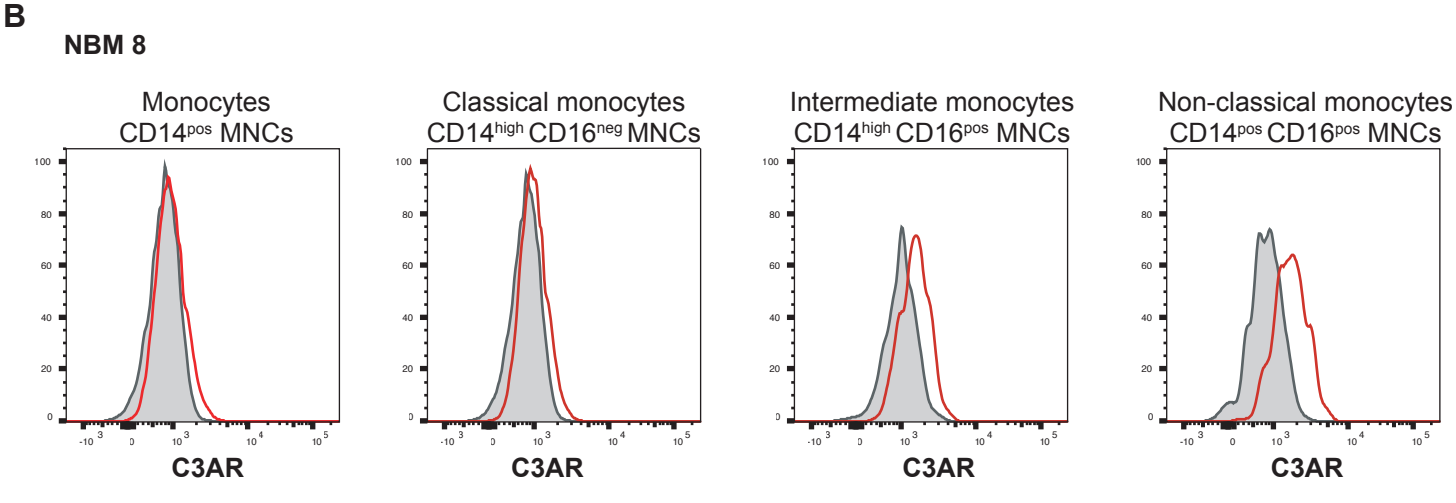

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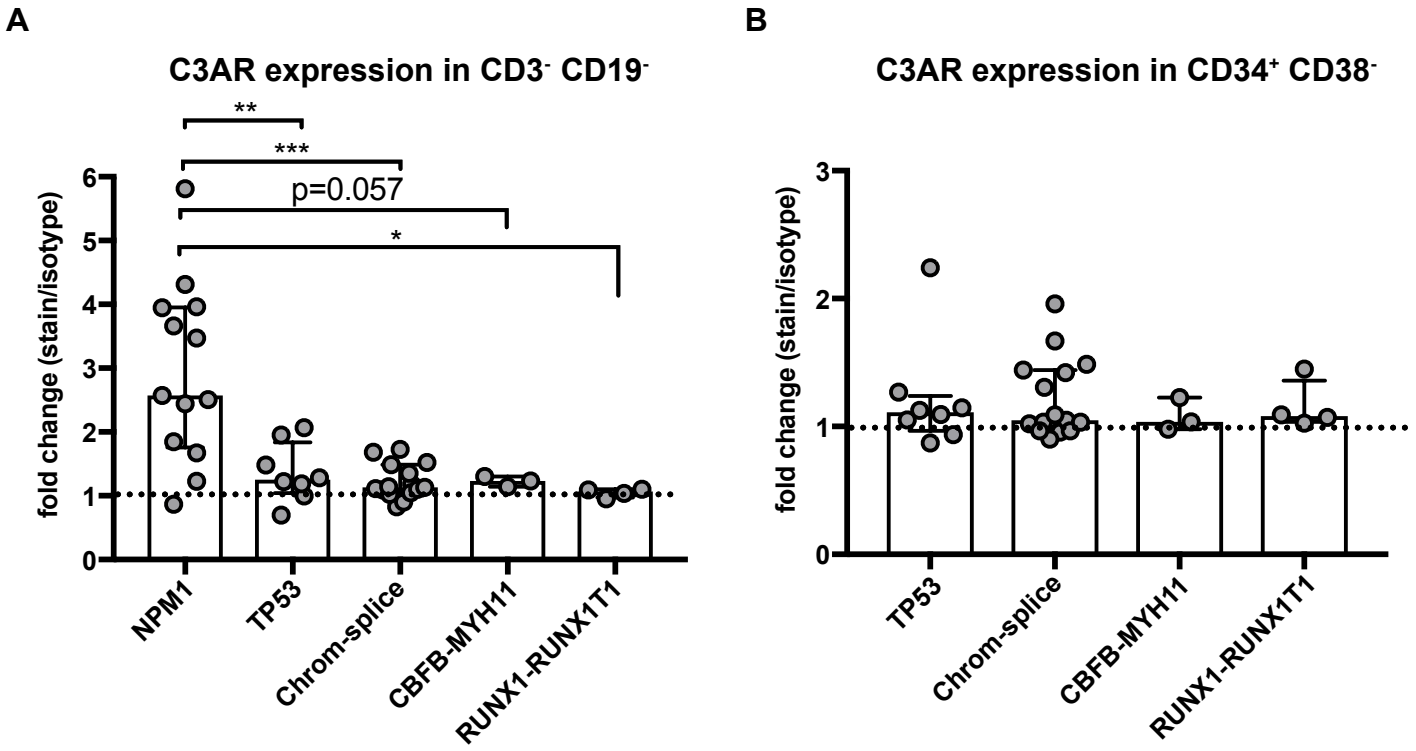

