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ORIGINAL RESEARCH

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NPM1 and *DNMT3A* mutations are associated with distinct blast immunophenotype in acute myeloid leukemia

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ABSTRACT

The immune system is important for elimination of residual leukemic cells during acute myeloid leukemia (AML) therapy. Anti-leukemia immune response can be inhibited by various mechanisms leading to immune evasion and disease relapse. Selected markers of immune escape were analyzed on AML cells from leukapheresis at diagnosis (N = 53). Hierarchical clustering of AML immunophenotypes yielded distinct genetic clusters. In the absence of *DNMT3A* mutation, *NPM1* mutation was associated with decreased HLA expression and low levels of other markers (CLIP, PD-L1, TIM-3). Analysis of an independent cohort confirmed decreased levels of HLA transcripts in patients with *NPM1* mutation. Samples with combined *NPM1* and *DNMT3A* mutations had high CLIP surface amount suggesting reduced antigen presentation. TIM-3 transcript correlated not only with TIM-3 surface protein but also with CLIP and PD-L1. In our cohort, high levels of TIM-3/PD-L1/CLIP were associated with lower survival. Our results suggest that AML genotype is related to blast immunophenotype, and that high TIM-3 transcript levels in AML blasts could be a marker of immune escape. Cellular pathways regulating resistance to the immune system might contribute to the predicted response to standard therapy of patients in specific AML subgroups and should be targeted to improve AML treatment.

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Introduction

The most important challenge in the treatment of acute myeloid leukemia (AML) is associated with frequent disease relapses after chemotherapy. The curative potential of allogeneic bone marrow transplantation indicates that a functional immune system is able to eradicate residual leukemia cells. However, similar to solid tumors, evidence of immune system impairment is available for AML.[1–6] Immunotherapy could complement the current treatment strategies in AML. The possible modalities include blocking antibodies against inhibitory receptors, bispecific antibodies, adoptive T-cell or natural killer (NK) cell therapy, or administration of T-cells with chimeric antigen receptors (CAR T-cells).[6–8]

In this study, we analyzed selected surface markers of different mechanisms involved in AML blast resistance to the immune system: (i) The inhibitory receptors PD-L1 and PD-L2 bind to PD-1 on cytotoxic T-lymphocytes and prevent target cell lysis. Blocking antibodies against these so-called checkpoint inhibitors are in clinical trials for many tumor types including AML, usually in combination with other treatment.[9] (ii) Reduced HLA expression impairs antigen-specific recognition of tumor cells by T-cells. (iii) The invariant chain peptide CLIP, which can be present on HLA molecules in place of antigens, indicates defective antigen presentation. In AML, CLIP presentation on the surface of leukemia blasts was related to worse outcome,[10] and CLIP down-modulation enhanced the immunogenicity of myeloid leukemic blasts and resulted in increased CD4+ T-cell responses.[11] (iv) TIM-3, a marker of exhausted T-cells,[12] is often present on AML blasts[13] but its function in leukemia cells is not clear. TIM-3 enhances leukemia cell proliferation and resistance to apoptosis.[14] It is also secreted in complex with galectin-9, which inhibits the activity of T-cells.[15] Sabatolimab, an antibody targeting TIM-3, is currently tested for AML treatment (trial NCT03066648). (v) CD47 provides a "don´t-eat-me" signal to macrophages, and its presence on AML blasts was associated with worse outcome.[16] CD47 targeting was suggested as a possible therapeutic anti-cancer approach,[17,18] and magrolimab, an anti-CD47 antibody, is being developed in several hematological cancers, including AML, as well as in solid malignancies.[9]

Activation of signaling pathways regulating the immune response can form an integral part of oncogenic transformation. For example, PD-L1 expression can be induced by extracellular stimuli but also by intracellular genetic defects.[19,20] Nucleophosmin 1 (*NPM1*) mutation, which is present in a third of AML patients, leads to neoantigen formation as well as to aberrant cytoplasmic localization of the protein product, possibly

