

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

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## Key Points

- C3AR is selectively expressed on *NPM1*-mutated primary AML cells compared to both *NPM1* wild type cases and normal hematopoietic stem cells.
- The C3a/C3AR signaling axis is active in *NPM1*-mutated AML cells, and C3AR can be therapeutically targeted with antibodies.

Mutated *nucleophosmin 1* (*NPM1*) is the most common genetic alteration in acute myeloid leukemia (AML), found in ~30% of cases. Although mutations in this gene are considered favorable according to current risk stratification guidelines, a large fraction of patients will experience relapse, demonstrating the urgent need for new treatment options. Therefore, we aimed to identify cell surface proteins specifically expressed on *NPM1*-mutated AML cells, allowing for potential targeting with antibody-based therapies. Herein, we report on an arrayed flow cytometry-based screen directed to 362 cell surface markers. In comparing the cell surface expression on *NPM1*-mutated AML cells with primitive (CD34<sup>+</sup> CD38<sup>-</sup>) normal bone marrow cells, we identified the complement receptor C3AR as being specifically expressed in *NPM1*-mutated AML. By flow cytometry and single-cell RNA sequencing, we further show that normal hematopoietic stem and progenitor cells lack detectable C3AR gene and protein expression, making it particularly suitable as a target for antibody therapy. We also demonstrate that C3AR in combination with GPR56 distinguishes the leukemic stem cells (LSCs) in *NPM1*-mutated AML from the normal hematopoietic stem cells, defining the LSC population, as shown by transplantation into immunodeficient mice. Mechanistically, the stimulation of C3AR-expressing cells with C3a, the ligand of C3AR, leads to the activation of ERK1/2 and increased survival of AML cells, suggesting that this is an important signaling axis in this subtype of AML. Finally, we show that antibodies directed against C3AR efficiently elicit natural killer cell-mediated killing of primary AML cells *ex vivo*, highlighting C3AR as a candidate therapeutic target in *NPM1*-mutated AML.

## Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous disease caused by acquired genetic alterations arising in a hematopoietic stem cell (HSC) or myeloid progenitor cell. This leads to aberrant self-renewal and faulty differentiation capacity, resulting in an expansion of immature malignant myeloid cells.<sup>1,2</sup> The overall prognosis of AML is poor, especially with older patients, with survival rates of <10% for patients

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The data sets generated during the current study fall under the GDPR regulations for sharing of personal data and will therefore be deposited at EGA-SE once this repository is complete. Until then, the data sets are available from the corresponding authors upon request through the following DOIs: <https://doi.org/10.17044/sciiflab.21557163> (RNA-seq data set) and <https://doi.org/10.17044/sciiflab.21557247> (scRNA-seq data set).

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The full-text version of this article contains a data supplement.

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aged >70 years.<sup>2,3</sup> Chemotherapy remains the standard treatment in AML,<sup>4</sup> but it often fails to eradicate the leukemic stem cells (LSCs), which serve as a reservoir for disease relapse.<sup>5</sup> Hence, novel therapies selectively targeting the LSC may provide new means to improve prognosis and achieve a cure. In this context, recombinant antibodies provide attractive therapies given their target antigen specificity and general minimal toxicity.<sup>6</sup> An ideal target for an antibody-based targeted treatment in AML should be selectively expressed on the LSCs but not on normal HSCs,<sup>6,7</sup> thereby allowing replenishment of normal hematopoiesis. In addition, restricted expression in nonhematopoietic tissues is critical for limiting potential side effects on other vital organs.

AML can be stratified into different genetic subgroups based on chromosomal aberrations and recurring mutations.<sup>8,9</sup> The most common genetic subgroup of AML, which constitutes ~30% of all AML cases, is defined by mutations in the *nucleophosmin 1* (*NPM1*) gene, encoding a nucleocytoplasmic shuttling protein.<sup>2,8,9</sup> *NPM1*-mutated AMLs are distinct from AMLs with other genetic alterations, as they are almost exclusively negative for the primitive marker CD34.<sup>5,10,11</sup> Two well-known AML cell surface markers that are highly expressed in the majority of *NPM1*-mutated AML blasts are CD33 and CD123 (IL3RA),<sup>12-14</sup> but they are not specific to this genetic subgroup and are also expressed on normal HSCs. Furthermore, very few cell surface markers that enrich for LSC activity in CD34-negative/*NPM1*-mutated AMLs have been described. Examples include the transmembrane tyrosine kinase c-Kit (CD117), especially in combination with the natural killer (NK) cell receptor 2B4 (CD244/NKR2B4),<sup>11,15</sup> CD200<sup>16</sup> and the adhesion G protein-coupled receptor G1 (GPR56).<sup>17-19</sup> However, as therapeutic targets, these are limited by their expression on normal HSCs.<sup>11,16,18</sup>

To identify novel cell surface markers specifically upregulated on *NPM1*-mutated AML cells compared with primitive normal bone marrow (NBM) cells, we performed an arrayed cell surface marker screen using 362 phycoerythrin (PE)-conjugated monoclonal antibodies. With this approach, we identified the complement component 3a receptor 1 (C3AR) as being specifically upregulated on *NPM1*-mutated AML cells and show that C3AR-expressing AML cells respond to stimulation with their ligand, C3a. Importantly, we show that anti-C3AR antibodies and NK cells mediate specific cell killing of primary *NPM1*-mutated AML cells *ex vivo*, through antibody-dependent cellular cytotoxicity (ADCC). Combined, our results demonstrate that C3AR is a candidate therapeutic target in *NPM1*-mutated AML.

## Materials and Methods

### Patient samples

Bone marrow (BM) or peripheral blood (PB) samples from patients with AML were collected after written informed consent in accordance with the Declaration of Helsinki. Mononuclear cells (MNCs) were isolated using Lymphoprep (GE Healthcare Bio-Sciences AB) and samples were viably frozen. Clinical and genetic characteristics of the *NPM1*-mutated samples from patients were characterized by flow cytometry are summarized in supplemental Table 1. In addition, BM samples from healthy donors were prepared in the same way. The study was approved by a regional ethics committee.

### Cell surface receptor screen

The LEGENDScreen Human PE Kit containing an arrayed library of 361 unique PE-labeled antibodies against human cell surface receptors was used as a base for the screen. The screen plates containing lyophilized antibodies were diluted in double-distilled water according to the manufacturer's instructions, and an antibody cocktail of either anti-CD3 and anti-CD19 (for the AML samples) or anti-CD34 and anti-CD38 (for normal BM samples) was added to each well. An anti-IL1RAP antibody was also added to the screen, because we have shown previously that IL1RAP is a therapeutic marker on AML LSCs,<sup>20,21</sup> bringing the total number of antibodies assessed to 362. BM or PB MNCs from *NPM1*-mutated AML samples ( $n = 4$ ), or MNCs from NBM ( $n = 4$ ) were thawed and resuspended in phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS) and added to the wells. Plates were incubated in the dark for 20 minutes at 4°C, washed twice, and resuspended in PBS with 2% FBS. Just before analysis on an LSR Fortessa with an HTS-sampler (BD Biosciences), the viability dye 7AAD (Biolegend) was added to each well. For details regarding data analysis, screen validation, and cell surface marker evaluation in NBM, see supplemental Methods. All antibodies used are listed in supplemental Table 2.

### C3AR GPR56 transplantation experiments

To assess the engraftment potential of primary *NPM1*-mutated AML cells expressing C3AR<sup>+</sup> GPR56<sup>+</sup> compared with C3AR<sup>+</sup> GPR56<sup>-</sup>, fluorescence-activated cell sorter-sorted cells were transplanted into irradiated NSGS immunodeficient mice. For a detailed description of xenograft experiments, see supplemental Methods.

### C3a stimulations

The C3AR-expressing leukemia cell line OCI-AML3 and the C3AR-negative cell line NALM-6 were both cultured according to the vendor's instructions (German Collection of Microorganisms and Cell Cultures GmbH) but were starved by resuspension in serum-free minimal essential medium- $\alpha$  (Merck) or serum-free RPMI 1640 medium (Thermo Fisher Scientific), respectively, the day before addition of C3a. Primary AML samples were resuspended in StemSpan serum-free expansion medium (Stem Cell Technologies, Canada) after thawing. Thereafter, OCI-AML3 cells, primary AML cells, and NALM-6 cells were cultured in 96-well plates at 37°C, 5% CO<sub>2</sub>, for 3 days in different concentrations of C3a (0, 10, 100, 1000, 5000, and 10 000 ng/mL) purified from human serum (Comp Tech or Merck). Viable cell number was evaluated using CountBright Absolute Counting Beads (Thermo Fisher Scientific) and the viability dye Draq7 (Biostatus) on a LSRFortessa (BD Biosciences).