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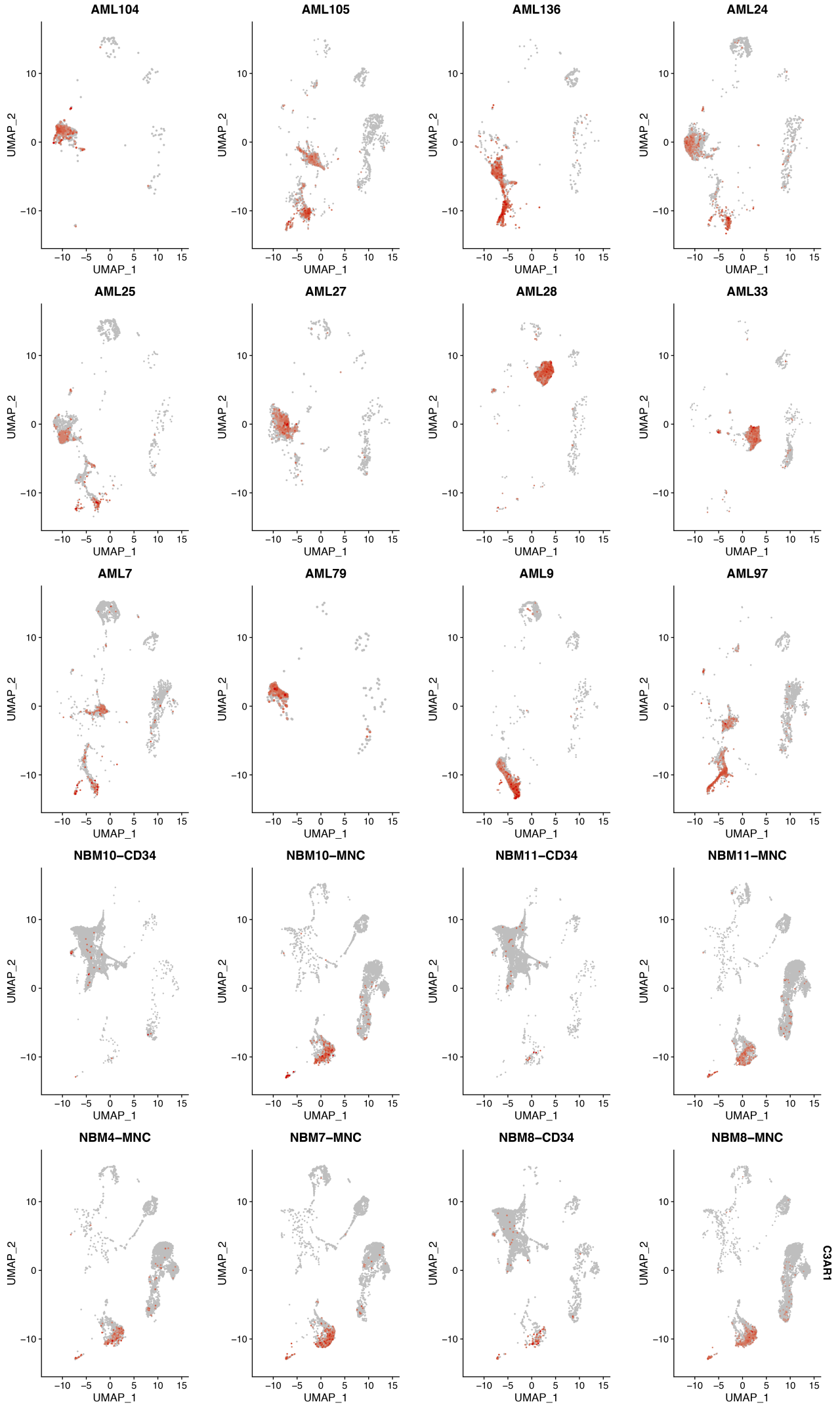
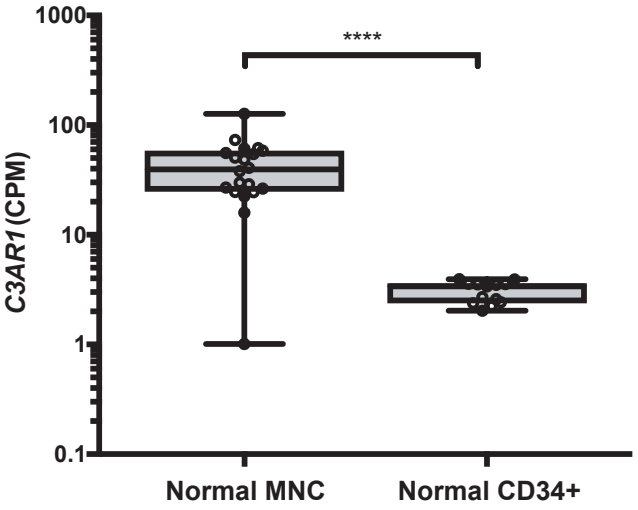