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resulting in more efficient NPM1 processing and presentation on HLA molecules. Several HLA class I alleles were underrepresented in patients with *NPM1* mutation,[21,22] possibly due to spontaneous cure of individuals with HLA types suitable for presentation of NPM1-derived immunopeptides. Such peptides were indeed found in the immunopeptidome of AML blasts,[23,24] and T-cells reactive against NPM1 were repeatedly detected.[25–28] *NPM1* mutation was also associated with lower HLA-DR expression.[29] Furthermore, NPM1 was described to interact with the PD-L1 promotor and to regulate PD-L1 gene transcription in a breast cancer model.[30] The DNA methyltransferase 3A (DNMT3A) regulates gene transcription by promotor methylation. Loss-of-function mutations occurring in about 20% of AML cases might enhance the transcription rate of genes involved in the immune escape. *DNMT3A* mutation was indeed associated with higher HLA-DR positivity[31] and with decreased methylation of PD-L1 promotor.[32] Reduced DNMT3A expression or inhibition of DNMT3A activity by decitabine correlated with higher PD-L1 expression in breast cancer,[33] resp. in melanoma.[34] In this work, we focused to possible impact of these frequent recurrent mutations on the immunophenotype of AML blasts.

Materials and methods

Material

Primary cells from peripheral blood of AML patients with hyperleukocytosis were obtained by leukapheresis before therapy initiation. The leukapheretic products were diluted 10-fold in phosphate buffered saline (PBS), and the mononuclear cell fraction was separated using Histopaque-1077 (Sigma, #H8889). The cells were resuspended in RPMI 1640 medium with 10% fetal calf serum and with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), and aliquots were used for analysis of surface markers by flow cytometry and for mRNA isolation.

The antibodies used were as follows: CD45-V450 (#560367), CD4-BUV395 (#564724), CD8-BUV395 (#563795), CD19-BUV737 (#564303), CD34-BV786 (#743534), and CD371-BB515 (#565926) from BD Biosciences; HLA-DR-FITC (#11-9952-42), TIM-3-APC (#17-3109-42), and PD-L2-APC (#17-5888-42) from eBioscience; CLIP-PE (sc-12725 PE) from Santa Cruz; PD-L1-PE (#1P-177-T100), CD47-FITC (#1F-225-T100), and CD38-PE (#1P-366-T100) from Exbio (Prague, Czech Republic). HLA class I antibody (Abcam, ab2217) was conjugated in house using the Lightning-Link Fluorescein Conjugation kit (#707-0010, Innova Biosciences).

Ethics statement

All patients included in the study provided their written informed consent as to the use of their biological material for research purposes. The project was approved by the Ethics Committee of the Institute of Hematology in June 2015. All procedures followed were in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

AML treatment

Analyses of karyotype and gene mutations were performed as routine clinical procedures. After cytoreduction combining apheresis and oral administration of hydroxyurea, patients were treated with a standard 3 + 7 induction regimen. All subjects with FLT3 mutation diagnosed after 2018 received the FLT3 inhibitor midostaurin in addition. Consolidation therapy consisted in at least one cycle of high-dose cytarabine and allogeneic stem cell transplantation if still in remission.

Flow cytometry

Mononuclear cells were washed once in PBS, five tubes containing 1 milion cells in 50 µl PBS were prepared, antibodies were added (2 µl of each), and the samples were incubated for 30 min at 5°C. The cells were washed once in PBS (300 g/5 min/4°C), resuspended in ice-cold PBS, placed on ice, and immediately analyzed on a BD Fortessa flow cytometer using Application Settings. The settings were maintained using calibration beads. The gating strategy is illustrated in Figure S1 (Supplemental Information). All the tubes contained CD45, CD4+CD8, and CD19 to discriminate blasts, T-cells, and B-cells. Leukemia blasts and lymphocytes were gated in CD45/SSC dotplots. The lymphocytes were further specified as T-cells using CD4 and CD8, or as B-cells using CD19 positivity. No other antibody was added to the first tube, which was used to determine the background fluorescence. The immune escape markers were distributed into three panels with minimal spillover using FITC, PE, and APC channels. The background was subtracted from the mean fluorescence values (MFI) in each channel, for each subpopulation separately.