### C3a phospho-flow cytometry

Primary AML cells were thawed and washed twice in RPMI 1640 medium (Thermo Fisher Scientific) without supplements and left to incubate for 15 minutes at 37°C, 5% CO<sub>2</sub>, before 1-minute or 5-minute stimulation with 100 ng/mL of human C3a (Comp Tech). Cells were fixed with 1.6% paraformaldehyde for 10 minutes at room temperature before permeabilization with 90% ethanol at -80°C. Subsequently, cells were thoroughly washed with PBS to remove any residual ethanol, resuspended in 2% FBS, and stained with anti-pERK1/2 antibody. Samples were analyzed on a

LSRFortessa (BD Biosciences) instrument. All antibodies used are listed in supplemental Table 2.

### ADCC assay

For the ADCC assays, KG-1 cells or primary AML samples from patients with AML were stained with PKH26 (Sigma-Aldrich) according to the manufacturer's instruction, before resuspension in RPMI 1640 medium (Thermo Fisher Scientific) with 20% heat-inactivated FBS (Nordic Biolabs, Sweden). Cells were plated in a flat-bottom 96-well plate with different concentrations of anti-C3AR antibody or isotype control antibody. The plates were incubated for 30 minutes at 37°C, 5% CO<sub>2</sub>, before addition of freshly thawed NK cells at a 10:1 ratio to target cells. NK cells were purified from leukocyte concentrates using magnetic beads (Miltenyi Biotec). Subsequently, the plates were incubated overnight. CountBright Absolute Counting Beads (Thermo Fisher Scientific) and 4',6-diamidino-2-phenylindole (Sigma-Aldrich) were added to the wells before analyses on a LSRFortessa (BD Biosciences). For details on the ADCC in vivo repopulation analysis, see supplemental Methods. All antibodies used are listed in supplemental Table 2

### Bulk RNA sequencing and external data

RNA was extracted from PB or BM samples from patients with AML at diagnosis or relapse (n = 112) using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions (H. Lilljebjörn, C. Orsmark-Pietras, H. Thorsson, M. Rissler, and T. Fioretos, unpublished data, December 2022). Complementary DNA libraries were prepared using the Truseq RNA library prep kit v2 (Illumina) and sequenced by paired-end 2 × 150 bp sequencing on a NextSeq 500 (Illumina). Details on the gene expression analysis of in-house and external sequencing data are provided in the supplemental Methods.

### Single-cell RNA sequencing (scRNA-seq)

In total, 20 BM or PB samples (12 *NPM1*-mutated AML diagnostic samples, 5 NBM MNC samples, and 3 CD34-enriched NBM samples) were subjected to scRNA-seq (H. Lilljebjörn, C. Orsmark-Pietras, H. Thorsson, M. Rissler, and T. Fioretos, unpublished data, December 2022). Cryopreserved cells were thawed and resuspended in Draq7-buffer (Biolegend) before sorting on an Aria Fusion (BD Biosciences). Samples were gated on viability; cell doublets and cell debris were excluded. The sorted cells were subjected to scRNA-seq by preparation of libraries using the Chromium Single Cell 3' Reagent Kits v3 (10X Genomics).<sup>22</sup> The libraries were sequenced on a NovaSeq 6000 System (Illumina). Details on the single-cell gene expression analysis are provided in the supplemental Methods.

### Complement factor D (CFD) enzyme-linked immunosorbent assay

*NPM1*-mutated AML samples (n = 4) and NBM MNC samples (n = 4) were thawed and washed twice in Iscove modified Dulbecco medium, before resuspension in S7 media.<sup>23</sup> One million cells per mL were plated and cell culture supernatants were collected for analysis after 24 hours. Samples were diluted 1:10 and the amount of CFD was evaluated using an enzyme-linked immunosorbent assay (Abcam), according to manufacturer's instructions.

## Results

### Antibody-based screening identifies cell surface markers specifically upregulated in *NPM1*-mutated AML samples

To identify cell surface molecules specifically upregulated in *NPM1*-mutated AML samples compared with healthy primitive BM cells, we performed a flow cytometry-based screen using antibodies directed to 362 cell surface proteins (Figure 1A). By comparing the CD3<sup>-</sup>CD19<sup>-</sup> cell fraction of 4 *NPM1*-mutated AML samples with the CD34<sup>+</sup>CD38<sup>low</sup> cell fraction of 4 NBM samples for each marker, a ranked list of cell surface markers with high expression in AML compared with NBM was obtained (Figure 1B). Several known AML cell surface markers were among the top 50 hits: CD371/CLL-1,<sup>24,25</sup> CD99,<sup>26</sup> IL1RAP,<sup>21,27</sup> CD33,<sup>28</sup> CD82,<sup>29</sup> CD43,<sup>30</sup> CD123,<sup>31</sup> CD184 (CXCR4),<sup>32</sup> and CD73,<sup>33</sup> thus validating our approach to select for relevant markers. Of the novel markers, C3AR displayed the highest selective expression in the AML samples (Figure 1B). CD22 and CD127 scored above C3AR in the ranked list (Figure 1B), but these markers did not show consistent receptor expression when samples were reanalyzed individually (supplemental Figure 1). In contrast, validations confirmed that C3AR displayed a consistent expression on all 4 *NPM1*-mutated AML samples included in the screen (Figure 1C). Thus, C3AR was selected for further studies.

Next, we measured C3AR expression in 9 additional *NPM1*-mutated AML samples by flow cytometry (Figure 1D). Overall, most of the samples (11/13) showed expression of C3AR, with a significantly higher median fluorescence intensity compared with the isotype control ( $P < .001$ ) (Figure 1E). These data suggest that C3AR is expressed in the vast majority of AML cases with *NPM1* mutations.

### C3AR is upregulated in *NPM1*-mutated AML cells but absent on normal hematopoietic stem and progenitor cells (HSPC)

Given the consistent expression of C3AR in *NPM1*-mutated AML, we next investigated its specificity by analyzing the expression in NBM cells. Different fractions of NBM MNCs were analyzed by flow cytometry, demonstrating that C3AR is not expressed in any of the immature cell fractions (Figure 2; supplemental Figure 2). A weak expression of C3AR was noted in a subpopulation of MNCs. Because it is known that C3AR is expressed on monocytes and other myeloid innate immune cells,<sup>34,35</sup> we investigated whether the C3AR-expressing MNCs in the NBM were indeed monocytes. Further flow cytometric analysis revealed that the bulk CD14<sup>pos</sup> monocyte population displayed a weak C3AR-expression. Out of the different monocyte subsets, CD14<sup>high</sup>CD16<sup>+</sup> (intermediate monocytes) and CD14<sup>+</sup>CD16<sup>+</sup> (nonclassical monocytes) had the highest expression, whereas the CD14<sup>high</sup>CD16<sup>-</sup> (classical monocytes) displayed weak expression (supplemental Figure 3).

Collectively, our data suggest that therapeutic targeting of C3AR would selectively target the *NPM1*-mutated leukemic cells and a minority of the monocytes that express CD16 (<10%), thus sparing the vital HSPC populations.

