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CD200 in acute myeloid leukemia: marked upregulation in CEBPA biallelic mutated cases

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Abstract

CD200 is a glycoprotein that binds with its receptor CD200R, providing immunosuppressive signals to T and NK cells. CD200 is expressed by normal stem cells and progenitors committed to B-lymphopoiesis and myeloid development. CD200 biological relevance in acute leukemias is only partially understood.

The study included a consecutive series of four hundred thirty-one patients with acute myeloid leukemia (AML). Immunophenotype was established by multiparametric flow cytometry, and the genetic diagnosis was performed by PCR-based methods and a targeted resequencing method covering 42 genes.

66% of AML patients expressed CD200 being significantly associated with CD34 reactivity. The frequency of CD200 positivity was higher in cases with core-binding factor genetic lesions such as *RUNX1-RUNX1T1* (81.3%) fusions and *CBFB-MHY11* (63.2%) rearrangements and also with biallelic *CEBPA* mutations (100%). The molecular AML group with the lowest CD200 reactivity (19.1%) corresponded to AML with *NPM1* mutations. RNA seq showed no uniform pattern of infiltrating cells in CEBPA mutated AML. Deconvolution analysis may be used to assess the immunoregulatory mechanisms of AML.

CD200 expression could help identify the more immature compartment and, combined with other markers, single out CEPA-mutated AML.

Keywords CD200, Acute myeloid leukemia- *RUNX1*, *CEBPA -NPM1*, *FLT3*

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of neoplastic disorders arising from malignant hematopoietic progenitors' malignant transformation. It accounts for almost 10% of all cancers. AML diagnosis is based on morphology, flow cytometry, immunophenotyping, and genetic analysis. Those techniques are combined to set the proper diagnosis and treatment [1–2].

AML is sustained by a minor population of leukemia stem cells (LSCs) characterized by self-renewal capacity, immunologic privilege, and resistance to apoptosis. LSCs in AML patients may be genetically, immunophenotypically, and functionally heterogeneous [3]. Treatments should be addressed to eliminate the LSC population. Some immunophenotypic studies suggested that LSC may be found within the CD34 + and CD34- fractions [4]. Further phenotypic delineation of the LSC cells could be clinically helpful.

The discovery of immune checkpoints and their inhibition is a recently developed modality for AML treatment. T-cell immunoglobulin mucin-3 (TIM3), expressed on the surface of LSCs, is involved in AML progression. TIM3 binding by Galectin-9 inhibits the AML cell-killing activity by NK and activates different cellular survival pathways [5–8]. The CD200 antigen is gaining importance in the diagnosis and prognosis of AML because it has been suggested that it may behave like TIM3 [9]. CD200 is a type-I membrane glycoprotein that contains two extracellular domains: a transmembrane and a cytoplasmic domain [10]. This protein is expressed in immune cells, endothelial cells, neurons, and normal hematopoietic stem cells (HSCs). CD200 binds with its receptor CD200R on T- and B- and myeloid cells, providing immunosuppressive signals [11–13]. CD200 is overexpressed in various solid and hematologic neoplasms, as in the surface of LSCs in AML [14]. It has been suggested that CD200 could promote the growth of the more immature leukemic cell compartment [15–18]. CD200 is also expressed by chronic lymphocytic leukemia (CLL) cells, and its analysis is gaining acceptance as one key marker to differentiate CLL from mantle-cell lymphoma [19–21].

This study investigates the pattern of CD200 expression in patients with AML and establishes phenotypic and genetic correlations.

Patients and methods

Patients

This study included 431 patients diagnosed with AML based on standard WHO criteria 2017 [22], from 2017 to 2020, at the Hospital de la Santa Creu I Sant Pau in Barcelona. Patients referred to flow cytometry and molecular analysis were included in this series.

In an additional series, 60 adult AML diagnosed from the Hospital de la Santa Creu I Sant Pau were enrolled in the study to perform RNA analysis and gene expression by RT-PCR. Cases were categorized into different groups according to the molecular lesions as follows: Group 1 (t(8,21), $n = 6$); group 2 (inv(16), $n = 6$); group 3 (*CEBPA*-m, $n = 8$), group 4 (*NPM1*-m, $n = 20$); group 5 (Other AML, $n = 20$). Two normal bone marrow samples were used as calibrator samples.

An additional series of 13 AML cases with biallelic CEBPA were used for RNA seq (Suppl. Table 1).

Flow cytometry analysis

Sample preparation

Immunophenotyping studies were performed upon diagnosis of erythrocyte-lysed bone marrow samples upon staining with monoclonal antibodies (MoAbs) directly conjugated with fluorochromes. Antigenic expression of leukemic cells was analyzed by four-color multiparametric flow cytometry; fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll protein (PerCP) or peridinin chlorophyll protein-Cyanine5.5 (PerCP-Cy5.5) and allophycocyanin (APC), in combination with MoAbs as follows: CD15/CD34/CD45/HLA-DR, CD10/CD20/CD34/ CD19, CD2/CD33/CD45/CD34, CD7/CD117/CD45/CD34, CD66/CD13/CD64/CD45, CD36/CD56/CD45/HLA-DR, CD14/CD123/CD45/CD34, CD36/Glycophorin A/CD45/HLA-DR, CD71/CD200/CD45/CD34, myeloperoxidase (MPO)/CD79a/CD3/CD34, TdT/MPO/CD45/CD34 and lysozyme/lactoferrin/CD45/CD34, CD2/CD4/CD8/CD3, CD34/CD117/CD45/HLA-DR and CD38/CD33/CD45/CD34.

The MoAbs used in the study were (antibody clone, conjugated fluorochrome): TdT (HT-6 FITC), CD117 (10402 PE), MPO (MPO-7 FITC and PE), CD45 (H130 PerCP and PerCP-Cy5.5), CD71 (Be-Tq FITC), CD20 (B-Ly1 PE), CD79a (HM57 PE) from DAKO, Glostrup, Denmark; CD66 (GI55-228 FITC), CD64 (MOPC-21 PerCP-Cy5.5), CD19 (TB28.2 PerCP-Cy5.5), CD34 (8G12 FITC, PE and Per-CP-Cy5.5), HLA-DR (L243 PerCP-Cy5.5), CD10 (W8E7 FITC), CD2 (S5.2 FITC), CD33 (P67.6 PE), CD7 (4H9 FITC), CD13 (L138 PE), CD14 (M0P9 FITC), CD3 (SK7 PerCP and PerCP-Cy5.5), CD4 (SK4 FITC), CD56 (MY31 PE), CD15 (MMA FITC), CD123 (MOPC-21 PE), CD8 (SK1 PerCP), GA (GAR-1 PE), CD200 (MRC OX-104 PE), Lysozyme (EC 3.2.1 FITC), Lactoferrin (4C5 PE), CD38 (HB7 FITC) and CD36 (CB38 FITC) purchased from Becton Dickinson, San José, CA, USA (BDIS).

Data acquisition and analysis

Leukemic cells were acquired and analyzed on a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA,

USA). We measured at least 10.000 events/tube. We used Infinicyt 2.0 software (Cytognos SL, Salamanca, Spain). We removed nonviable cells, doublets, and debris. Blasts were then identified based on CD45 + dim/low SSC properties. We determined the percentage of positive cells and the mean fluorescence intensity (MFI) values for CD200 and the rest of the antigens within the blast cell gate for each case. The positivity threshold was set at 20% except for CD117, MPO, TdT, and CD79a, for which a 10% value was used [19, 23]. We differentiated the threshold positivity of CD200 into 3 groups dependent on the percentage of CD200 expression: Pattern 0 or negative (0–20%), Pattern 1 or partially positive (20–50%), and Pattern 2 or fully positive (> 50%) corresponded to a homogeneous CD200 + cell population. P1 and P2 being positive cases with a progressive increase in the MFI and a simultaneous loss of negative cells (Fig. 1).