Supplemental Figure 6



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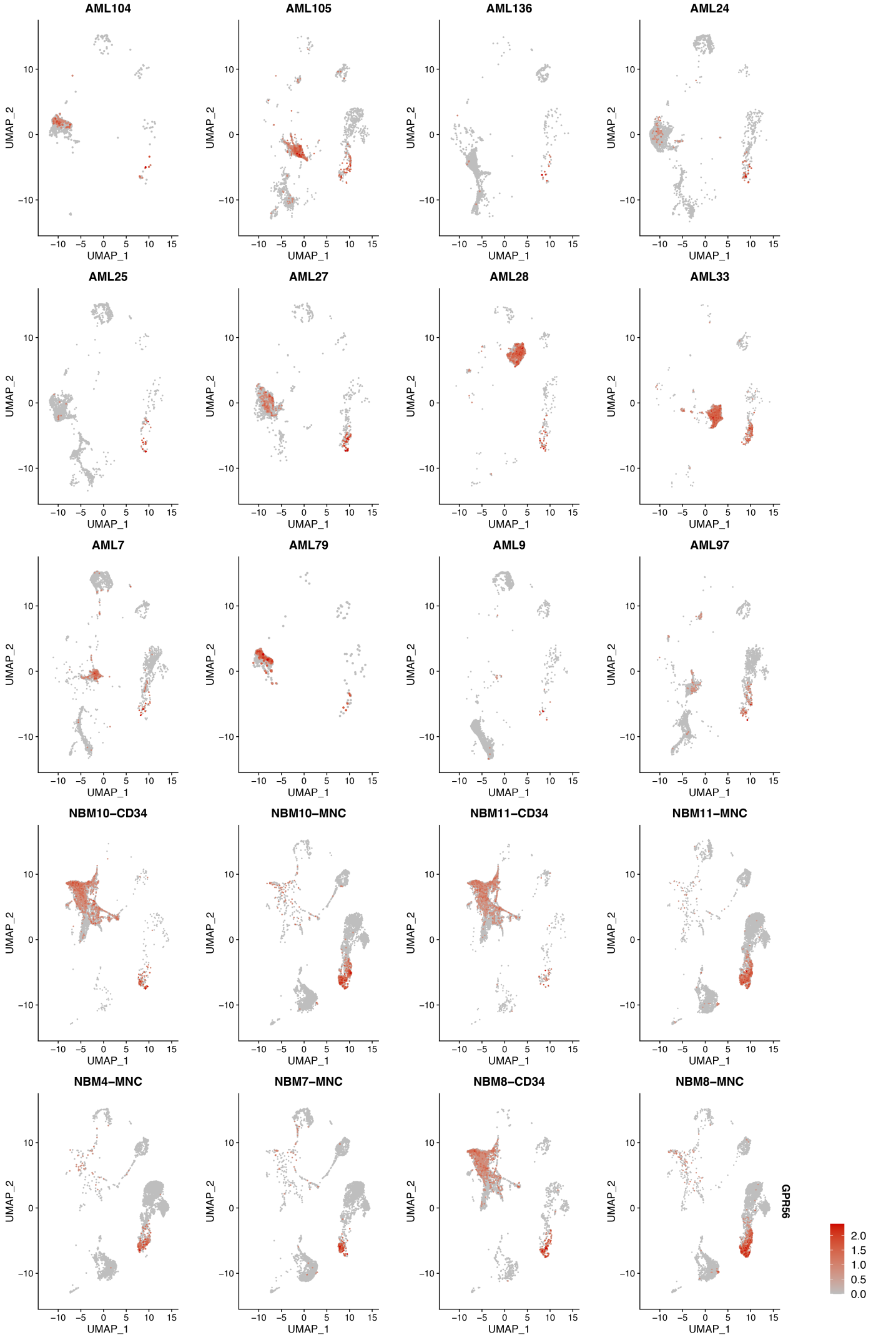