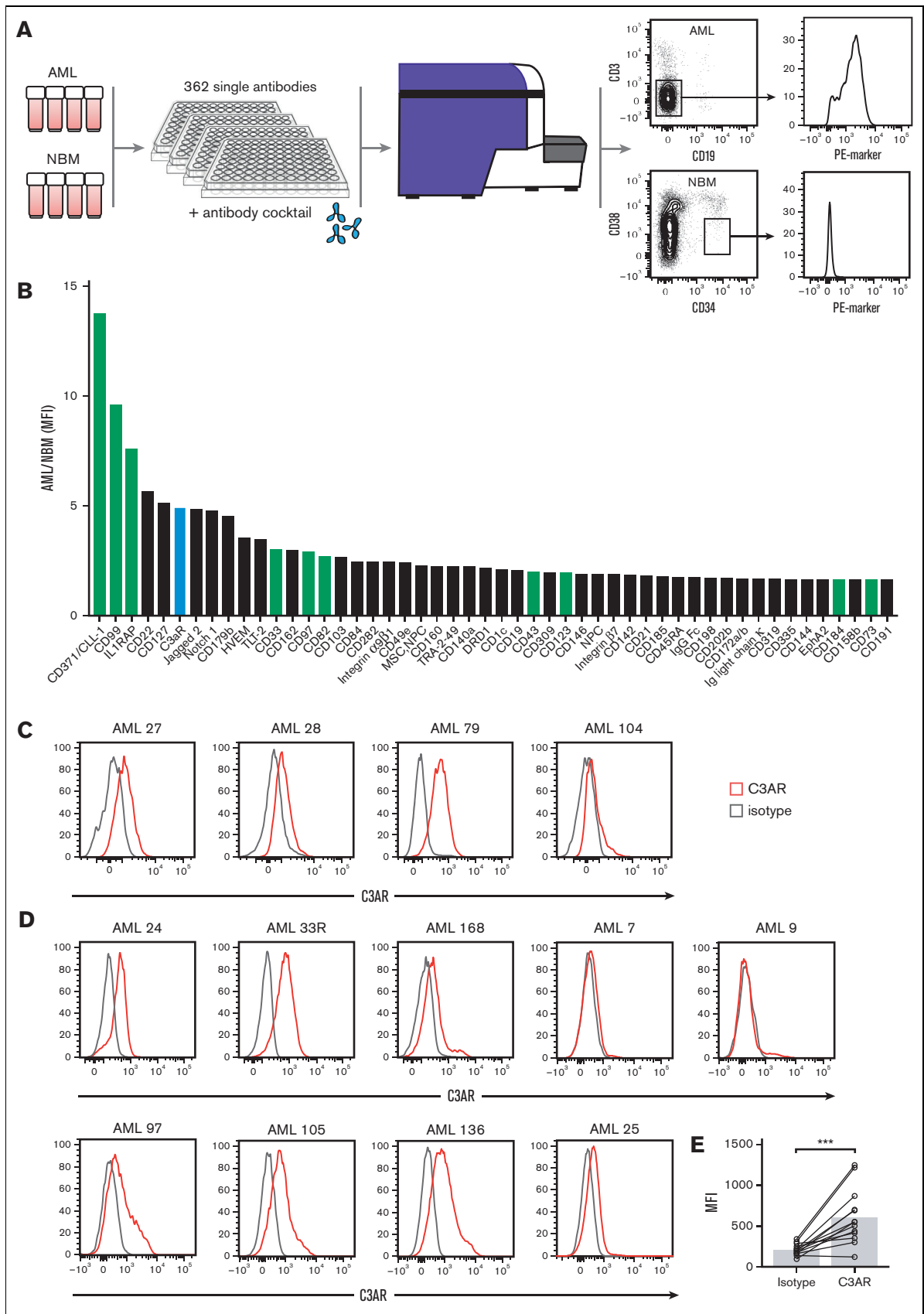


Figure 1.

## RNA-seq reveals *C3AR1* to be specifically upregulated in *NPM1*-mutated AMLs

Next, we investigated whether the gene expression of *C3AR* (*C3AR1*) is specific to *NPM1*-mutated AMLs, or whether it is also expressed in AMLs characterized by other genetic alterations. We compared the *C3AR1* expression in different genetic subgroups of AML using a cohort of AML samples analyzed by RNA-seq (referred to as the Lund cohort; H. Lilljebjörn, C. Orsmark-Pietras, H. Thorsson, M. Rissler, and T. Fioretos, unpublished data, December 2022). We divided the samples into 2 groups, *C3AR1* high ( $n = 54$ ) and *C3AR1* low ( $n = 55$ ) and determined the number of *C3AR1*-high and *C3AR1*-low cases in each genetic subgroup (Figure 3A). This analysis showed that *C3AR1* high is most common in AML cases with mutated *NPM1* ( $P \leq .0001$ ). The opposite was observed for AML samples classified into the chromatin-spliceosome subgroup, which had a higher proportion of *C3AR1*-low-expressing samples ( $P \leq .0001$ ).

Furthermore, we compared the expression of *C3AR1* in *NPM1*-mutated with all *NPM1* wild-type (WT) cases in the Lund cohort and in 2 external data sets from The Cancer Genome Atlas<sup>36</sup> and Beat AML<sup>37</sup> (Figure 3B-D). This revealed a significantly higher expression of *C3AR1* in *NPM1*-mutated compared with WT cases ( $P \leq .0001$  all cohorts).

Finally, to validate the correlation at the protein level, we assessed C3AR expression in *NPM1* WT AML cases ( $n = 30$ ) and compared it with the expression in *NPM1*-mutated cases ( $n = 13$ ) (Figure 3E). This analysis demonstrates that the expression of C3AR is significantly higher in *NPM1*-mutated compared with WT cases ( $P < .0001$ ). Looking at individual AML subgroups, none of the other genetic subtypes consistently express C3AR, either in the myeloid ( $CD3^- CD19^-$ ) or stem cell-enriched ( $CD34^+ CD38^-$ ) compartments (supplemental Figure 4). We thus conclude that C3AR expression correlates with mutations in *NPM1*.

## scRNA-seq confirms expression of *C3AR1* in *NPM1*-mutated AML cells and monocytes

Because bulk RNA-seq does not fully recapitulate the heterogeneity of the samples sequenced, we performed scRNA-seq to investigate the expression of *C3AR1* in AML and NBM. We sequenced *NPM1*-mutated AML samples ( $n = 12$ ), along with NBM MNC samples ( $n = 5$ ) and NBM MNC samples enriched for CD34 ( $n = 3$ ). Gene expression signatures of all samples revealed distinct cell clusters of normal hematopoietic cells as well as major AML-specific clusters (Figure 4A). To determine which clusters contained the malignant cells, we performed mutation calling for all cells that had reads covering the *NPM1*-mutation site. This analysis showed that the cells with detected *NPM1* mutations were mainly located within the AML-specific cell clusters (Figure 4B), confirming that these

clusters constitute the actual leukemic cells whereas other cell clusters likely comprise residual normal hematopoiesis (predominantly NK cells, T cells, B cells, and erythroid cells). Assessing *C3AR1* expression in all samples sequenced (Figure 4C), 2 main clusters expressing *C3AR1* were observed, an AML-specific cluster and a monocyte cluster. No expression was seen in the HSC or progenitor populations (which almost exclusively contained cells from the NBM samples; Figure 4C; supplemental Figure 5). In concordance with this, the NBM samples analyzed by bulk RNA-seq in the Beat AML data set, showed much lower expression of *C3AR1* in the  $CD34^+$  cells compared with the MNCs (supplemental Figure 6), in which the monocytes are found.

These data are consistent with the protein expression data assessed by flow cytometry, showing C3AR expression on *NPM1*-mutated AML cells (Figure 1C-D) and monocytes (supplemental Figure 3) but not on immature NBM cells (Figure 2; supplemental Figure 2).

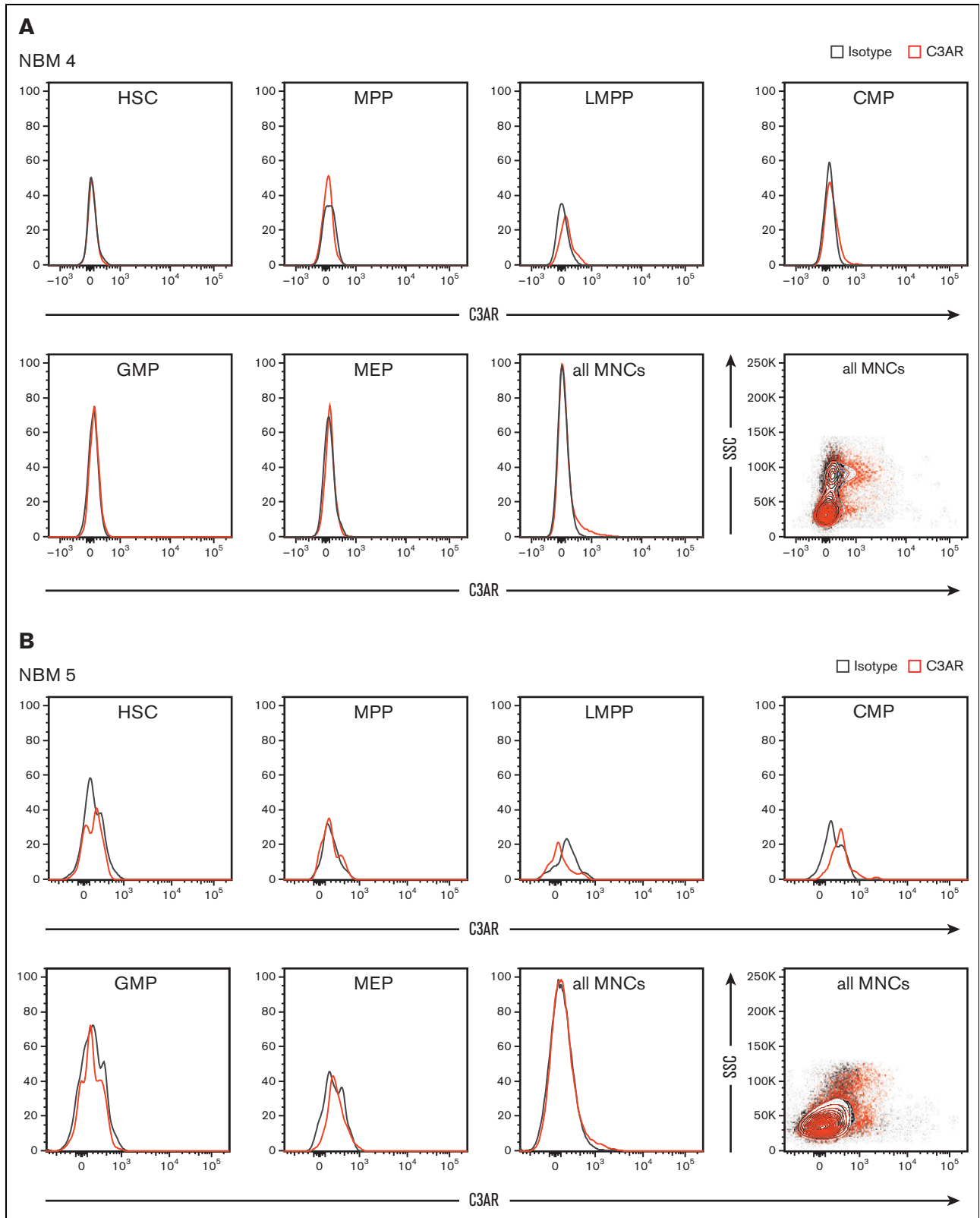
## Coexpression of GPR56 and C3AR defines *NPM1*-mutated AML cells with leukemia-initiating capacity