To quantify HLA class I expression, we used the mean fluorescence intensity (MFI) values after background subtraction. MFI for blasts were comparable with MFI for T-cells, whereas the values for B-cells were usually substantially higher (Supplemental Information, Figure S1c). As the surface amount of HLA class I and/or the affinity of the pan-HLA class I antibody might vary among individual HLA alleles, we expressed HLA class I values on blasts relatively to the autologous T-cells. MFI was also used to quantify CD47 surface expression. For the remaining markers, only a fraction of cells was usually positive, and these markers were quantified using the positive cell fraction (%) values.

Real-time PCR

RNeasy Mini Kit (Qiagen) was used for RNA isolation from 2 × 10^7 cells, and cDNA was generated by reverse transcription on CFX96 real-time system (BioRad) using SensiFAST cDNA Synthesis Kit (Bioline). The quality and concentration of template RNA and of cDNA were assessed with NanoDrop One^C Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). The relative amount of mRNA transcripts was measured by real-time PCR using SensiFAST SYBR N-ROX Kit (Bioline) and calculated by Bio-Rad CFX Manager Software. Primers were designed using the PrimerBLAST software (Table 1). For the relative quantification by $2^{-\Delta\Delta Ct}$ method, GAPDH expression was measured as a reference, using GAAACTGTGGCGTGATGGC and CCGTTCAGCT CAGGGATGAC as the forward and reverse primer, respectively.

Table 1. Primer sequences.

Gene	Forward primer	Reverse primer	NCBI Ref Seq
PD-L1 variant 1	ATGGTGGTGCCGACTACAAG	GGAATTGGTGGTGGTGGTCT	NM_014143.4
PD-L1 variant 2	TTGCTGAACGCCCCATACAA	TCCAGATGACTTCGGCCTTG	NM_001267706.2
Tim-3	CTACTGCTGCCGGATCCAAA	GTCCCCTGGTGGTAAGCATC	NM_032782.5
CD47	TCCACTGTCCCCACTGACTT	CCTGTGTGTGAGACAGCATCA	NM_001777.4

Test of natural killer (NK) cell cytotoxicity

The sensitivity of AML primary cells to lysis by NK cells was tested using cryopreserved samples of mononuclear cells obtained from leukapheresis. A total of 12 samples included one group with low HLA class I expression (N = 7) and one group with high HLA class I expression (N = 5). HLA amount was checked on defrozen samples and was found to be closely similar to that measured on fresh samples. K562 cells were used as a positive control. Although the target cell samples also contained other cells, the percentage of leukemia blasts was always very high.

NK cells were isolated and expanded from the buffy coat of a healthy donor. NK cells were separated from 1×10^8 PBMC using NK Cell Isolation Kit (130-092-657, Miltenyi Biotech) and MACS column in the magnetic field (130-042-401, Miltenyi Biotech). They were expanded two weeks in CellGro GMP SCGM (20802 Cell Genix) with 5% inactivated human AB serum (AK9340, Akron Biotech), hIL-2 (1000 U/ml) (Proleukin S, Novartis), hIL-15 (20 ng/ml) (130-095-765, Miltenyi Biotech), and gentamycin (40 ug/ml) (G1272, Sigma). Cells were stimulated with Activation/Expansion Kit (130-094-483, Miltenyi Biotech) consisting of Bead Particles loaded with NKp46 and CD2 antibodies. The KIR genotype of NK cells included 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5, 3DL2, 3DL3, 3DS1, 2DP1, and 3DP1 genes.