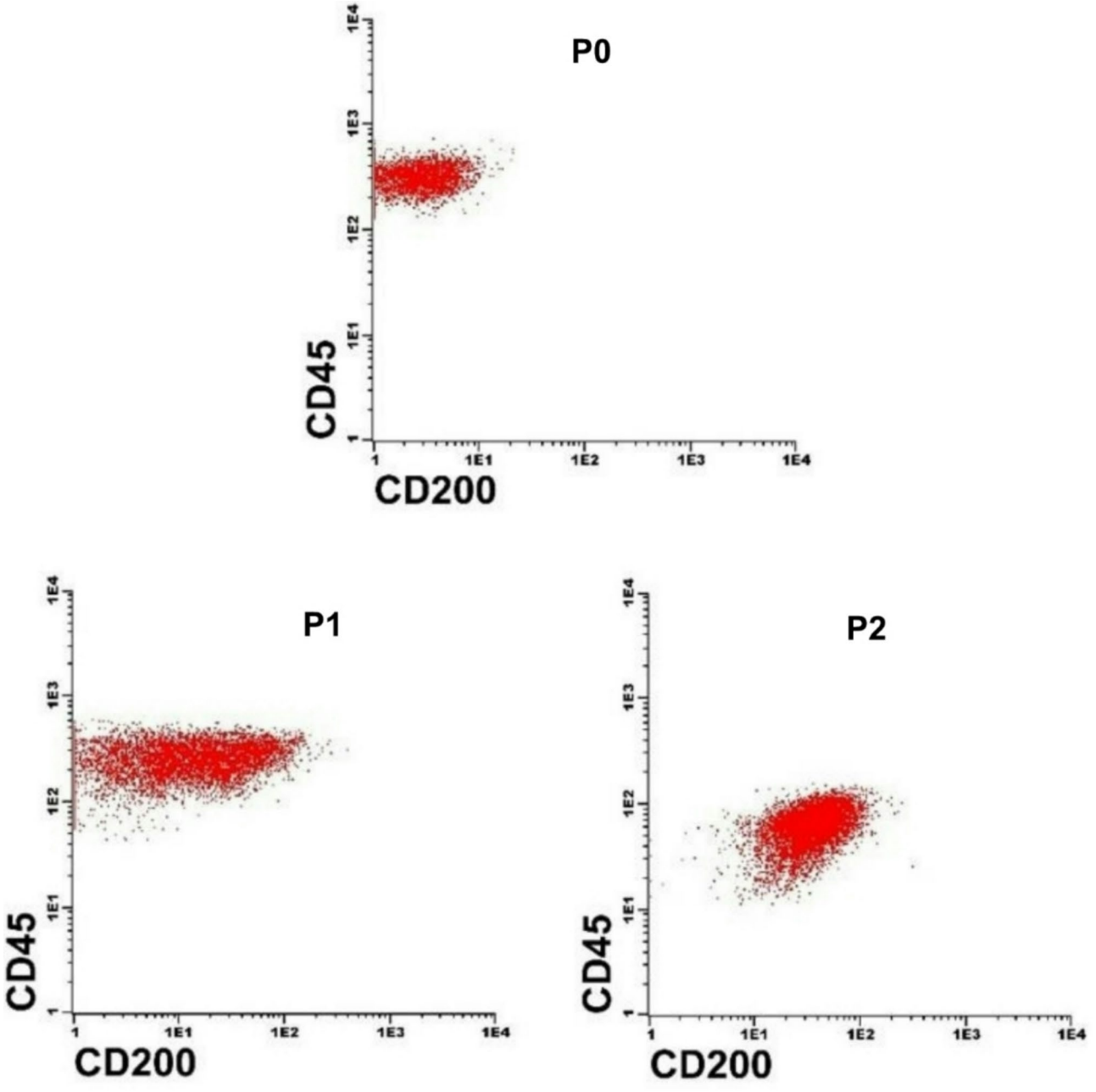

Fig. 1 CD200 positivity patterns. Arbitrary positivity patterns were established based on the presence of negative cells and MFI. Pattern 0 or negative (P0) 0–20% expression of CD200 in blast CD45 + gate; Pattern 1 or partially positive (P1) 20–50% expression of CD200; Pattern 2 or fully positive (P2) > 50% expression of CD200

Table 1 Markers associated with CD200 expression in AML

	Positive cases/total cases MFI ± SD*	Correlation coefficient with CD200 MFI	*p*-value
CD34+	127/431 166.7 ± 147.1	**0.650**	**< 0.001**
HLA-DR+	208/431 1312.9 ± 1321.4	0.502	**< 0.001**
CD13+	203/431 217.1 ± 546.4	0.492	**< 0.001**
CD117+	255/431 122.4 ± 90.5	0.488	**< 0.001**
CD71+	202/431 55.0 ± 117.9	0.456	**< 0.001**
CD123+	238/431 127.8 ± 108.4	0.428	**< 0.001**
CD38+	205/431 48.7 ± 35.7	0.402	**< 0.001**

AML, acute myeloid leukemia; *MFI ± SD, mean MFI of each marker in positive cases ± Standard derivation. MFI, mean fluorescence intensity. CD15+, CD7+, CD36+, CD64+, and CD33 + also showed statistically significant correlations; however, their correlation coefficients with CD200 MFI were all below 0.4

Gene sequencing using NGS

Next-generation sequencing of 42 genes was performed with a customized panel using HaloPlexHS (Agilent Technologies®) and MiSeq platform (Illumina®). Library preparation and sequencing were performed according to the manufacturer's instructions. The median reading depth was around 1000x, and the medium variant allele frequency (VAF) for variants was 5%. Only variants with a read depth > 100x and a minimum of 25 reads were analyzed. Pathogenic variants were classified using Varsome, COSMIC, ClinVar, PolyPhen2, and SIFT.

Real-time PCR

Total RNA was extracted from bone marrow or peripheral blood samples. One µg of RNA was retrotranscribed in a total reaction volume of 20 µl. Samples were incubated for 2 min at 37ºC, 10 min at 25ºC, 50 min at 37ºC, and 15 min at 70ºC.

*Meis*1, *Hox*A9, and *CD200* gene expression were monitored by quantitative real-time RT-PCR using the Assays on Demands on a QuantStudio 5 (Applied Biosystems, Foster City, CA, USA) and calculated using the DDCT method. PCR reactions were set up in MicroAmp optical 96-well reaction plates. After 2 min at 50ºC and 10 min at 95ºC, the amplification was carried out by 40 cycles at 95ºC for 15 s and 60ºC for 60 s. Each sample was analyzed in duplicate and normalized to the *ABL* levels, and a mix of two normal bone marrow samples was used as a calibrator.

RNA-Seq

cDNA was sequenced using the Illumina platform, obtaining ~ 34 to 46 million 75 bp paired-end reads per sample. Adapter sequences were trimmed with Trim Galore v.0.4.4. Raw sequencing reads in the fastq files were mapped with STAR (v.2.7.8) [24]. Gencode release 41 based on the GRCh38.p13 reference genome and the corresponding GTF file. The table of counts was obtained with feature Counts function in the package subread (v.2.0.3) [25]. Genes having more than 10 counts in 11 or more samples were kept to filter out lowly-expressed genes considering all samples (CEBPA and CEBPA_control).

Immune cell deconvolution was performed over the 13 AML cases with biallelic CEBPA with the CIBERSORT tool using as a reference the LM22 signature. CIBERSORTx was run with setting the permutations to 100. Expression data was imputed using TPMs considering all genes. Deconvolution was also performed over The Cancer Genome Atlas (TCGA) and BEAT2.0 cohorts. TCGA data was downloaded from cBioPortal [26–28], specifically, mRNA RSEM expression data from Acute Myeloid Leukemia (TCGA, PanCancer Atlas)

Table 2 CD200 expression is strongly associated with core-binding factor AML, CEBPA and RUNX1 mutations

N = 431	CD200 expression			%Positive cases	*p*-value
	P0	P1	P2		
	N	*N*	*N*		
FLT3	86	35	17	37.6%	ns
NPM1	118	21	7	19.1%	**< 0.001**
RUNX1	21	15	10	54.3%	**< 0.05**
RUNX1/RUNX1T1	3	11	2	81.3%	**< 0.001**
CBFB-MYH11	7	12	0	63.2%	**< 0.001**
IDH1/2	58	18	6	29.3%	ns
Biallelic CEBPA	0	4	4	100%	**< 0.001**
KMT2A	15	7	2	37.5%	ns
TP53	22	6	1	24.1%	ns
DAT					
DNMT3A	82	20	7	24.8%	ns
TET2	33	11	9	37.8%	ns
ASXL1/2	41	17	7	36.9%	ns
Spliceosome					
SF3B1	5	3	2	50.0%	ns
SRSF2	20	6	2	28.6%	ns
U2AF1	10	0	2	16.7%	ns
ZRSR2	4	3	0	42.9%	ns
WT1	15	9	4	46.4%	ns
GATA2	5	5	4	64.2%	**< 0.05**
ETV6	1	3	2	83.3%	**< 0.05**
PHF6	3	1	3	57.1%	**< 0.05**
ZBTZ7A	3	6	1	70.0%	**< 0.05**
Other alterations	219	73	26	31.1%	ns
No mutations	20	5	0	20.0%	ns