Because the cell surface protein GPR56 has been identified as an important LSC marker in *NPM1*-mutated AML,<sup>17-19</sup> we investigated its coexpression with C3AR. Analysis of the scRNA-seq data set showed that *GPR56* is expressed in certain AML cell populations but also in normal HSCs, NK cells, and some T cells (Figure 4D; supplemental Figure 7). Further flow cytometric analysis of 3 *NPM1*-mutated AML samples showed coexpression of C3AR and GPR56 in a portion of the AML cells (Figure 5A). GPR56 is known to enrich for LSC activity in AML cells<sup>18</sup> but is also highly expressed on normal HSPCs. Considering our observation that C3AR is expressed on AML cells but not on normal immature cell populations, we speculated that  $GPR56^+ C3AR^+$  could provide a combination of markers to define AML cells with increased LSC activity within the *NPM1*-mutated subtype. Therefore, we sorted and transplanted  $C3AR^+ GPR56^+$  and  $C3AR^+ GPR56^-$  primary *NPM1*-mutated AML cells and assessed human engraftment in BM 19 weeks after transplantation (Figure 5B). Remarkably, all mice that underwent transplantation with  $C3AR^+ GPR56^+$  cells showed human engraftment, whereas the  $C3AR^+ GPR56^-$  cells did not engraft in any of the mice (Figure 5C; supplemental Figure 8). In summary, these data show that the combination of C3AR and GPR56 can be used to enrich for LSCs in *NPM1*-mutated AML.

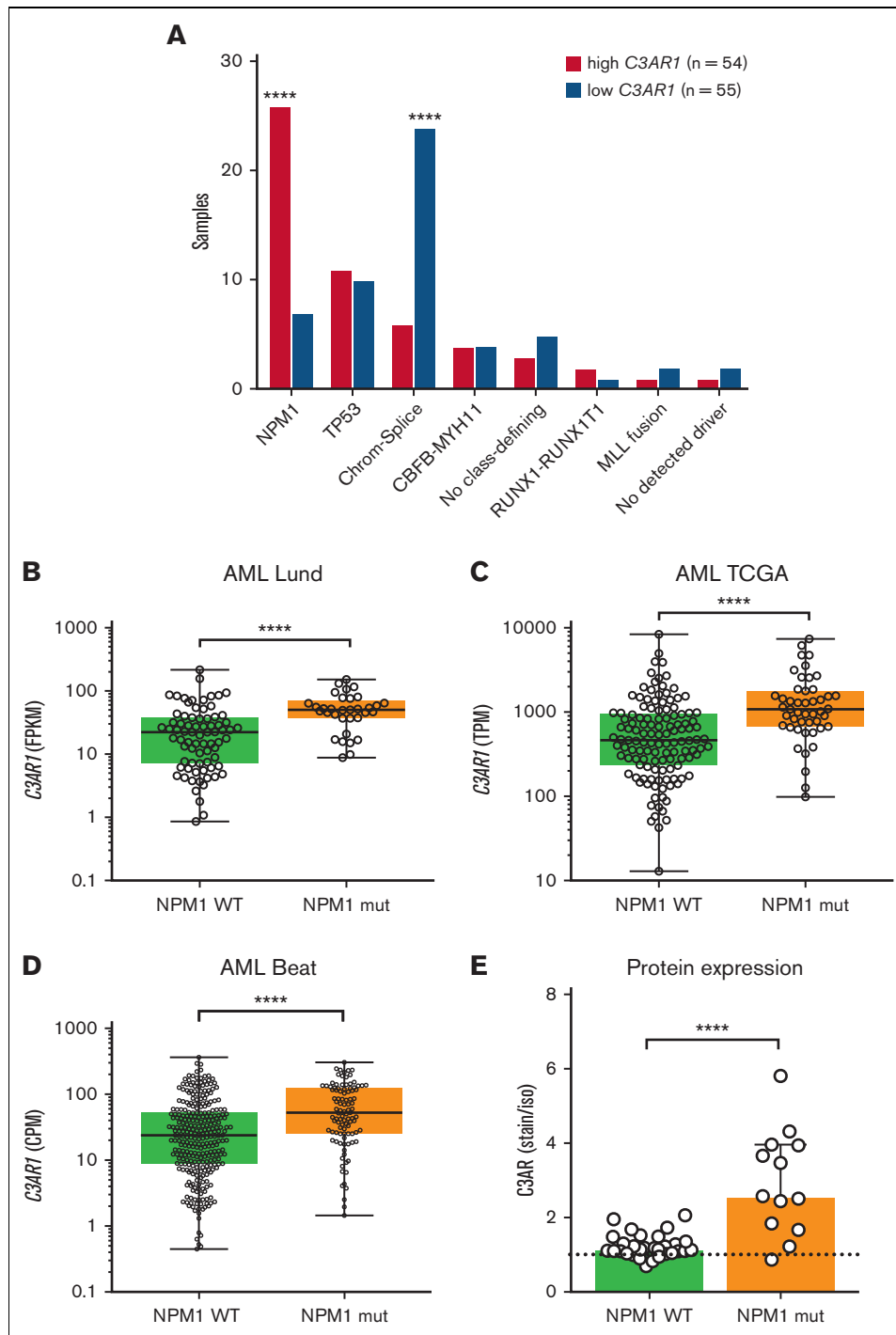
## Stimulation with C3a leads to activation of ERK1/2 in primary *NPM1*-mutated AML cells and increases survival of OCI-AML3 cells

Next, we wanted to investigate whether C3AR-expressing AML cells respond to stimulation with the ligand C3a. Because it is