Target cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, 565082 BD Biosciences) and mixed with the effector NK cells in 1:10 or 1:20 ratio. The experiment was performed in triplicate with 10,000 target cells per well (96-well plate). Wells without effector NK cells were used to determine the fraction of spontaneously dead cells (% spont dead). The plate was incubated for 4 h at 37°C, in a CO_2 incubator. Then, the CFSE + cell viability was assessed by flow-cytometry using propidium iodide staining. The cell debris was outgated from scattergrams, the singlet events were selected from FSC-A vs FSC-H dotplots, and CFSE+ target cells were gated from CFSE vs SSC-A dotplots. The cytotoxicity was calculated from the following formula:

% cytotoxicity = (% dead in the sample with NK – % spont dead)/(100 – % spont dead)

Data analysis

Hierarchical clustering was performed using the McQuitty clustering method in the free Wessa software (www.wessa.net). GraphPad Prism software (version 7.03, GraphPad Software, San Diego, California USA) was used for statistical evaluation of experimental data (t-test, Mann–Whitney test, Spearman correlation) and R Statistical Software version 4.1.0. for univariate and multivariate survival analyses. The measured values were usually not normally distributed, and we thus used the Mann–Whitney non-parametric test to assess differences between patient groups. For univariable survival analyses, patients dead from other cause than AML (N = 1), before therapy initiation (N = 2) or during induction therapy (N = 5) were excluded. Group comparison by contingency tables was performed using the Epi Info 7.1.5.2. software (Centers for Disease Control and Prevention). The p value limit for significant differences between groups was set to 0.05.

Results

The basic characteristics of the patient cohort are given in Table 2, the individual details including the types of NPM1 and DNMT3A mutations, as well as information about additional mutations found during the diagnostic procedures are specified in the Supplementary Table. Mononuclear cell samples were isolated from leukapheresis products obtained at diagnosis (N = 53), prior to treatment. The surface amounts of HLA class I, HLA-DR, CLIP, PD-L1, PD-L2, TIM-3, and CD47 were measured by flow cytometry. PD-L2 was not detected in any of the first 20 samples, and the antibody was subsequently removed from the panel. As it is described in the Methods section, HLA class I and CD47 were quantified using the mean fluorescence intensity (MFI), whereas the other parameters are given as the fraction of positive cells (%). Flow cytometry results were well reproducible when analyses were repeated from defrozen sample aliquots, except for TIM-3. Due to a low stability of this marker, we assessed TIM-3 also at the transcript level using quantitative real-time polymerase chain reaction (qPCR). A summary of all the obtained values is also given in the Supplementary Table.

Hierarchical clustering analysis

Hierarchical clustering was performed using HLA class I, HLA-DR, CLIP, PD-L1, and TIM-3 values obtained from flow cytometry measurements. One group of closely similar samples (cluster 1 in Figure 1a) had low amounts of all the input parameters (Figure 1b). On the opposite side, the cluster 2 contained samples with high expression of all the markers. Although TIM-3 mRNA levels were not used for cluster analysis, they were clearly distinct in the cluster 1 (usually low levels) compared to the other two

Table 2. AML patient cohort characteristics. Basic characteristics of the patient cohort is given in this table. Individual specification of all the patients is given in supplementary table. The mutation status of nucleophosmin 1 (NPM1), Fms-like tyrosine kinase 3 (FLT3), and DNA methyltransferase 3A (DNMT3A) were determined as a part of routine clinical procedures.

	Total number N = 53
Age	Median 51, range 21–69
Sex (male/female)	27/26 (50.9/49.1%)
Karyotype	Available in 44 of 53 cases (83%)
normal	27 of 44 (61.4%)
1 to 3 aberrations	15 of 44 (34.1%)
complex karyotype	2 of 44 (4.5%)
Molecular markers	
NPM1 mutation	28 (52.8%)
DNMT3A mutation	20 (37.7%)
FLT3-ITD	23 (43.3%)
Hematopoietic stem cell transplantation	30 (56.6%)

groups (Figure 1b). The majority of the remaining samples belonged to an intermediate group, except for one sample (ID 18), which had high levels of TIM-3 and HLA class I. This patient had an altered karyotype with duplicated chromosomes 5 and 7 (Supplementary Table).