AML, acute myeloid leukemia; P0, pattern 0 = CD200 negative; P1, pattern 1 = CD200 positive; P2, pattern 2 = CD200 strong positive; DAT, DNMT3A, ASXL and TET2; ns, not significant. % CD200 positivity, included P2 and P3. No genetic data: 39 cases. Other alterations included: BCR-ABL1 (5), monoallelic CEBPA (12), TP53 (29), C-MYC (7), PTPN11 (25), RAD21 (16), CSF3R (10), CALR (5), NRAS (38), PML-RARA (5), CBL (12), KIT (18), NF1 (20), EZH6 (10), JAK2 (11), ANKRD26 (12), KDM6A (11), BCOR (21), DDX41 (8), PPM1D (6), MPL (6), SMC1A (7), KRAS (20), SETBP1 (1) and BRAF (3)

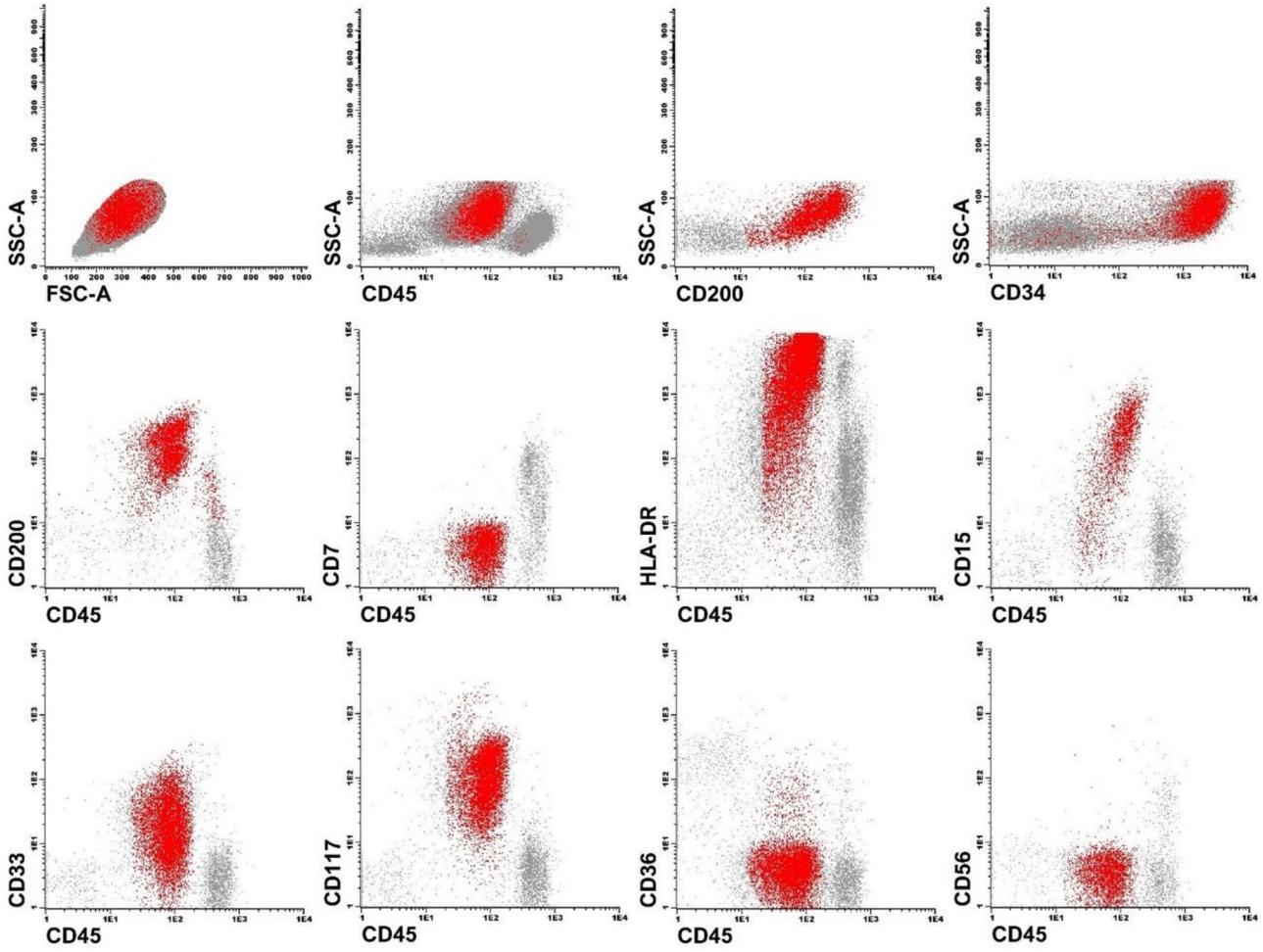

Fig. 2 CD200 expression in AML with CBF genetic lesions. Immunophenotype from one AML patient with a RUNX1/RUNX1T1 rearrangement (UPN 213) which is associated with P1 (partially positive), expressed **44.18%** CD200-positive cells. Red: Leukemic cells expressing CD200

was retrieved ($n = 173$). CEBPA mutation status was also retrieved com cBioportal. Any CEBPA status other than "*No alterations*" was considered as CEBPA-mutated. RSEM and mutation data merged led to a total of $n = 166$ samples. BEAT2.0 data was downloaded from Beat AML 2.0 project [29]. Specifically, normalized expression and clinical data were used. Only patients with expression data were used for the analysis ($n = 671$). CEBPA Biallelic variable (status "*bi*") was used to classify 19 patients as CEBPA mutated. CD200 expression was compared between CEBPA-mutated and CEBPA WT patients (Wilcoxon test). To study differences of cell type abundances between CEBPA-mutated and CEBPA WT a meta-analysis of the two cohorts was performed with the meta package in R (v6.2.1) using a random effects model using the mean difference as summary measure. *P*-values of the overall random effects models are reported for each cell type.

Weighted correlation network analysis (WGCNA) package (v.1.72-5) [30] was employed to identify clusters (modules) of highly correlated genes using the log2(CPM) for the CEBPA mutated samples. These modules were subsequently correlated with CD200 expression and CEBPA status (either mutated or control) respectively. The top 5000 most variable genes were used for the analysis. CD200 was added to the 5000 most variable genes as it was a gene of interest.

Gene network plots were constructed using the igraph package (v.1.6.0). Spearman's correlation was utilized to generate correlation matrices from the log2(CPM) expression values obtained from CEBPA patients ($n = 13$) and CEBPA_control patients ($n = 11$) independently. The analysis focused on genes within modules identified through WGCNA analysis that exhibited statistically significant correlations with CD200 expression ($n = 903$) solely in CEBPA patients. Additionally, CD200, CD200R1, and the downstream genes DOK2 and RASA1 from the CD200-CD200R pathway were included in the correlation analysis, despite not being part of the identified modules [18], resulting in a total of 907 genes. A