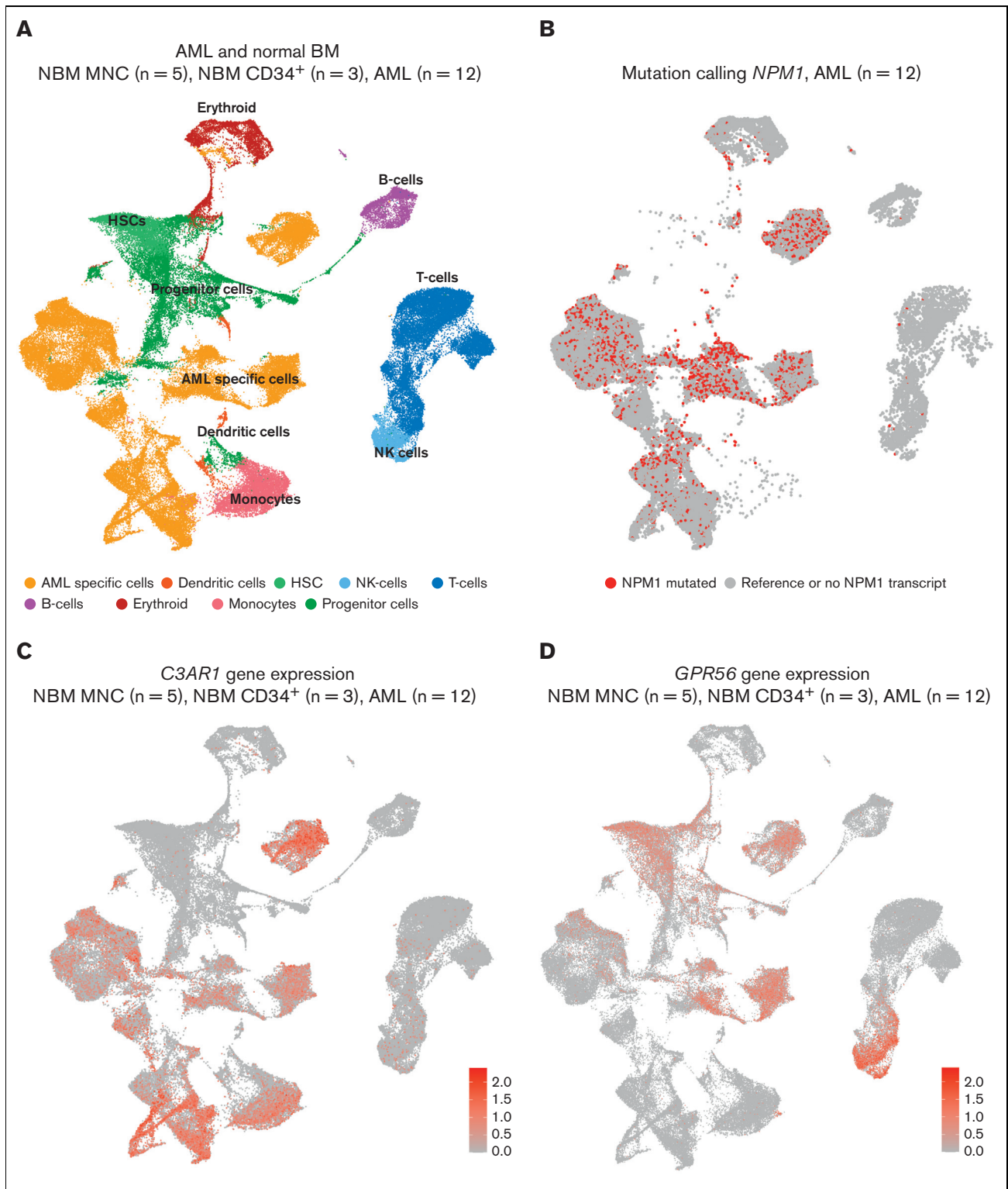
**Figure 1. Antibody-based screening identifies cell surface markers specifically upregulated on *NPM1*-mutated AML cells.** (A) Schematic illustration showing the arrayed antibody screen performed on  $CD3^- CD19^-$  myeloid-enriched blasts from *NPM1*-mutated AML samples and  $CD34^+ CD38^{low}$  stem cell-enriched NBM samples. The antibody library contained 362 PE-marked antibodies. In total, 4 primary AML samples and 4 NBM samples were used in the screen. (B) Screening results showing the top-ranked cell surface markers in AML compared with NBM cells, calculated as the ratio of the median MFI of the  $CD3^- CD19^-$  AML cells over the median MFI of the NBM  $CD34^+ CD38^{low}$  cells for each marker. The green bars illustrate known LSC markers, the lavender bars illustrate novel markers, and the red bar shows C3AR that was chosen for further investigation. (C) Expression of C3AR in the primary *NPM1*-mutated samples used in the screen. (D) Validation of C3AR expression in 9 additional *NPM1*-mutated AML samples. C3AR stain in red and isotype control in gray. (E) MFI of C3AR in all *NPM1*-mutated AML samples ( $n = 13$ ), compared with isotype control. \*\*\* $P \leq .001$  in a Wilcoxon nonparametric paired test. MFI, median fluorescence intensity.



**Figure 2. C3AR is not expressed on normal HSPCs.** (A-B) flow cytometric analysis of C3AR expression in different fractions of NBM MNCs from 2 healthy donors. C3AR stain in red and isotype control in gray. The MNCs are shown both as a histograms and contour plots. CMP, common myeloid progenitor ( $\text{Lin}^- \text{CD34}^+ \text{CD38}^+ \text{CD123}^+ \text{CD45RA}^-$ ); GMP, granulocyte-macrophage progenitor ( $\text{Lin}^- \text{CD34}^+ \text{CD38}^+ \text{CD123}^+ \text{CD45RA}^+$ ); HSC, hematopoietic stem cell ( $\text{Lin}^- \text{CD34}^+ \text{CD38}^- \text{CD90}^+ \text{CD45RA}^-$ ); LMPP, lymphoid-primed multipotent progenitor ( $\text{Lin}^- \text{CD34}^+ \text{CD38}^- \text{CD45RA}^+$ ); MEP, megakaryocyte-erythroid progenitor ( $\text{Lin}^- \text{CD34}^+ \text{CD38}^+ \text{CD123}^- \text{CD45RA}^-$ ); MPP, multipotent progenitor ( $\text{Lin}^- \text{CD34}^+ \text{CD38}^- \text{CD90}^- \text{CD45RA}^-$ ).