In the search for possible associations between genetic aberrations and immunophenotype, we compared the frequencies of the most common recurrent mutations found in AML among the three patient groups (Figure 1c). The cluster 1 (N = 16) was characterized by high incidence of *NPM1* mutations without concomitant *DNMT3A* mutation (Figure 1c, right). The frequency of *NPM1* mutations without *DNMT3A* mutation was significantly higher in the cluster 1 compared to the other two clusters (p less than 0.0001 by Fisher exact two-tailed test of 2 × 2 contingency table). In the cluster 2 (N = 18), a half of samples had both *NPM1* and *DNMT3A* mutation, and the incidence of this combined mutation was lower in the other two clusters, although the difference was not statistically significant (p = .068 by Fisher exact two-tailed test). The occurrence of *FLT3-ITD* was closely similar in all the groups (Figure 1c, left).

Impact of NPM1 and DNMT3A mutations on the immunophenotype

The whole cohort was subdivided according to the presence of mutations in *NPM1* or *DNMT3A*, and the flow cytometry results were statistically evaluated. As shown in Figure 2, several statistically significant differences were found: *NPM1* mutation was associated with decreased HLA-DR expression (Figure 2b, p = .0225). Patients with *DNMT3A* mutation had higher levels of all the tested markers, the difference being statistically significant for CLIP (p = .0132) and PD-L1 (p = .027).

The impact of *NPM1* mutation on HLA expression was opposed to that of *DNMT3A* mutation (Figure 2a,b) and we thus stratified the whole cohort into four groups according to *NPM1* and *DNMT3A* mutation status (Figure 3a). Isolated mutation in *NPM1* was indeed associated with a decrease of HLA class I surface amount. Concomitant *DNMT3A* mutation prevented the effect of *NPM1* mutation: the double-mutated group had similar HLA class I levels as the unmutated group. In the absence of *DNMT3A* mutation, the difference between *NPM1* wild-type (wt) and *NPM1* mutated (mut) samples was statistically significant (p = .0049), as it was for the difference between *DNMT3A*-wt and *DNMT3A*-mut in the presence of *NPM1* mutation (p = .0046).

HLA class I and *HLA-DR* genes are located at the same DNA locus and their transcription is thus probably correlated. Consistently, reduced HLA-DR expression was observed in *NPM1*-mut group: p = .0225, resp. 0.0008 for comparison between *NPM1*-wt and *NPM1*-mut patients from the whole cohort (Figure 2) or from *DNMT3A*-wt subgroup (Figure 3a, right), respectively. Low HLA-DR expression usually correlated with low HLA class I expression (Figure 3b).

As expected, samples with low HLA expression also had low amounts of CLIP, which usually forms part of HLA class II (Figure 3b). In samples with high HLA-DR levels, however, CLIP positivity was highly variable. We noted a marked difference between *NPM1*-wt and *NPM1*-mut samples with high HLA-DR expression: in the subset of samples with more than 85% HLA-DR positivity, the amount of CLIP was strongly associated with *NPM1* and/or *DNMT3A* mutation (Figure 3c, p = .0005 for comparison between DNMT3A-wt and DNMT3A-mut, p less than 0.0001 for comparison between *NPM1*-wt and *NPM1*-mut). In our cohort, *DNMT3A* mutation was mostly found in combination with *NPM1* mutation (Figure 1c, right), and the effects of these two mutations thus could not be separated.

Transcript analysis and correlation with surface protein amounts

TIM-3 protein is removed from the cell surface by proteolytic cleavage.[15] Nevertheless, the surface TIM-3 positivity correlated with higher mRNA content (Figure 4a). In addition, TIM-3 transcript positively correlated with surface expression of CLIP (Figure 4b) and PD-L1 (Figure 4c). This suggests that different mechanisms of immune escape could be activated simultaneously.