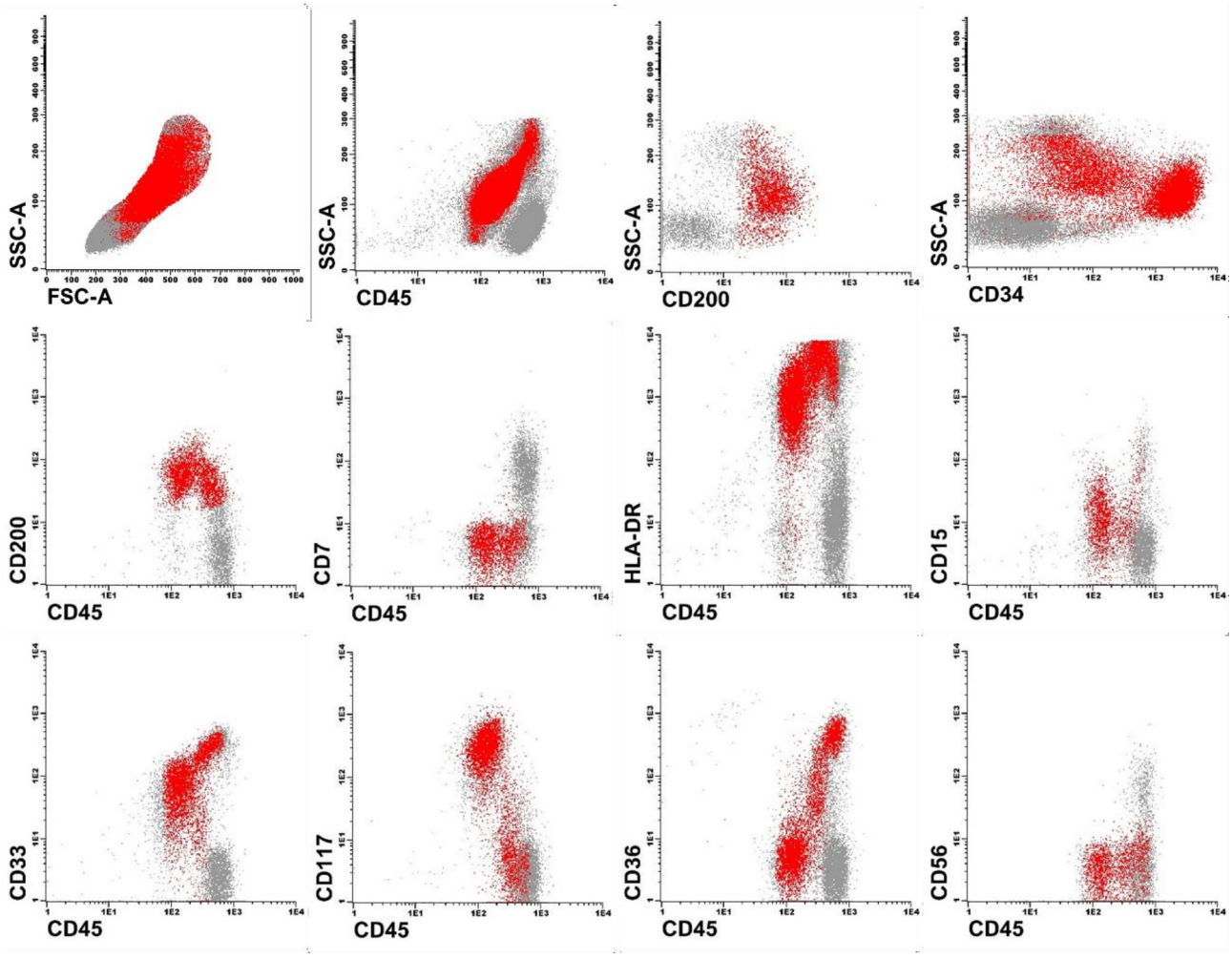

Fig. 3 CD200 expression in AML with CBF genetic lesions. Immunophenotype obtained from one AML patient with a CBFB-MYH11 rearrangement (UPN: 347) which is associated with P1 (partially positive), expressed **28.53%** CD200-positive cells. Red: Leukemic cells expressing CD200

correlation threshold of 0.75 was defined encompassing all genes. Only vertices (genes) connected to CD200 and/or CD200R1 were retained for visualization. Analyses were performed under R version 4.2.1.

Statistical methods

The Student's t-test compared quantitative variables, and categorical variables were compared using Chi-square (X2) or Fisher's exact test. Spearman coefficient correlation was performed to analyze markers related to CD200 with Bonferroni correction for multiple comparisons. Analysis was carried out using the statistical package (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). The One-way ANOVA followed by the Tukey HSP post-hoc test was employed to compare the gene expression levels.

Results

Immunophenotypic findings

66% of patients with AML expressed CD200. The immunophenotype analysis of the 431 cases showed that CD200 antigen was significantly associated whit CD34, CD117, HLA-DR, CD33, CD123, CD15, CD7, CD71, CD38, CD13 with a p-value < 0.001, and with CD36 and CD64 with a p-value < 0.05 (Table 1).

Next, we tried to establish correlations between CD200 positivity and genetic lesions in the AML group. (Table 2 and Suppl. Figure 1.1–1.5).

The strongest correlation corresponded to chimeric fusions at *RUNX1/RUNX1T1* and *CBFB-MYH11* and biallelic *CEBPA* mutations. Other mutations commonly associated with these categories, such as genetic lesions in *RUNX1*, *GATA2*, *PHF6*, *ETV6*, and *ZBTB7A*, were also associated with CD200 expression with p-values of < 0.05.

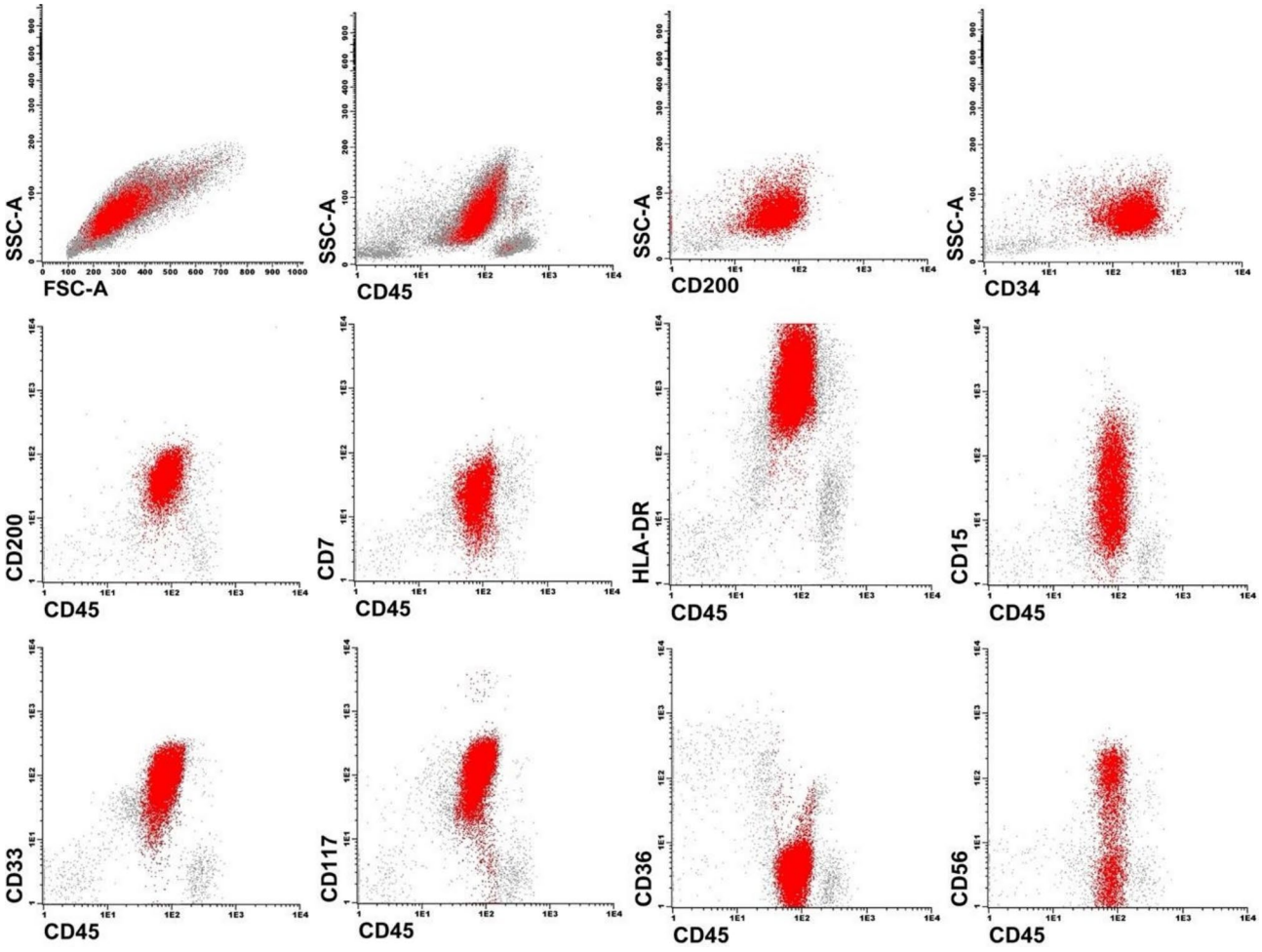

Fig. 4 CD200 expression in AML with biallelic *CEBPA* mutation. Immunophenotype from one AML patient with biallelic *CEBPA* mutation (UPN: 102; c.68delC // P23fsX137) which is associated with P2 (fully positive), expressed **80.08%** CD200-positive cells. Red: Leukemic cells expressing CD200

Thirteen out of 16 patients with the *RUNX1/RUNX1T1* rearrangement were CD200+, with a predominant pattern 1 of CD200 expression. Moreover, 56% were CD34+, 87% were HLA-DR+, 75% CD33+, 93% were CD117+, 62% were CD15, four cases were CD56 + and only 1 case was CD7 + and CD36+ (Fig. 2).

Most *CBFB-MYH11* cases (12/19) were also CD200+, most having a pattern 1. 63% of the cases with CBFB-MYH11 were HLA-DR + and CD33+, 73% were CD117+, 52% were CD15+, seven cases were CD34+, four cases were CD36+, and two cases were CD7+. No single case was CD56+ (Fig. 3).