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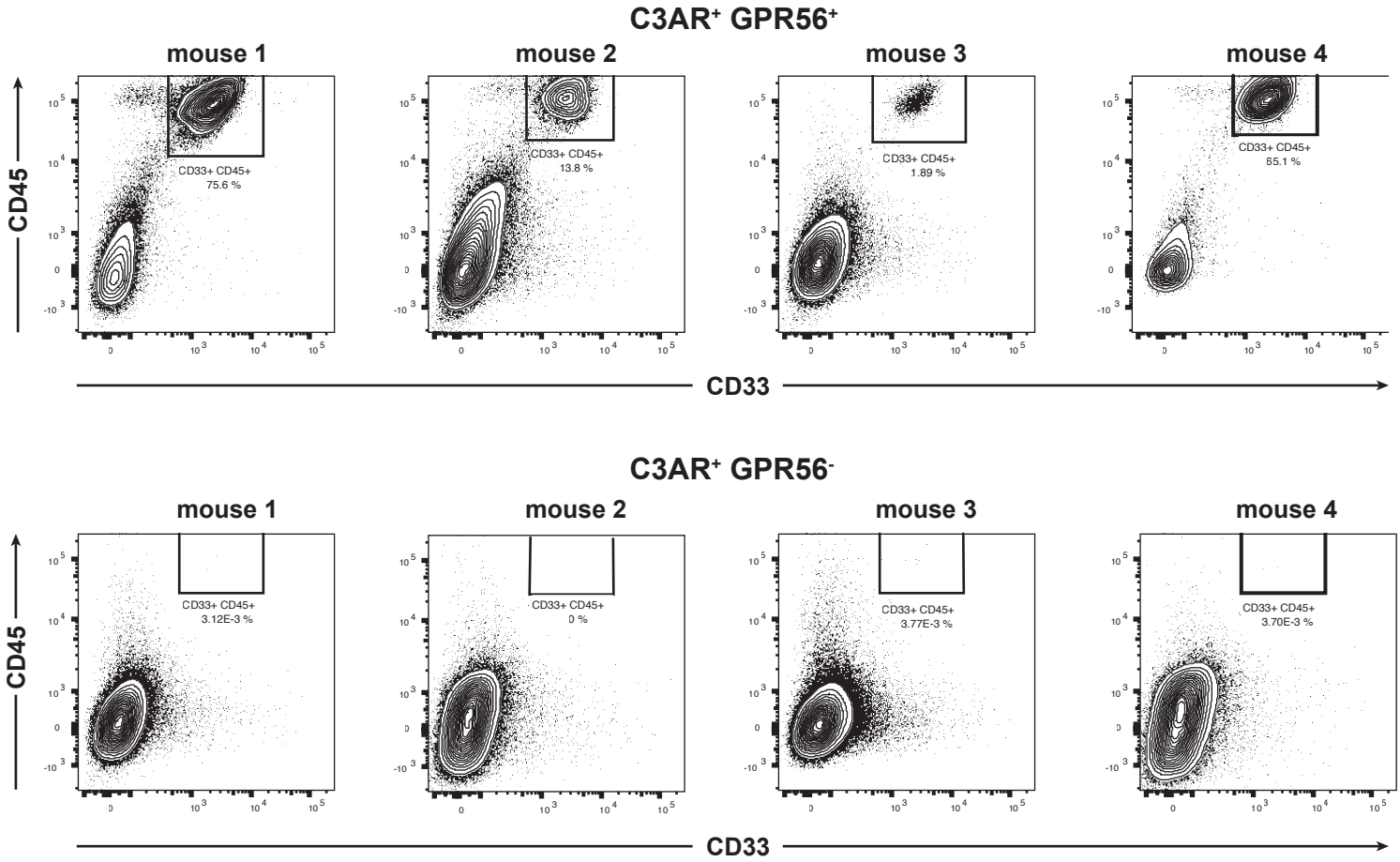