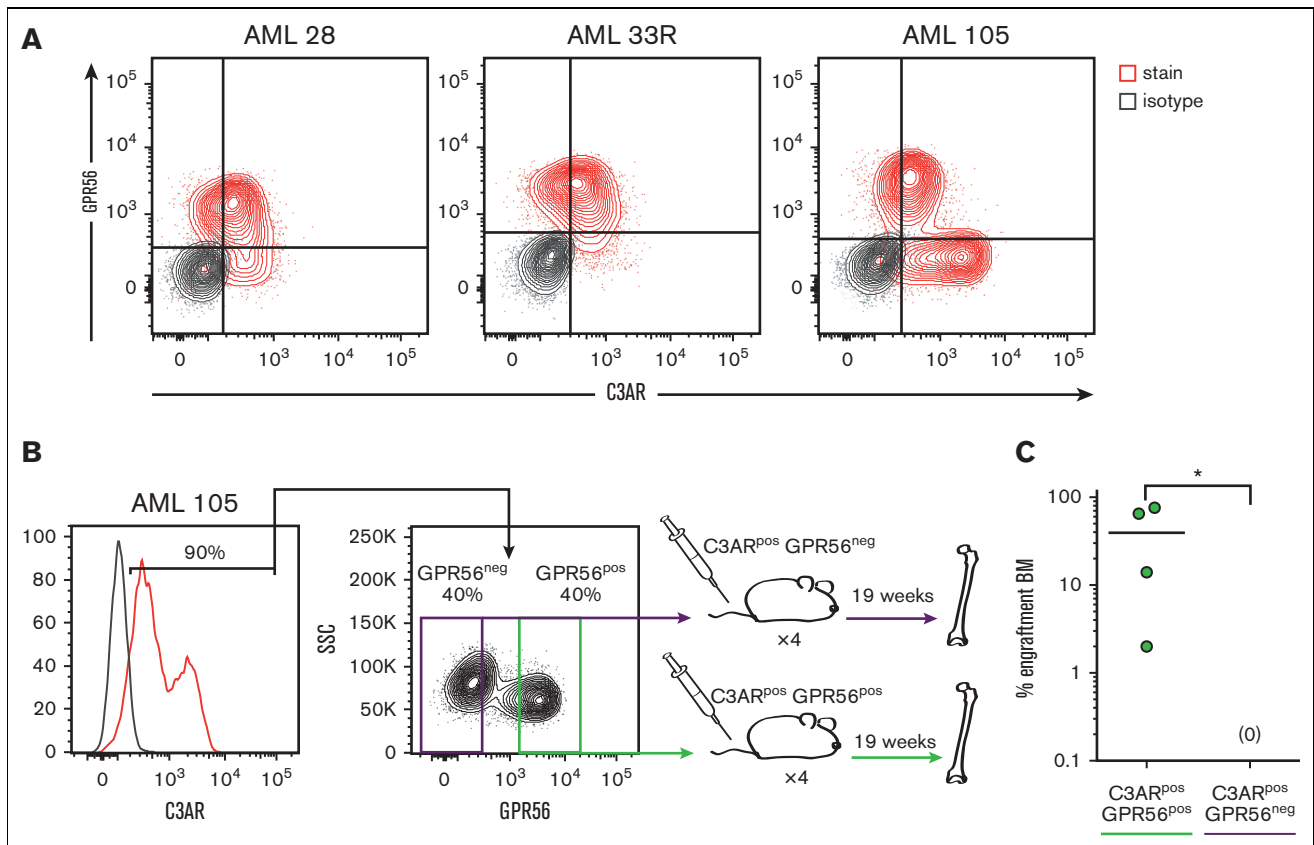


**Figure 3. *C3AR1* is specifically upregulated in *NPM1*-mutated AML cells.** (A) Number of cases with high vs low *C3AR1* mRNA expression in each genetic subgroup in a cohort of AML samples (n = 109) diagnosed and analyzed by RNA-seq in Lund, Sweden. AML subgroups with <3 patients were excluded from the analysis. High *C3AR1* in magenta and low *C3AR1* in purple. (B) FPKM values for *C3AR1* in *NPM1*-mutated cases vs cases with *NPM1* WT in the entire AML Lund cohort (n = 112). (C) TPM values for *C3AR1* in *NPM1*-mutated cases vs cases with *NPM1* WT, in the publicly available TCGA AML data set (n = 173). (D) CPM values of *C3AR1* in *NPM1*-mutated cases vs cases with *NPM1* WT, in the Beat AML data set (n = 400). *C3AR1* expression in panels B-D are represented as box plots. (E) *C3AR1* protein expression calculated as fold change of *C3AR* MFI over isotype control MFI in *NPM1*-mutated AML cases and *NPM1* WT cases, analyzed by flow cytometry, in the CD3<sup>-</sup> CD19<sup>-</sup> myeloid-enriched compartment (n = 43). Bars show median with interquartile range. \*\*\*\**P* ≤ .0001 using a Fisher exact test in panel A. \*\*\*\**P* ≤ .0001 using a nonparametric Mann-Whitney *U* test in panels B-E. Chrom-splice, chromatin-spliceosome; CPM, counts per million mapped reads; FPKM, fragments per kilobase of exon model per million reads mapped; MFI, median fluorescence intensity; mRNA, messenger RNA; TCGA, The Cancer Genome Atlas; TPM, transcripts per million.



**Figure 4.** scRNA-seq confirms *C3AR1* expression in *NPM1*-mutated AML blasts and in normal monocytes. (A) UMAP showing *NPM1*-mutated AMLs (n = 12), NBM MNCs (n = 5), and NBM CD34<sup>+</sup> (n = 3) samples combined. (B) UMAP showing mutation calling of *NPM1* in the same AML cells and projection as in panel A. Red: cells with detected *NPM1* mutations. Gray: cells with WT *NPM1* or no detected *NPM1* transcript. (C) UMAP showing the expression of *C3AR1* in same cells and projection as in panel A. Red indicates high expression. (D) UMAP showing the expression of *GPR56* in same cells and projection as in panel A. Red indicates high expression. UMAP, uniform manifold approximation and projection for dimension reduction.





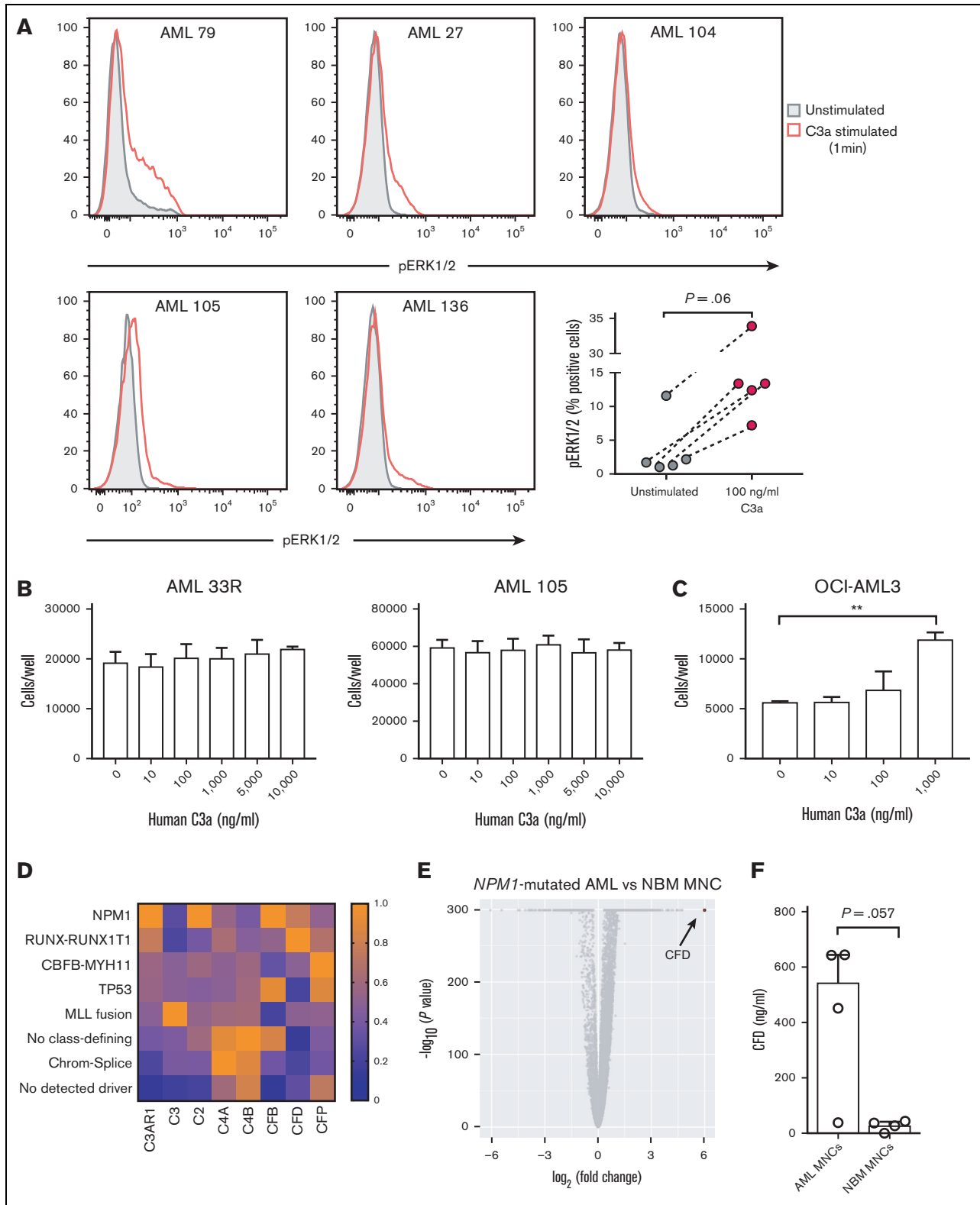
**Figure 5. Coexpression of GPR56 and C3AR marks *NPM1*-mutated AML cells with leukemia-initiating capacity.** (A) Protein coexpression of C3AR and GPR56 in 3 *NPM1*-mutated AML samples. Marker stains in red and isotype in gray. (B) Schematic illustration showing the experimental setup for the C3AR/GPR56 transplant experiments; the myeloid-enriched (CD3<sup>-</sup>CD19<sup>-</sup>) cells expressing C3AR from a *NPM1*-mutated AML case were sorted based on GPR56 expression and transplanted into immunodeficient NSGS mice, which were sacrificed at 19 weeks. (C) Leukemic engraftment in the BM of mice that had received transplantation with C3AR<sup>+</sup> GPR56<sup>+</sup> and C3AR<sup>+</sup> GPR56<sup>-</sup> cells, as the percentage of BM cells positive for human CD45 and CD33. Line at median; \**P* ≤ .05 using a nonparametric Mann-Whitney *U* test.

known that binding of C3a to C3AR leads to activation of the MAP kinase pathway in immune cells,<sup>38,39</sup> we investigated whether this was also true in primary AML cells. By phospho-flow cytometry, we found increased phosphorylation of ERK1/2 in C3AR-expressing AML cells following 1 minute of C3a stimulation (Figure 6A). After 5-minute stimulation, this phosphorylation was abolished in all but 1 sample in which the phosphorylation had decreased compared with the 1-minute time point (supplemental Figure 9A), indicating that C3AR-mediated activation by C3a is very rapid but transient.