We have shown previously that the surface amount of PD-L1 correlates with the ratio of two transcription variants: the full-length v1 and a shorter v2 lacking the exon 2.[35,36] Similar result was obtained in the present study (Supplemental Information, Figure S2). Whereas the surface amount of PD-L1 did not display significant correlation with any of the individual transcript variants, the v1/v2 ratio correlated with the fraction of surface-positive cells. Interestingly, the positive correlation between surface protein and v1/v2 transcript was better in *FLT3-ITD*-positive samples (Supplemental Information, Figure S2).

In the case of CD47, only weak, not significant correlation between transcript and protein was found (Supplemental Information, Figure S3).

Analysis of Vizome/BeatAML dataset

As our patient cohort was relatively small, we analyzed an additional dataset obtained from the Vizome/BeatAML database, which contains transcriptomic data stratified according to different parameters.[37] The analysis confirmed transcript levels of all available *HLA* genes as well as that of *TIM-3* (denoted as *HAVCR2*) to be significantly lower in the group with *NPM1* mutation (N = 108) than in the group with wild-type *NPM1* (N = 340). The graphs and p values are given in Figure S4 (Supplemental Information).

Survival analyses

Although our patient cohort was relatively small and heterogenous, we examined the possible impact of blast immunophenotype on patient outcome using standard survival analysis. Curves obtained for overall survival (OS) and for relapse-free survival (RFS) are shown in Figure 5. The results of univariate analyses indicated that patients with decreased HLA class I expression had significantly better survival (p = .0393, resp. 0.0184 for OS, resp. RFS, Figure 5a). This group mostly consisted of patients with *NPM1* mutation and without *DNMT3A* mutation, who also had low levels of the other markers of immune escape. In agreement with the fact that CLIP, PD-L1, and TIM-3 were mutually correlated (Figure 4b,c), their impact on the survival was similar, and high levels were always associated with worse prognosis (Figure 5b-d). Alternatively, TIM-3 transcript could also be used as a prognostic factor reflecting a complex immune resistance

associated with worse outcome (Supplemental Information, Figure S5). In addition, PD-L1 can be measured as a ratio of v1/v2 transcript variants.[35] Indeed, similar results were obtained when v1/v2 mRNA PD-L1 levels were used for patient stratification (Supplemental Information, Figure S6).

In multivariable Cox regression analysis all markers retained statistical significance also after adjustement for age and NPM1 mutation (Supplemental Information, Figure S7).

NK cell cytotoxic assay

Reduced HLA class I expression on leukemia cells could enhance the activity of natural killer (NK) cells, which are inhibited through binding of their inhibitory receptors (KIR) to HLA-C molecules on the target cell. We thus tested if the observed decrease of HLA expression could increase leukemia cell sensitivity to lysis by NK cells from a healthy donor. The effector cells used in the experiment had both 2DL1 and 2DL2 inhibitory KIR genes and were thus supposed to be inhibited by any HLA-C allele expressed on the target cells. NK cell-mediated killing of the positive control, i.e., K562 cells with very low HLA class I expression, occurred with 94% efficiency after 4 h co-incubation at the effector:target ratio 10:1. In contrast, the cytotoxicity against primary AML cells was limited (4% to 30%), and no significant difference was found between samples with low versus high HLA class I levels (Figure 6).

Correlation with CD34 and FAB groups

In our study, expression of surface molecules were measured on gated bulk leukemia blasts regardless of cell stemness. The percentage of CD34-positive cells was measured in a separate tube, and we thus cannot directly correlate the blast characteristics with CD34 as a stem cell marker. Nevertheless, we found no correlation between the fraction of CD34-positive cells and the expression of HLA, CLIP, PD-L1, or TIM-3 in individual samples (Supplemental Information, Figure S8).