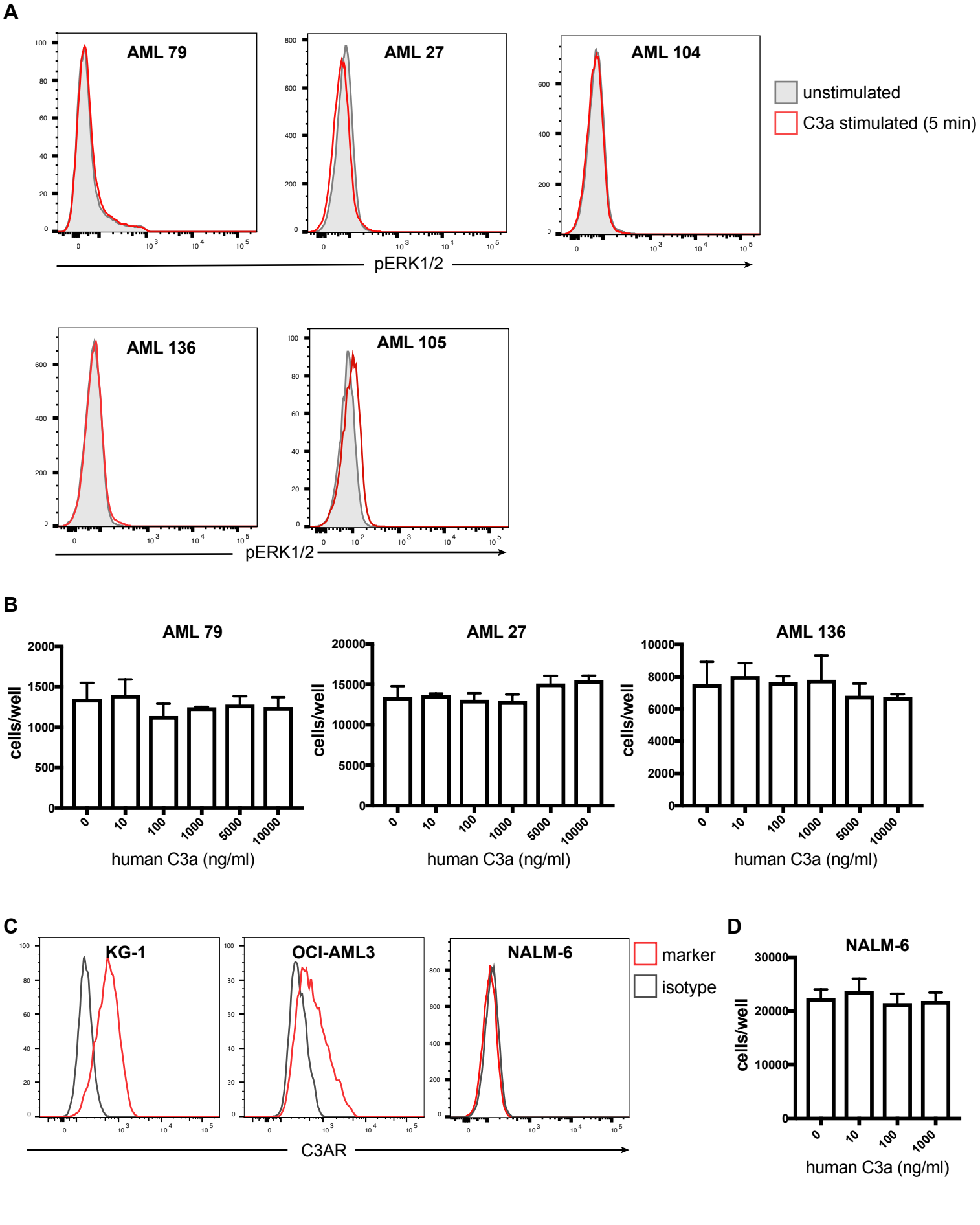
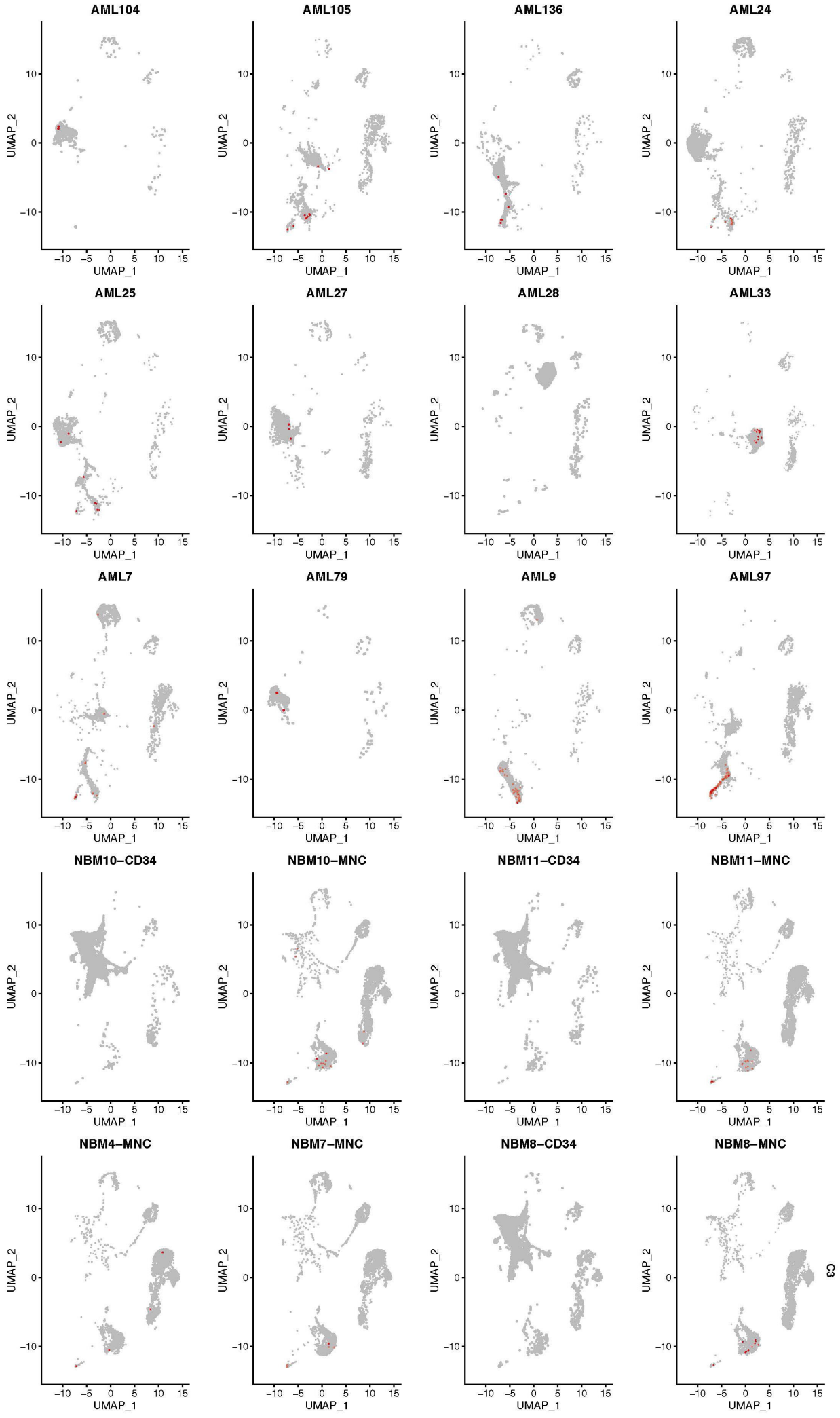