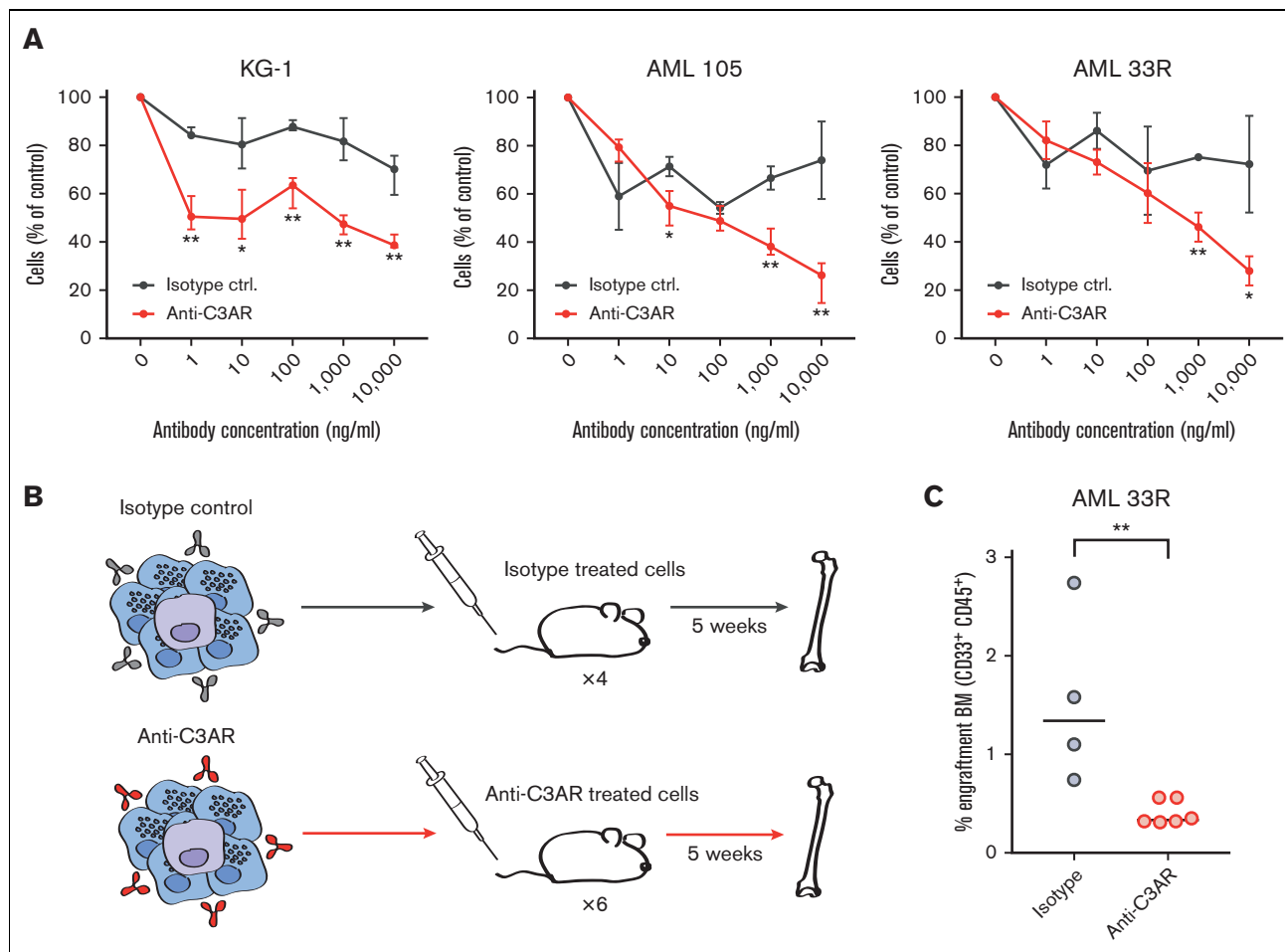
To evaluate whether C3a-stimulation also leads to expansion of C3AR-expressing AML cells, we cultured 5 primary *NPM1*-mutated AML samples in increasing concentrations of human C3a (Figure 6B; supplemental Figure 9B). After 3 days in culture, no differences in cell numbers were seen, possibly owing to poor survival of primary AML cells in culture. To circumvent this problem, we repeated the experiment using serum-starved *NPM1*-mutated OCI-AML3 cells, which also express C3AR (supplemental Figure 9C). After 3 days in culture, the number of OCI-AML3 cells was more than twofold higher in the wells that had received 1000 ng/mL of C3a compared with control (Figure 6C). For the *NPM1* WT cell line NALM-6, which does not express C3AR (supplemental Figure 9C), no increase in cell

number after C3a stimulation was observed (supplemental Figure 9D).

Because C3AR-expressing AML cells were shown to respond to C3a stimulation by increased phosphorylation of ERK1/2, we also investigated whether the *NPM1*-mutated AML cells could produce C3a in an autocrine manner. First, we analyzed expression of C3, the precursor for C3a, in our scRNA-seq data (supplemental Figure 10). In this data set, the expression of C3 in normal hematopoietic cells and *NPM1*-mutated AML cells was low or absent. However, a lack of detectable gene expression using this method could be explained by limited sensitivity (a median of 2097 genes/cell were detected in each sample investigated). Therefore, we also analyzed the expression of C3 in the bulk RNA-seq data from the Lund cohort. This revealed expression of C3 in the vast majority of AML samples and in all analyzed genetic subtypes (Figure 6D; supplemental Figure 11). Although these data do not suggest high autocrine expression of C3 in AML, C3 is one of the most abundant plasma proteins in the blood<sup>40</sup> and can thus be provided externally. Therefore, we next investigated whether AML cells can produce components of C3-convertases, the enzyme complexes that cleave C3 into its biologically active counterparts, C3a and C3b. To address this, we analyzed genes encoding the components of the classical pathway C3-convertase (C2 and



**Figure 6. C3AR is biologically functional in *NPM1*-mutated AML.** (A) Expression of pERK1/2 after 1-minute stimulation with 100 ng/mL of human C3a in 5 primary AML samples. Unstimulated shown in filled blue and stimulated in red.  $P = .06$  using a Wilcoxon nonparametric paired test. (B) Number of primary *NPM1*-mutated AML cells and OCI-AML3 cells (C) after a 3-day culture with increasing concentrations of human C3a. Primary AML cells (100 000) and OCI-AML3 cells (20 000) were seeded per well in serum-free medium. The experiments were performed in triplicate. Bars in panels B-C show mean cell numbers with standard deviation. (D) Heat map showing the median FPKM value of C3-related complement genes in each AML subgroup, relative to the highest median value of that gene, in the Lund AML Cohort. AML subgroups with <3 patients



**Figure 7. C3AR can be specifically targeted with anti-C3AR antibodies.** (A) ADCC assay on AML cell line KG-1 and 2 primary *NPM1*-mutated AML samples. The red line shows the percentage of AML cells after overnight incubation with a polyclonal anti-C3AR antibody and NK cells, compared with control without antibody. The black line shows the isotype-treated cells under the same experimental conditions. Error bars show mean with standard deviation. (B) Schematic illustration of the ADCC in vivo repopulation assay; residual cells from an ex vivo ADCC experiment on a primary AML sample were transplanted into immunodeficient NSG mice, which were sacrificed after 5 weeks. AML cells are depicted in purple and NK cells in blue. (C) Leukemic engraftment after ADCC, as the percentage of CD45<sup>+</sup>CD33<sup>+</sup> human cells in the BM of mice that received transplantation with isotype-treated or anti-C3AR-treated AML cells, as in panel B. Antibody (10 µg/mL) was used. Line at median. \* $P \leq .05$  and \*\* $P \leq .01$  using a Student *t* test in panel A and a nonparametric Mann-Whitney *U* test in panel C.

*C4A/C4B*), as well as the alternative pathway C3-convertase (complement factor B [*CFB*] and *CFD*) (Figure 6D; supplemental Figure 11).

Notably, *NPM1*-mutated AMLs showed a relatively high expression of alternative pathway C3-convertase genes, *CFB* and *CFD*. In fact, *CFD*, encoding the protein CFD was the most upregulated gene in *NPM1*-mutated AML blasts compared with NBM NCs in our scRNA-seq data set (Figure 6E). Interestingly, CFD is known to be the rate-determining enzyme in the alternative complement pathway.<sup>41</sup>

To investigate whether the *NPM1*-mutated AML cells and NBM MNCs also produce the CFD protein, we measured the amount of CFD in the cell culture media after 24 hours (Figure 6F). Expression of CFD by NBM MNCs ( $n = 4$ ) was detected in all but 1 sample (3/4), with a median CFD expression of 31.8 ng/mL. However, in the supernatants from the cultured AML cells ( $n = 4$ ), the median CFD expression was ~17 times higher (median = 547.4 ng/mL). One AML sample had notably lower expression than the other 3, possibly owing to poor survival of this sample in culture or because of biological differences between samples. Nevertheless, these data suggest that *NPM1*-mutated AMLs cells may have

**Figure 6 (continued)** were excluded from the analysis ( $n = 109$ ). (E) Relative gene expression of bulk *NPM1*-mutated AML cells ( $n = 12$  cases) compared with NBM MNCs ( $n = 5$  cases), as assessed by scRNA-seq. (F) Amount of CFD in the cell culture supernatant 24 hour after seeding. One million cells per mL were seeded from 4 *NPM1*-mutated AML samples and 4 NBM MNC samples in S7 media. Bars show median with interquartile range. \*\* $P \leq .01$  using a Student *t* test in panel C. A nonparametric Mann-Whitney *U* test was used in panel F. chrom-splice, chromatin-spliceosome; FPKM, fragments per kilobase of exon model per million reads mapped.

the ability to generate active C3a from the bloodstream by their own production of the alternative pathway C3-convertase.