All the AML cases with biallelic *CEBPA* mutations showed positivity of CD200+, four with a pattern 1, and the remaining 4 cases with a pattern 2. All eight cases also highly expressed HLA-DR, CD33, and CD117. CD7 was expressed in seven of the eight samples. CD15 and CD34 were expressed in six of the eight samples. Two samples expressed CD36, and only one expressed CD15. The presence of CD200 was correlated with CD117, CD33, CD15, HLA-DR ($p < 0.001$) and CD7 ($p < 0.05$) (Fig. 4 and Suppl. Figure 2.1–2.7).

The lowest CD200 reactivity was found in the *NPM1* group, with only 19.1% showing CD200 positivity. Most *NPM1* mutated cases were also CD34- but it has been suggested that those cases with CD34 + positivity may represent a subgroup with a larger LSC compartment given that CD34 + NPM1 + cells can repopulate

Table 3 Bone marrow lymphoid ratios and leukemic CD200 positivity

	N **= 486**	**Correlation coefficient with CD200**	***p*-value**
CD4/CD8		-0.172	**< 0.001**
> 1	357/486		
< 1	132/486		
NK		-0.150	**< 0.001**
> 10% (High NK)	467/486		
< 10% (Low NK)	22/486		

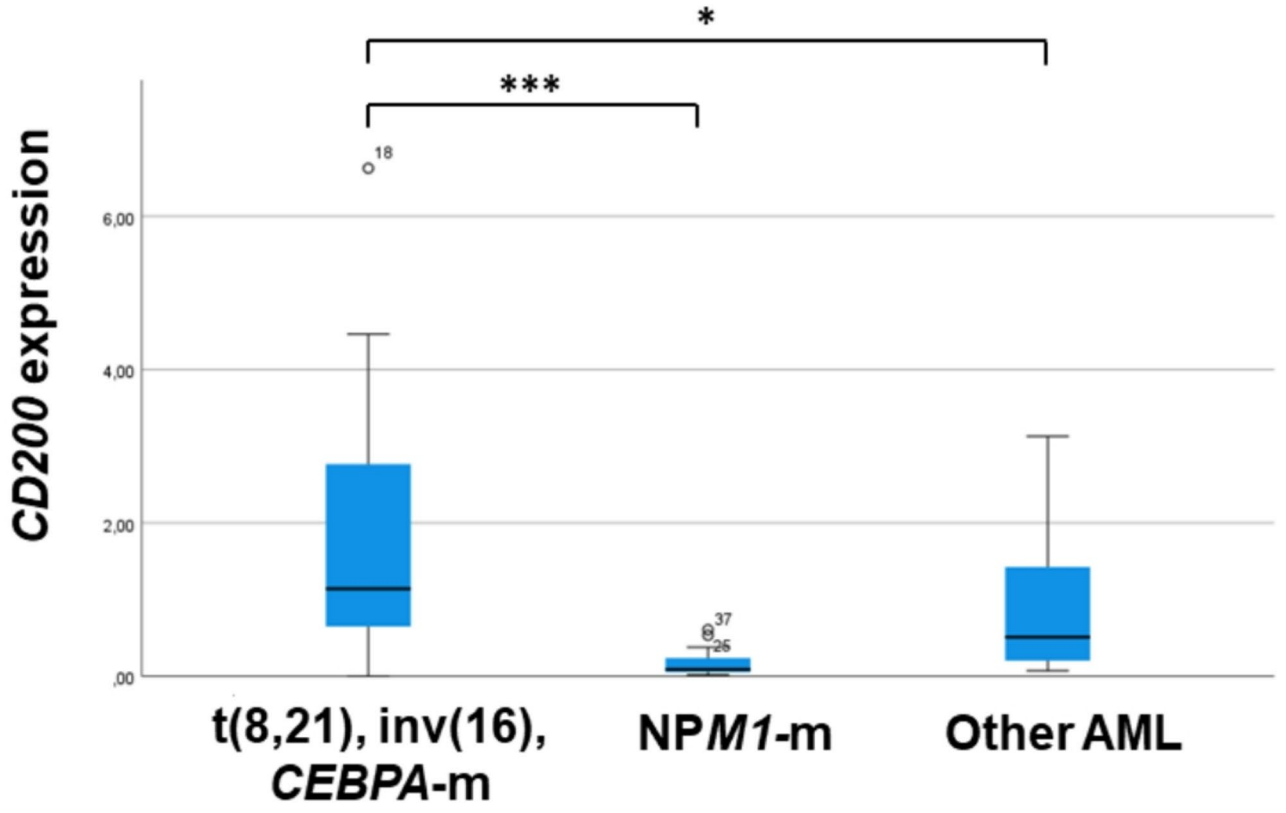

Fig. 5 CD200 expression by RT-PCR. CD200 expression comparing 3 groups: t(8,21), inv(16) and CEBPA-m vs. NPM1-m vs. Other AML. $p < 0.001$ (***); $p < 0.05$ (*)

immunodeficient mice [31]. *NPM1* + AML cases with high *FLT3*-ITD allelic ratios expressed more commonly CD34 and CD200 than the remaining *NPM1* AML (Suppl. Table 2).

We assessed the lymphoid marrow populations using CD2, CD3, CD4, and CD8. If the CD4/CD8 ratio was > 1, we assumed a helper predominance, whereas cases with less than < 1 were considered cytotoxic dominant. The difference between CD2 and CD3 estimated the presence of Natural Killer (NK) cells. Most patients had more than 10% of CD2 + CD3-lymphocytes in the bone marrow. Leukemic CD200 expression was correlated with a lower percentage of T-Helper and NK lymphoid cells (Table 3), as it could be the case of an active immunosuppression mediated by the CD200-CD220R loop.

RNA analysis

CD200 expression was significantly higher in the t(8,21), inv(16) and *CEBPA*-m group than in *NPM1*-m and Other AML ($p < 0.001$ and $p < 0.05$ respectively, (Fig. 5). Down-regulation of *Meis1* and *HoxA9* expression in t(8,21), inv(16) and *CEBPA*-m group compared with *NPM1*-m and Other AML groups supports the observed findings on gene expression analysis (Suppl. Figure 3.1–3.2).

We were interested in the lymphoid populations observed in AML cases with CEBPA mutations, given that this category typically overexpresses CD200. Indeed, CD200 expression was higher in CEBPA-mutated patients than WT in external cohorts (Fig. 6A). We applied deconvolution protocols to investigate the immune cells present in CEBPA-mutated AML cases and in TCGA-LAML and BEAT data (Fig. 6B). Deconvolution showed similar overall cell type composition between cohorts. Cell abundances were compared between CEBPA-mutated and CEBPA WT patients performing a meta-analysis of both cohorts. Our CEBPA-mutated AML cases were not included in this comparison as no WT group was available. Random effect models showed statistical differences between CEBPA-mutated and WT patients of B cells memory ($p = 3.53e\text{-}5$) and dendritic cells activated ($p = 0.0117$) proportions. B cells naive ($p = 0.0867$) and monocytes ($p = 0.074$) showed a trend for significance (Fig. 6C and Suppl. Figure 4.1). Interestingly, CD200 expression correlated with B cells naïve abundance only in WT patients (TCGA $R = 0.3$, $p = 0.00018$, BEAT $R = 0.26$, $p = 9.1e-12$) whereas it correlated with B cells memory only in CEBPA mutated patients (TCGA $R = 0.57$, $p = 0.067$; $R = 0.38$, $p = 0.11$) (Fig. 6D). CD200 expression correlation with dendritic cells was weak (TCGA $R = 0.2$, $p = 0.013$, BEAT $R = -0.062$, $p = 0.12$) and monocytes showed a negative correlation in WT patients (Suppl. Figure 4.2). Despite not establishing a