Supplemental Figure 9



Supplemental Figure 10



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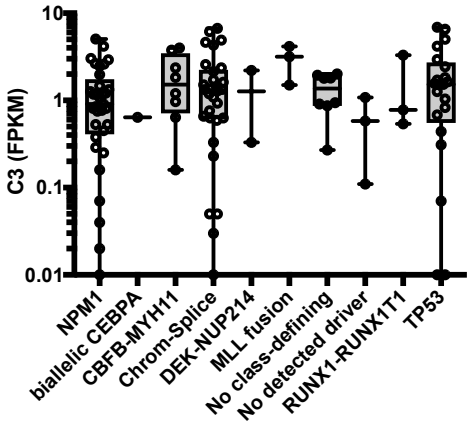
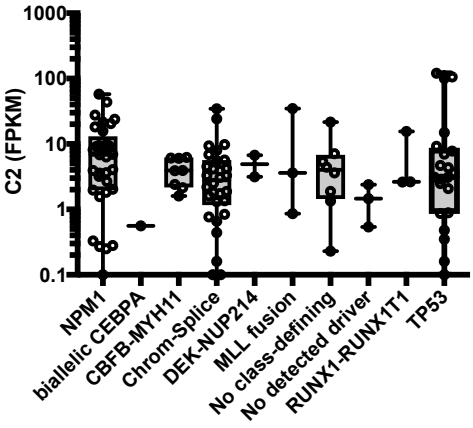
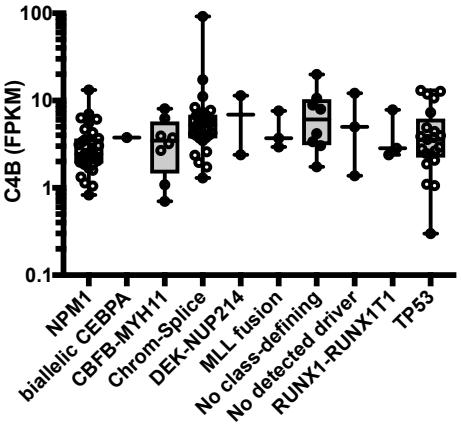
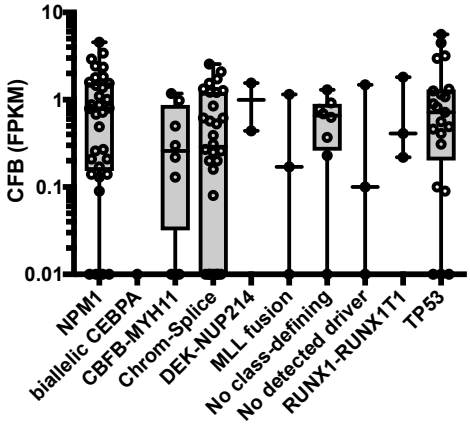
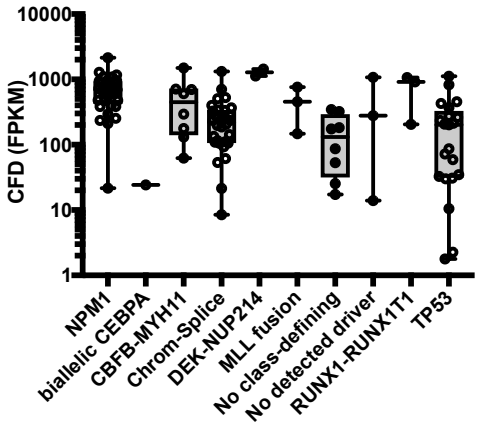
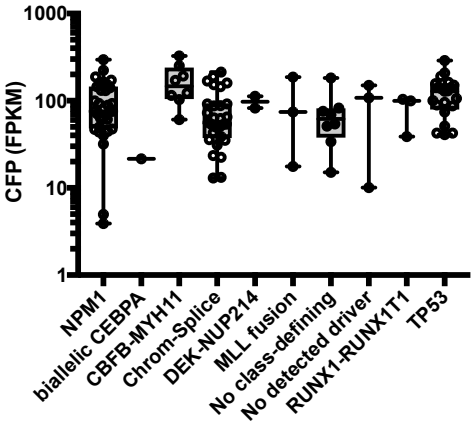