In conclusion, our data show that C3AR-expressing AML cells respond to C3a-stimulation with activation of the MAP kinase pathway and that these cells produce CFD, the rate-limiting enzyme of alternative pathway complement activation.

### C3AR can be specifically targeted with an anti-C3AR antibody

Finally, we evaluated the therapeutic potential of C3AR. The selective expression of this marker on *NPM1*-mutated AML cells but not on normal HSPCs or AMLs with *NPM1* WT, suggests that C3AR can be utilized for antibody-based targeting in this subgroup of AML. Hence, we investigated, using an ADCC assay, whether C3AR-antibodies would be able to elicit cell killing by attracting human NK cells. For the C3AR-expressing AML cell line KG-1 (supplemental Figure 9C), and 5 primary *NPM1*-mutated AML samples, in 7 individual experiments, we observed selective killing of AML cells compared with treatment with an isotype control (Figure 7A; supplemental Figure 12A). Importantly, when residual primary AML cells from an ADCC assay were transplanted into immunodeficient mice (Figure 7B), the cells treated with C3AR-antibodies engrafted at lower levels than isotype control-treated cells (Figure 7C; supplemental Figure 12B), demonstrating that C3AR-antibodies are able to efficiently kill AML cells with leukemia-initiating capacity. Collectively, our results suggest that C3AR constitutes a novel therapeutic target in *NPM1*-mutated AML, representing, to the best of our knowledge, the first selectively expressed marker in this subtype that is targetable with antibodies while sparing normal HSPCs.

### Discussion

AML with mutated *NPM1* is the most common molecular subgroup in AML, comprising ~30% of all AML cases.<sup>8,9</sup> When *NPM1* mutations are observed without *FLT3*-ITD in AML with a normal karyotype, this is associated with a favorable prognosis according to current risk stratification guidelines.<sup>9</sup> However, a recent study based on the Swedish AML Registry showed that the favorable prognosis of *NPM1*-mutated cases without *FLT3*-ITD is only seen in patients aged <60 years. For patients aged 60 to 75 years, the median overall survival is only 3.3 years. Moreover, if *NPM1* mutations and *FLT3*-ITD are present, the prognosis is poor regardless of age (median overall survival <2 years).<sup>42</sup> Hence, new treatment alternatives are urgently needed to improve survival rates for *NPM1*-mutated AML.

In this study, we searched for novel cell surface markers in *NPM1*-mutated AML that could form the basis for the development of an antibody-based treatment. In most subtypes of AML, the LSCs are enriched within the primitive (CD34<sup>+</sup>CD38<sup>-</sup>) population.<sup>43</sup> However, *NPM1*-mutated AML typically lacks CD34-expression,<sup>44,45</sup> making it impossible to use CD34 as an LSC maker. Furthermore, most studies of LSCs in AML have focused on AML samples with >10% CD34<sup>+</sup> blasts,<sup>5</sup> hence disregarding CD34-negative AML samples with *NPM1* mutations. Therefore, we, instead, focused on identifying markers with expression within the entire myeloid compartment of *NPM1*-mutated AMLs but not in the primitive NBM cells. Using an arrayed antibody screen, we identified the complement protein C3a receptor C3AR as the highest

and most consistently expressed selective marker in *NPM1*-mutated AMLs, with nondetectable expression in NBM HSPCs.

C3AR is a G protein-coupled receptor with high expression on myeloid innate immune cells, especially on granular cells and monocytes.<sup>35</sup> It binds to the anaphylatoxin, C3a, an inflammatory molecule released following complement activation.<sup>46</sup> In cancer, increased inflammation as a result of dysregulated complement has been suggested to play an important role in disease initiation and progression,<sup>39,46-50</sup> and autocrine signaling involving tumor-derived complement factors has been identified in solid tumors.<sup>51</sup> We show that genes encoding 2 of the complement proteins that make up the C3-convertase of the alternative pathway, *CFB* and *CFD*, are expressed in *NPM1*-mutated AML. In fact, the gene encoding the serine protease CFD, which is the rate-limiting enzyme of the alternative complement pathway, was the most upregulated gene in the *NPM1*-mutated AML cells analyzed by scRNA-seq, compared with NBM MNCs. This is remarkable as CFD usually has the lowest plasma concentration of all complement proteins and is predominantly produced by adipocytes.<sup>41</sup> However, we show that *NPM1*-mutated AML cells can readily produce CFD. This suggests that the AML cells have the ability to generate active C3a from the abundant amounts of C3 in the blood<sup>40</sup> by producing the alternative pathway C3-convertase, thus providing a previously unexplored mechanism of complement activation in this subtype of AML. As of now, complement involvement in leukemia has not been as extensively studied, as for many solid tumors<sup>51</sup>; herein, however, we provide evidence that the C3a/C3AR signaling axis is functional in primary *NPM1*-mutated AML cells, suggesting that cells would benefit from a proinflammatory environment with high levels of C3a.

We, and others, have shown that LSCs expressing, for example, IL1RAP<sup>20,21</sup> or CD123<sup>52</sup> on the cell surface can be specifically targeted with antibodies and killed by ADCC, with several clinical trials currently ongoing. Similarly, we show herein that C3AR-expressing *NPM1*-mutated AML cells can be specifically targeted ex vivo using C3AR antibodies and NK cells. Given that we observed a consistent expression of C3AR on the bulk leukemic cell population, an antibody targeting C3AR would be anticipated to kill AML cells, including the critical LSCs, while sparing normal HSPCs. Indeed, our results support the ability to target LSCs as demonstrated by reduced ability of C3AR-antibody-treated AML cells to initiate leukemia in immunodeficient mice.

In concordance with previous studies, we also found expression of C3AR on normal monocytes.<sup>34,35</sup> However, cell surface expression of C3AR was mainly limited to CD16-expressing monocytes, suggesting that C3AR-antibodies would spare the classical (CD16<sup>neg</sup>) monocytes that constitute the majority of the monocytic population. Furthermore, as normal HSPCs do not express C3AR, the affected monocytes would be expected to rapidly replenish after the end of treatment.

Recently, the G protein-coupled receptor GPR56 was identified as a cell surface marker of LSCs on both CD34<sup>+</sup> and CD34<sup>-</sup> cells.<sup>18</sup> In particular, *NPM1 FLT3*-ITD *DNMT3A* triple-mutated AML shows an enrichment for LSCs in the CD34<sup>-</sup> GPR56<sup>+</sup> compartment.<sup>19</sup> However, although GPR56 may provide a marker to study LSCs, GPR56 is also expressed on normal CD34<sup>+</sup> cells,<sup>18</sup> making it less ideal as a therapeutic target. Herein, we show that C3AR<sup>+</sup>GPR56<sup>+</sup> cells but not C3AR<sup>+</sup>GPR56<sup>-</sup> cells from a patient with primary *NPM1*-mutated AML engrafted in

immunodeficient mice. Collectively, these data demonstrate that C3AR can discriminate AML cells from normal HSPCs, whereas GPR56 can be used to enrich for cells with LSCs activity within this population.

In conclusion, our results demonstrate that complement receptor C3AR is specifically upregulated on *NPM1*-mutated AML cells. Importantly, targeting C3AR by antibodies could provide new therapeutic opportunities in this AML subgroup, possibly through a dual mode of action, by inhibition of C3a signaling and by marking leukemic cells for effector-cell-mediated immune destruction.

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## Authorship

Contribution: S.v.P. and T.F. designed the study; S.v.P. made the figures; S.v.P., H.T., P.P.-M., N.P.-M., C.S., N.L., H.Å., and M.R. performed experiments; S.v.P., H.T., P.P.-M., N.P.-M., C.S., R.H., V. R., M.J., C.O.-P., H.L., and T.F. analyzed the data; G.J., V.L., H.L., and C.O.-P. collected patient material and clinical data; A.M.B. and B.K. provided intellectual input; S.v.P. and T.F. wrote the manuscript; and all other authors reviewed the manuscript.

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