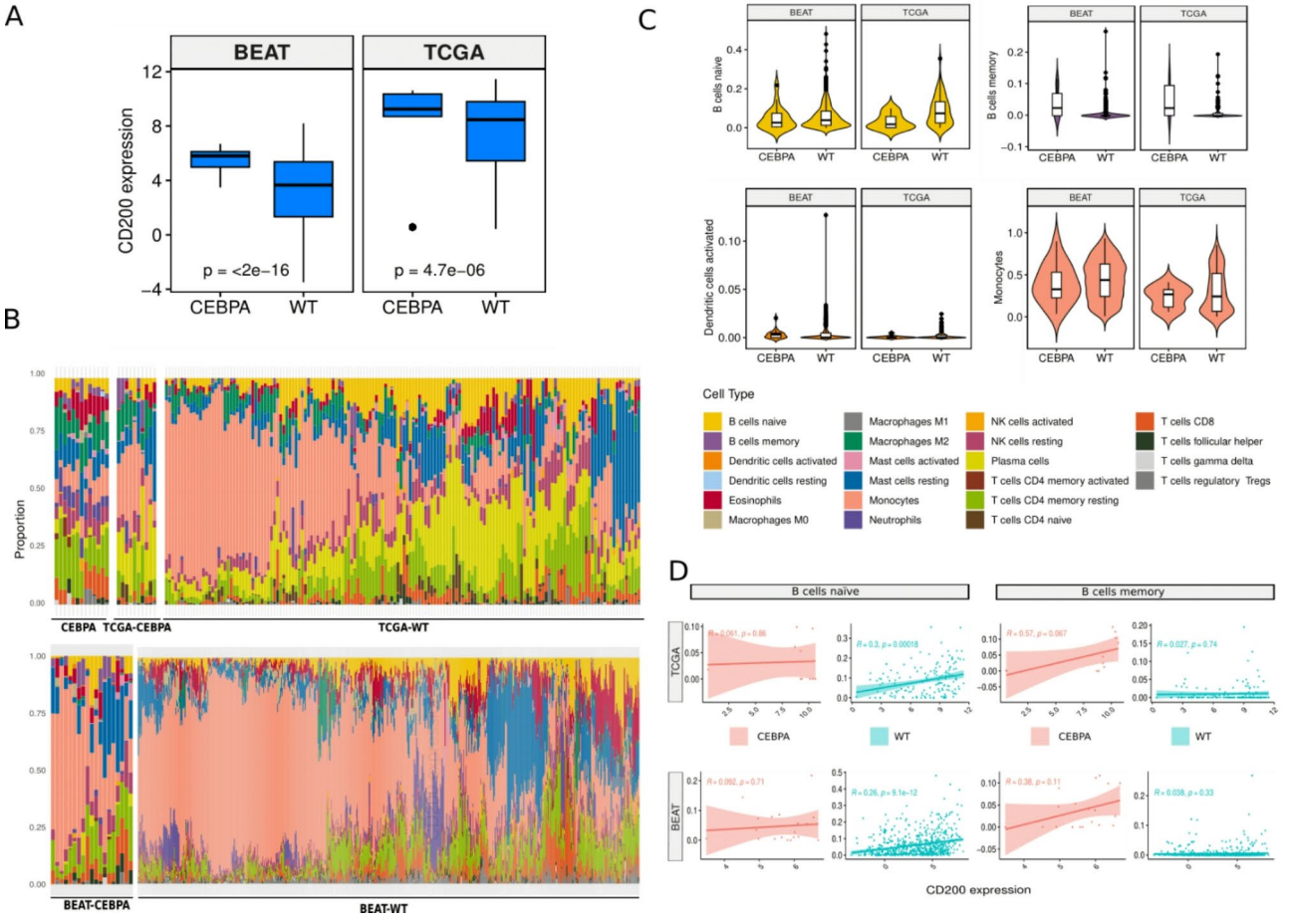

Fig. 6 Immune cell deconvolution of AML biallelic *CEBPA* patients, TCGA-AML and BEAT patients. **(A)** CD200 expression (log2 normalized) in CEBPA mutated patients and WT patients in BEAT and TCGA cohorts. *P*-values from Wilcoxon rank sum test. **(B)** Deconvolution results of the RNA-seq data from 13 AML cases with biallelic CEBPA (top left panel), TCGA-AML CEBPA-mutated patients (top center panel) and TCGA-AML CEBPA WT (top right panel), BEAT CEBPA-mutated patients (bottom left panel) and BEAT CEPBA-WT (bottom right panel). Cell type proportions are shown in the y axis. Samples were clustered with euclidean distance and complete method. **(C)** Cell types with significantly different proportions between CEBPA-mutated and CEBPA-WT in the meta-analysis of the TCGA and BEAT cohorts. Random effects models *p*-value: B cells naive (*p*-value = 0.0867), B cells memory (*p*-value = 3.53E-5), dendritic cells activated (*p* = 0.0117) and Monocytes (*p*-value = 0.074). Meta-analysis plots can be found in Suppl. Figure 4.1. **(D)** Correlation of CD200 expression (log2 normalized) with B cells naïve and memory. Pearson correlation coefficient (R) and *p*-values are shown separately for CEBPA mutated and WT patients

clear predominant cell population, uneven distribution of lymphoid cells may be used to analyze leukemic-immune interactions. We also checked the CD200 regulatory pathways in these samples. We found that the inhibitory effects of CD200 on lymphoid cells could be mediated through CD200R1 and RASA1. This protein behaves as a RAS inhibitor and allows control of cellular proliferation and differentiation. At the same time, CD200 overexpression in CEBPA leukemic cells was inversely correlated with PNMA3, this protein shares homology with retroviral Gag proteins, MMP19, a protein that plays a major role in the breakdown of extracellular matrix, and ARHGAP22, an insulin-dependent protein which regulates cell motility (Fig. 7).

Discussion

In this work, we consecutively analyzed the expression of CD200 in a series of 431 patients with AML. We showed that 66% of AML patients unequivocally expressed CD200, and its expression was significantly associated with simultaneous CD34 reactivity. We also identified the molecular subgroups with the highest probability of CD200 positivity: cases with *RUNX1/RUNX1T1*, *CBFB-MHY11* rearrangements, and AML with biallelic *CEBPA* mutations. Conversely, we found the lowest CD200 in AML with *NPM1* mutations. However, in this AML group, CD200 + was related to *NPM1*+/*FLT3*-ITD^{high} ratios, suggesting that this marker could indicate an enlarged leukemic stem cell compartment and be a surrogate marker of a bad outcome.

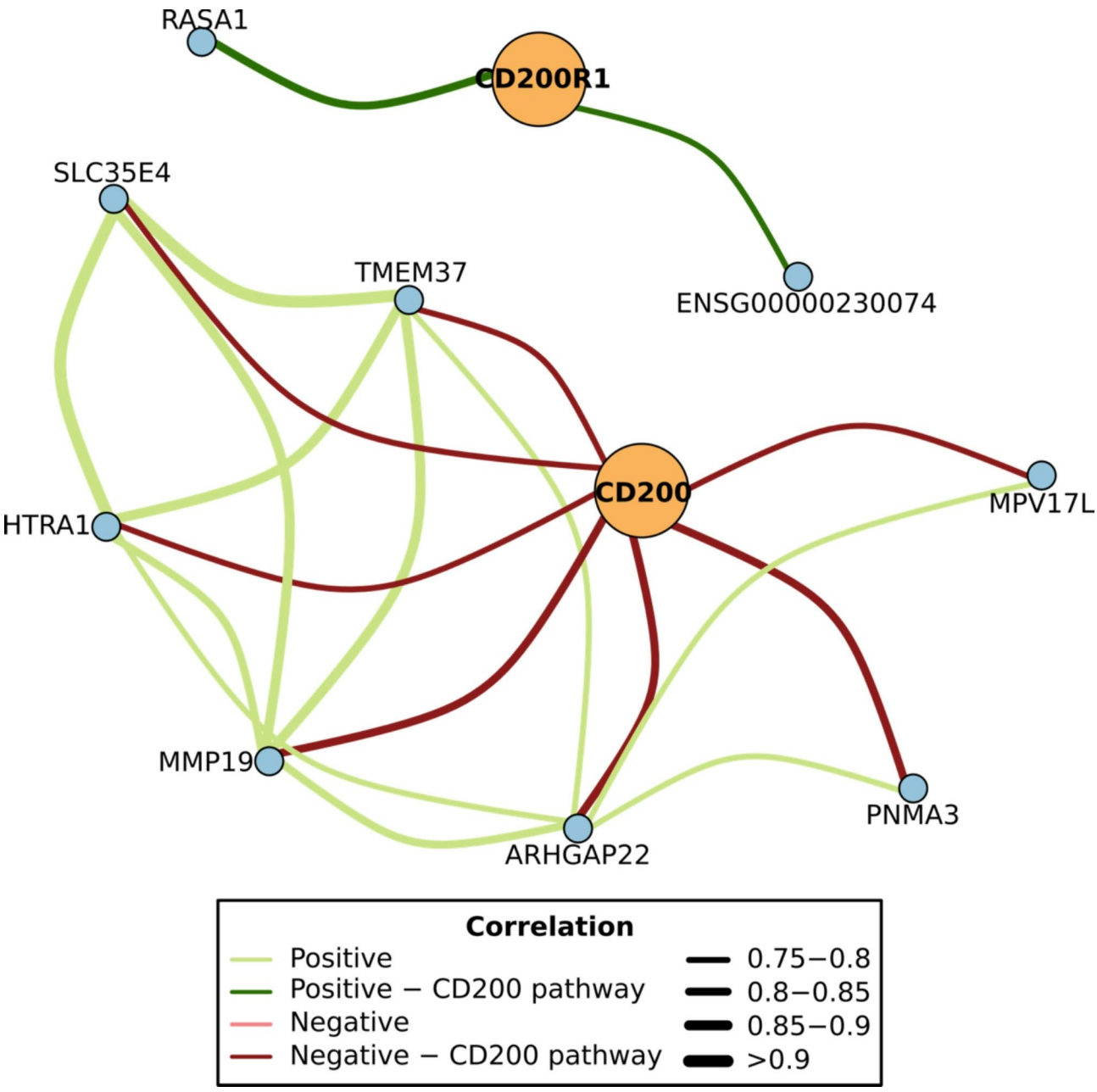

Fig. 7 Pathway analysis on bulk RNAseq experiments from CEBPA mutated AML. Gene network plot of CD200-CD200R1 related genes. Genes analyzed were previously identified as WGCNA modules that significantly correlated with CD200 expression in CEBPA mutated patients. CD200, CD200R1, DOK2 and RASA1 from the CD200-CD200R pathway were included in the correlation analysis. Only genes connected to CD200 and/or CD200R1 were retained for visualization. A correlation threshold of 0.75 was set for the network