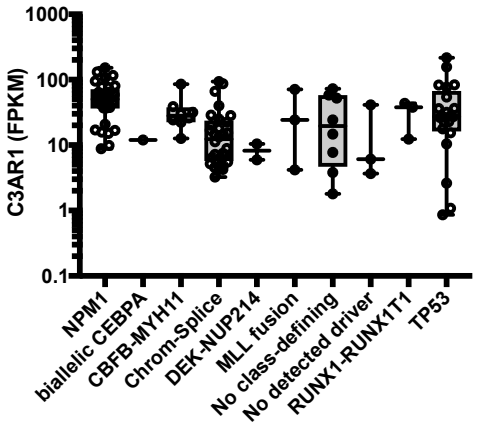


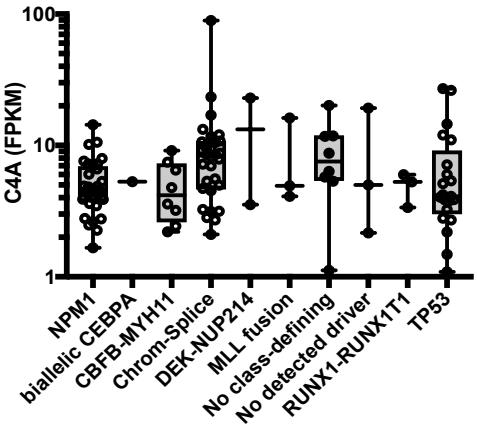




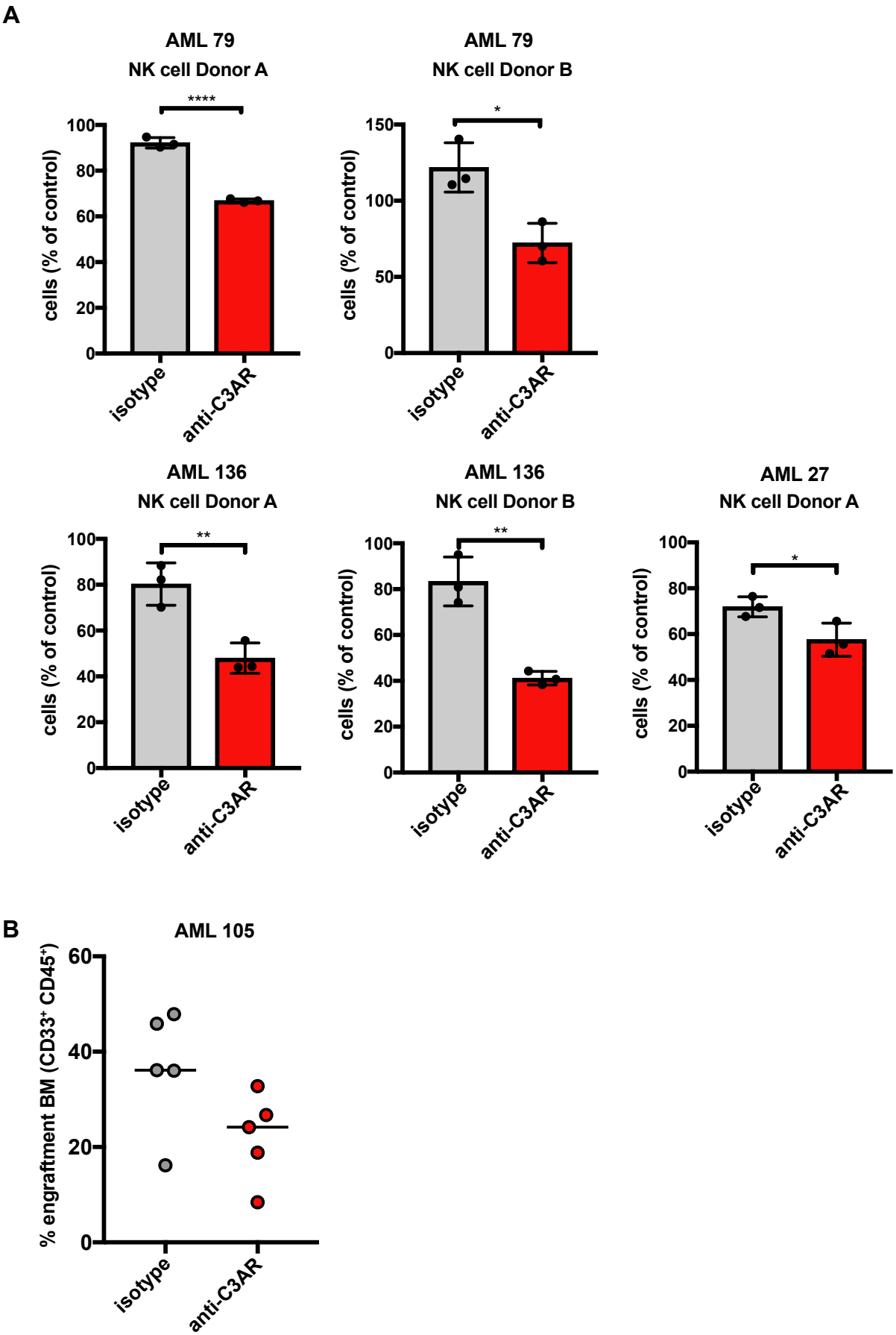








Supplemental Figure 12



Supplemental Table 1. Mutation status and clinical parameters

Sample ID	*NPM1*	*FLT3-ITD*	*DNMT3A*	*C3AR1* (FPKM)	Clinical FAB	Clinical Karyotype	Clinical Tissue	Gender	Papaemmanuil Description	Sampling timepoint	Age
AML 27	Mut	Mut	Wt	50.33	M1	46,XY	BM	M	AML with NPM1 mutation	Diagnosis	60
AML 28	Mut	Mut	Wt	116.46	M2	46,XY	BM	M	AML with NPM1 mutation	Diagnosis	68
AML 79	Mut	Mut	Wt	52.5	M4	46,XY	PB	M	AML with NPM1 mutation	Diagnosis	59
AML 104	Mut	Mut	Mut	58.6	M0	46,XY	PB	M	AML with NPM1 mutation	Diagnosis	74
AML 24	Mut	Mut	Wt	64.02	M2	46,XY	BM	M	AML with NPM1 mutation	Diagnosis	73
AML 33R	Mut	Mut	Mut	52.0	NA	NA	PB	F	AML with NPM1 mutation	Relapse	44
AML 168	Mut	Mut	Wt	80.19	M2	NA	PB	M	AML with NPM1 mutation	Diagnosis	86
AML 7	Mut	Wt	Mut	45.44	M4	46,XX	BM	F	AML with NPM1 mutation	Diagnosis	61
AML 9	Mut	Wt	Mut	46.63	M5	46,XX	BM	F	AML with NPM1 mutation	Diagnosis	48
AML 97	Mut	Mut	Mut	107.06	M4	46,XX	BM	F	AML with NPM1 mutation	Diagnosis	64
AML 105	Mut	Mut	Mut	78.06	M4	46,XX	PB	F	AML with NPM1 mutation	Diagnosis	48
AML 136	Mut	Wt	Wt	152.43	M4	46,XX	BM	F	AML with NPM1 mutation	Diagnosis	62
AML 25	Mut	Wt	Wt	15.83	M2	45,X,-Y	BM	M	AML with NPM1 mutation	Diagnosis	87

Abbreviations: Mut, mutation; WT, wild type; BM, bone marrow; PB, peripheral blood; M, male; F, female.

Supplemental Table 2. Antibodies and Reagents

Name of Product	Target	Fluorochrome	Company	Isotype	Catalog #
LEGENDScreen™ Human PE Kit	SCREEN	PE	BioLegend	10 different	700007
PE/Cy7 anti-human CD3 Antibody	CD3	PE-Cy7	BioLegend	mouse IgG2a,k	300316
APC/Cy7 anti-human CD19 Antibody	CD19	APC-Cy7	BioLegend	mouse IgG1,k	302218