Regarding CD200 in AML, we found that two-thirds of cases were positive. Damiani D. et al. [16] analyzed a cohort of 244 patients, finding that CD200 was expressed in 136 out of 244 (56%). Tribelli M. et al. [32] CD200 was expressed in 67/139 patients (48%). In both studies and in line with our findings, CD200 was most frequently expressed in CD34-positive blast cells.

We established correlations between CD200 expression and molecular findings. The most remarkable association was with core-binding factor molecular alterations. The study of Tonks A. et al. [33], using mainly transcriptomic data, reported that, in AML, there was a correlation between CD200 expression and the presence of core-binding factor-associated abnormalities such as t(8;21) and inv(16) ($p = 0.0001$). Coustan-Smith E. et al. [34] analyzed 370 bone marrow samples from patients with de novo or secondary AML and found that CD200 may be helpful to MRD studies. CD200 was significantly

overexpressed in patients with RUNX1/RUNX1T1 alterations in this mainly pediatric cohort. The survey of Ho JM. et al. [14], with a series of 65 AML patients, also showed that 5/65 patients presented *RUNX1* mutation, and four out of the five had high levels of CD200 expression (more than 86% of the total of myeloblasts). Herein, we add biallelic *CEBPA* mutation as typically CD200 overexpressing categories [35]. Our findings suggest that the immunophenotypic pattern at diagnosis (CD7, CD34, CD117, CD33, CD123), including CD200 (bright positivity), may be a reliable way to identify AML with biallelic *CEBPA* mutation. The study of Dentesano G. et al. [36] reported a relationship between the CD200 and *CEBPB* in microglial cells and suggested that CEBPB could regulate the expression of CD200; it remains to be tested if *CEBPA* is also involved in regulating CD200 in hematopoietic cells.

We have seen that AML patients with *NPM1* mutation have lower CD200 expression levels than other genetic alterations [37–38]. It is known that most NPM1 mutated AML are CD34 negative [39–40]; the association between CD200 and CD34 could explain why both antigens are low in *NPM1* mutated AML. *FLT3*-ITD mutations are subclonal events that provide an adverse prognosis, especially *in NPM1* + patients with high *FLT3*-ITD allelic ratios (> 0.5). These patients have been included in the ELN high-risk group [41–42]. CD200 differentiated a subgroup of 27 AML patients with *NPM1+/FLT3*-ITD^{high} ratios in our study. Tribelli M. et al. [32] showed that CD200 expression identified a group *NPM1+/FLT3*-ITD- ($n = 37$) characterized by poor prognosis. These observations suggest that CD200 reactivity needs to be tested in *NPM1* mutated AML cases.

Several studies have described the immunosuppressive effects through the CD200/CD200R signal pathway in solid cancers and hematological malignancies [43–44]. In our research, we suggest the role of CD200 by inhibiting NK cell activation and cytotoxic T-cell functions in AML patients. We detected a potential inhibitory loop on lymphoid populations mediated by RASA1, but these findings need to be confirmed by additional experiments.

Furthermore, we validate our cytometry results by analyzing 60 more AML patients with RT-PCR, and our results confirm the relation between CD200 expression and core-binding factors and the inverse correlation between CD200 expression and NPM1 mutation [45].

Our study has some limitations. Most of our population corresponded to adults, so the meaning CD200 in pediatric patients needs to be clarified. Also, we have yet to study the clinical outcomes of AML patients.

The discovery that CD200 plays a vital role in human neoplasia prompted the use of therapies to block the CD200-CD200R binding and suppress the overexpression of this antigen in leukemia. The study of Rastogi N. et al. [46] proposed a fully human anti-CD200 antibody (TTI-CD200) that can block the interaction of CD200 with its receptor and restore AML immune responses *in vitro and in vivo.* Another study [47] suggested that a recombinant humanized monoclonal antibody called Samalizumab targeted CD200 and was associated with reduced tumor burden in advanced CLL. It remains to be tested if these therapeutic tools help target the AML molecular subgroups with high CD200 expression.

In conclusion, CD200 is a valuable addition to a flow cytometry marker panel and is commonly expressed in AML patients, especially those with core-binding factor alterations. Its upregulation in some categories may suggest that it may be an indirect measure of the leukemic stem cell size or the development of inhibitory immune mechanisms. Further studies are needed to fully understand the prognostic role of CD200 in AML, its association with clonal evolution, and its effects on immunoregulatory cells.

Supplementary Information

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Supplementary Material 1

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Author contributions
LGG, HC, CM and JFN designed the study. LGG, HC, CM, NG, FG, NL, MP, PB, JP, LZ, MM, IG, AS, AGG, SV, MT, MA, NV and JFN obtained clinical and biological data. LGG, HC, NG and JFN wrote the manuscript with input from all authors. The study received approval from the CEIC (Ethical Committee number IIBSP-LEU-2021-120).

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study received approval from the CEIC (Ethical Committee number IIBSP-LEU-2021-120).

Consent for publication
The authors consent to the publication of this document.

Competing interests
The authors declare no competing interests.

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References

1. Döhner H, Wei AH, Appelbaum FR, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. Blood. 2022;140(12):1345–77. https://doi.org/10.1182/blood.2022016867.
2. George TI, Bajel A. Diagnosis of rare subtypes of acute myeloid leukaemia and related neoplasms. Pathology. 2021;53:312–27. https://doi.org/10.1016/j.pathol.2021.02.001.
3. Thomas D, Majeti R. Biology and relevance of human acute myeloid leukemia stem cells. Blood. 2017;129:1577–85. https://doi.org/10.1182/blood-2016-10-696054.
4. Hanekamp D, Cloos J, Schuurhuis GJ. Leukemic stem cells: identification and clinical application. Int J Hematol. 2017;105:549–57. https://doi.org/10.1007/s12185-017-2221-5.
5. Roth CG, Garner K, Eyck S, Ten, et al. TIM3 expression by leukemic and non-leukemic myeloblasts. Cytom Part B - Clin Cytom. 2013;84 B:167–72. https://doi.org/10.1002/cyto.b.21080.
6. Kikushige Y, Miyamoto T, Yuda J, et al. A TIM-3/Gal-9 autocrine stimulatory loop drives Self-Renewal of human myeloid leukemia stem cells and leukemic progression. Cell Stem Cell. 2015;17:341–52. https://doi.org/10.1016/j.stem.2015.07.011.
7. Gonçalves Silva I, Yasinska IM, Sakhnevych SS, et al. The Tim-3-galectin-9 secretory pathway is involved in the immune escape of human acute myeloid leukemia cells. EBioMedicine. 2017;22:44–57. https://doi.org/10.1016/j.ebiom.2017.07.018.
8. Dama P, Tang M, Fulton N, et al. Gal9/Tim-3 expression level is higher in AML patients who fail chemotherapy. J Immunother Cancer. 2019;7:1–7. https://doi.org/10.1186/s40425-019-0611-3.
9. Zahran AM, Mohammed Saleh MF, Sayed MM, et al. Up-regulation of regulatory T cells, CD200 and TIM3 expression in cytogenetically normal acute myeloid leukemia. Cancer Biomarkers. 2018;22:587–95. https://doi.org/10.3233/CBM-181368.
10. CD200 Gene. - GeneCards| OX2G Protein| OX2G Antibody. https://www.genecards.org/cgi-bin/carddisp.pl?gene=CD200#protein_expression. Accessed 17 Feb 2021.
11. Wright GJ, Cherwinski H, Foster-Cuevas M, et al. Characterization of the CD200 receptor family in mice and humans and their interactions with CD200. J Immunol. 2003;171:3034–46. https://doi.org/10.4049/jimmunol.171.6.3034.
12. Barclay AN, Wright GJ, Brooke G, et al. CD200 and membrane protein interactions in the control of myeloid cells. Trends Immunol. 2002;23(6):285–90. https://doi.org/10.1016/s1471-4906(02)02223-8.
13. Coles SJ, Hills RK, Wang ECY, et al. Increased CD200 expression in acute myeloid leukemia is linked with an increased frequency of FoxP3 Þ regulatory T cells. Leukemia. 2012;26:2146–8. https://doi.org/10.1038/leu.2012.75.
14. Ho JM, Dobson SM, Voisin V, et al. CD200 expression marks leukemia stem cells in human AML. Blood Adv. 2020;4:5402–13. https://doi.org/10.1182/bloodadvances.2020001802.
15. Coles SJ, Hills RK, Wang ECY, et al. Expression of CD200 on AML blasts directly suppresses memory T-cell function. Leukemia. 2012;26:2148–51. https://doi.org/10.1038/leu.2012.77.
16. Damiani D, Tiribelli M, Raspadori D, et al. Clinical impact of CD200 expression in patients with acute myeloid leukemia and correlation with other molecular prognostic factors. Oncotarget. 2015;6:30212–21. https://doi.org/10.18632/oncotarget.4901.
17. Coles SJ, Wang ECY, Man S, et al. CD200 expression suppresses natural killer cell function and directly inhibits patient anti-tumor response in acute myeloid leukemia. Leukemia. 2011;25:792–9. https://doi.org/10.1038/leu.2011.1.
18. Ngwa C, Liu F. CD200-CD200R signaling and diseases: a potential therapeutic target? Int J Physiol Pathophysiol Pharmacol. 2019;11:297–309.
19. Nomdedéu JF, Mateu R, Altès A, et al. Enhanced myeloid specificity of CD 117 compared with CD13 and CD33. Leuk Res. 1999;23:341–7. https://doi.org/10.1016/S0145-2126(98)00185-4.
20. Wong KK, Brenneman F, Chesney A, et al. Soluble CD200 is critical to engraft chronic lymphocytic leukemia cells in immunocompromised mice. Cancer Res. 2012;72:4931–43. https://doi.org/10.1158/0008-5472.CAN-12-1390.
21. Mora A, Bosch R, Cuellar C, et al. CD200 is a useful marker in the diagnosis of chronic lymphocytic leukemia. Cytom Part B - Clin Cytom. 2019;96:143–8. https://doi.org/10.1002/cyto.b.21722.
22. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129:424–47. https://doi.org/10.1182/blood-2016-08-733196.
23. Matutes E, Pickl WF, Van't Veer M, et al. Mixed-phenotype acute leukemia: clinical and laboratory features and outcome in 100 patients defined according to the WHO 2008 classification. Blood. 2011;17(11):3163–71. https://doi.org/10.1182/blood-2010-10-314682.
24. Dobin A, Davis CA, Schlesinger, et al. STAR: ultrafast universal RNA-seq aligner. Bioinf (Oxford England). 2013;29(1):15–21. https://doi.org/10.1093/bioinformatics/bts635.
25. Yang Liao GK, Smyth W, Shi. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. Nucleic Acids Res. 2019;47(8):e47. https://doi.org/10.1093/nar/gkz114
26. Cerami E, Gao J, Dogrusoz, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012;2(5):401–4. https://doi.org/10.1158/2159-8290.CD-12-0095.
27. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013;6(269):pl1. https://doi.org/10.1126/scisignal.2004088.
28. De Bruijn I, Kundra R, Mastrogiacomo B, et al. Cancer Res. 2023;83(23):3861–7. https://doi.org/10.1158/0008-5472.CAN-23-0816. Analysis and Visualization of Longitudinal Genomic and Clinical Data from the AACR Project GENIE Biopharma Collaborative in cBioPortal.
29. Bottomly D, Long N, Schultz, et al. Integrative analysis of drug response and clinical outcome in acute myeloid leukemia. Cancer Cell. 2022;40(8):850–e8649. https://doi.org/10.1016/j.ccell.2022.07.002.
30. Langfelder P, Horvath SWGCNA. An R package for weighted correlation network analysis. BMC Bioinformatics. 2008;9:559. https://doi.org/10.1186/1471-2105-9-559.
31. Martelli MP, Pettirossi V, Thiede, et al. CD34 + cells from AML with mutated NPM1 harbor cytoplasmic mutated nucleophosmin and generate leukemia in immunocompromised mice. Blood. 2010;116(19):3907–22. https://doi.org/10.1182/blood-2009-08-238899.
32. Tiribelli M, Geromin A, Cavallin M, et al. ABCG2 and CD200 define patients at high risk of relapse in ELN favorable subgroup of AML. Eur J Haematol. 2017;99:269–74. https://doi.org/10.1111/ejh.12915.
33. Tonks A, Hills R, White P, Rosie B, Mills KI, RD. CD200 as a prognostic factor in acute myeloid leukaemia. Leukemia. 2007;21:566–8. https://doi.org/10.1038/sj.leu.2404559.
34. Coustan-Smith E, Song G, Shurtleff S, et al. Universal monitoring of minimal residual disease in acute myeloid leukemia. JCI Insight. 2018;3:1–14. https://doi.org/10.1172/jci.insight.98561.
35. Hoyos M, Nomdedeu JF, Esteve J, et al. Core binding factor acute myeloid leukemia: the impact of age, leukocyte count, molecular findings and minimal residual disease. Eur J Haematol. 2013;91:209–18. https://doi.org/10.1111/ejh.12130.
36. Dentesano G, Straccia M, Ejarque-Ortiz A, et al. Inhibition of CD200R1 expression by C/EBP beta in reactive microglial cells. J Neuroinflammation. 2012;9:1–13. https://doi.org/10.1186/1742-2094-9-165.
37. Papaemmanuil E, Ph D, Gerstung M et al. (2016) Europe PMC funders group genomic classification and prognosis in acute myeloid leukemia. 374:2209–21. https://doi.org/10.1056/NEJMoa1516192.Genomic
38. Nomdedeu J, Bussaglia E, Villamor N, et al. Immunophenotype of acute myeloid leukemia with NPM mutations: prognostic impact of the leukemic compartment size. Leuk Res. 2011;35:163–8. https://doi.org/10.1016/j.leukres.2010.05.015.
39. Taussig DC, Vargaftig J, Miraki-moud F et al. (2016) Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34 ⧗ fraction. 115:1976–85. https://doi.org/10.1182/blood-2009-02-206565.The
40. Alcalay M, Ph D, Rosati R, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3):254–66. https://doi.org/10.1056/NEJMoa041974.
41. Daver N, Schlenk RF, Russell NH, Levis MJ. Targeting FLT3 mutations in AML: review of current knowledge and evidence. Leukemia. 2019;33:299–312. https://doi.org/10.1038/s41375-018-0357-9.
42. Pratcorona M, Brunet S, Nomdedéu J, et al. Favorable outcome of patients with acute myeloid leukemia harboring a low-allelic burden FLT3-ITD mutation and concomitant NPM1 mutation: relevance to post-remission therapy. Blood. 2013;121:2734–8. https://doi.org/10.1182/blood-2012-06-431122.

43. Nip C, Wang L, Liu C. CD200/CD200R: bidirectional role in Cancer progression and immunotherapy. Biomedicines. 2023;11. https://doi.org/10.3390/biomedicines11123326.
44. Oda SK, Daman AW, Garcia NM, Wagener F, Schmitt TM, Tan X, Chapuis AG, Greenberg PD. A CD200R-CD28 fusion protein appropriates an inhibitory signal to enhance T-cell function and therapy of murine leukemia. Blood. 2017;130:2410–9. https://doi.org/10.1182/blood-2017-04-777052.
45. Nagy Á, Ősz Á, Budczies J, et al. Elevated HOX gene expression in acute myeloid leukemia is associated with NPM1 mutations and poor survival. J Adv Res. 2019;20:105–16. https://doi.org/10.1016/j.jare.2019.05.006.
46. Rastogi N, Baker S, Man S, et al. Use of an anti-CD200-blocking antibody improves immune responses to AML in vitro and in vivo. Br J Haematol. 2021;193:155–9. https://doi.org/10.1111/bjh.17125.
47. Mahadevan D, Lanasa MC, Farber C, et al. Phase i study of Samalizumab in chronic lymphocytic leukemia and multiple myeloma: Blockade of the immune checkpoint CD200. J Immunother Cancer. 2019;7:1–13. https://doi.org/10.1186/s40425-019-0710-1.

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