Alexa Fluor 488 anti-human CD34 Antibody	CD34	AF488	BioLegend	mouse IgG1,k	343518
BV711 Mouse Anti-Human CD38 Clone HIT2 (RUO)	CD38	BV711	BD	mouse IgG1,k	563965
PE anti-human CD177 Antibody	CD177	PE	BioLegend	mouse IgG1,k	315806
Human IL-1 RAcP/IL-1 R3 PE-conjugated Antibody	IL1RAP	PE	RnD Systems	mouse IgG1,k	FAB676P
APC/Cy7 anti-human CD34 Antibody	CD34	APC-Cy7	BioLegend	mouse IgG2a,k	343614
PE-Cy7 Mouse Anti-Human CD45RA	CD45RA	PE-Cy7	BD	mouse IgG2b,k	560675
Alexa Fluor 488 anti-human CD123 Antibody	CD123	AF488	BioLegend	mouse IgG1,k	306036
APC anti-human CD90 (Thy1) Antibody	CD90	APC	BioLegend	mouse IgG1,k	328114
Pacific Blue™ anti-human Lineage Cocktail (CD3, CD14, CD16, CD19, CD20, CD56)	Lineage neg	Pacific Blue	Biolegend	mouse IgG1, κ; Mouse IgG2b, κ	348805
PE/Cyanine7 anti-human CD45 Antibody	CD45	PE-Cy7	Biolegend	Mouse IgG1, κ	368532
Brilliant Violet 421™ anti-human CD33 Antibody	CD33	BV421	BioLegend	Mouse IgG1, κ	303416
APC anti-human GPR56 Antibody	GPR56	APC	Biolegend	Mouse IgG1, κ	358204
Brilliant Violet 421™ anti-human CD14 Antibody	CD14	BV421	Biolegend	Mouse IgG2a, κ	301830
APC anti-human CD16 Antibody	CD16	APC	Biolegend	Mouse IgG1, κ	302012
PE anti-human C3AR Antibody	C3AR	PE	BioLegend	mouse IgG2b	345804
APC Mouse IgG1, κ Isotype Ctrl (FC) Antibody	Isotype	APC	BioLegend	Mouse IgG1, κ	400122
PE Mouse IgG2b, κ Isotype Ctrl Antibody	Isotype	PE	BioLegend	mouse IgG2b	400314
Anti-C3a R antibody	C3AR	-	Abcam	rabbit polyclonal	ab103629
Rabbit IgG, polyclonal - Isotype Control (Low Endotoxin, Azide-Free)	Isotype	-	Abcam	rabbit polyclonal	ab176094
Alexa Fluor® 647 anti-ERK1/2 Phospho (Thr202/Tyr204) Antibody	pERK1/2	AF647	BioLegend	Mouse IgG2b, κ	675504
APC anti-human CD45 Antibody	CD45	APC	BioLegend	mouse IgG1,k	982304