Comparison of the blast immunophenotype among patient groups according to French-American-British (FAB) classification revealed significantly higher CLIP and TIM-3 positivity in the groups with monocytic features (M4+M5) versus M1+M2. Reduced HLA class I and HLA-DR surface amounts were associated with M1+M2 subtype. These results are shown in detail in Figure S9 (Supplemental Information).

Discussion

AML therapy failure is mostly due to frequent relapses arising from the residual disease, and the immune system is considered crucial for elimination of leukemia cells surviving after chemotherapy and hematopoietic cell transplantation. Compared to solid tumors, AML has a low mutational burden, but several driver mutations with high incidence produce immunogenic peptides[38] and AML is susceptible to attack by both the innate and adaptive immune system.[6] However, multiple molecular mechanisms can mediate immune evasion or active immune suppression. Although the standard induction chemotherapy has the potential to normalize the T-cell phenotype, reduce regulatory T-cells and restore T-cell and NK-cell effector function by eliminating the bulk leukemia,[2] oncogenic mutations may render the persisting leukemia cells inherently resistant to immune attack. For example, AML-associated mutation in the C/EBPα transcription factor decreases leukemia cell susceptibility to NK-mediated lysis.[39] Individual targeting of the pathways providing such inherent resistance could improve the unsatisfactory outcome of AML patients. Besides specialized immunotherapeutic procedures, many clinically used therapeutics have promising immunomodulatory effects. Sorafenib, a multi-potent kinase inhibitor, prompted leukemic blasts to release IL-15, which in turn potentiated responses mediated by early-differentiated T and NK cells.[40] The broad cytotoxic agent doxorubicin, administered at low doses, reduced expression of multiple immune checkpoints including PD-L1 and TIM-3 specifically in chemoresistant leukemia stem cells.[41] Venetoclax, a Bcl-2 inhibitor, increased T-cell effector functions through reactive oxygen species generation without apoptosis induction.[42] However, the effect of drugs on the immune system function and/or on the leukemia cell immunophenotype may also be context-dependent or ambivalent. Hypomethylating agents, which are also used for AML therapy, enhance immune response to tumors by promoting improved tumor antigen expression and presentation and enhanced effector T-cell function while concomitantly dampening the immune response by promoting upregulation of inhibitory coreceptors on T-cells and activation of regulatory T-cells.[43] Similarly, the interferon γ can restore surface expression of HLA molecules on relapsed leukemia, but is also known to promote expression of PD-L1 and other inhibitory ligands.[44,45] Implementation of combinatory therapeutic protocols will require biomarkers to select the patients who will most likely benefit from the treatment while minimizing the possible adverse effects. Associations between recurrent genetic aberrations and immune escape markers may help to identify inherent mechanisms of immune escape activation, which will presumably persist under residual disease conditions.

In this work, we analyzed selected markers of the possible immune escape on AML blasts at diagnosis: HLA class I and HLA-DR as markers of decreased HLA expression, CLIP as a marker of reduced antigen presentation, the inhibitory ligand PD-L1 preventing T-cell cytotoxicity, TIM-3 as a secreted immunosuppressive molecule, and CD47 as an inhibitory ligand for macrophages. The majority of samples displayed at least one of these markers and our results thus confirmed that the mechanisms of immune escape known from solid tumors are also relevant for AML. The study was performed using cells obtained from leukapheresis, and was thus limited to patients with hyperleukocytosis. The cohort was thus enriched in cases with severe immune dysfunction and high proliferative activity. Not surprisingly, the incidence of *FLT3-ITD* mutations, which drive leukemic proliferation, was high in our cohort (Table 2).

HLA surface expression was reduced in association with *NPM1* mutation (Figures 2a, 2b, 3a). Decreased HLA-DR expression in AML with *NPM1* mutation has been reported previously,[29] and HLA-DR positivity in this context was associated with worse survival.[46] Our results show that HLA class I follows similar expression pattern as HLA-DR (Figure 3b), suggesting lower transcription rate of the whole DNA locus in the presence of *NPM1* mutation. This finding was supported by analysis of an independent large cohort (data available from Vizome/BeatAML

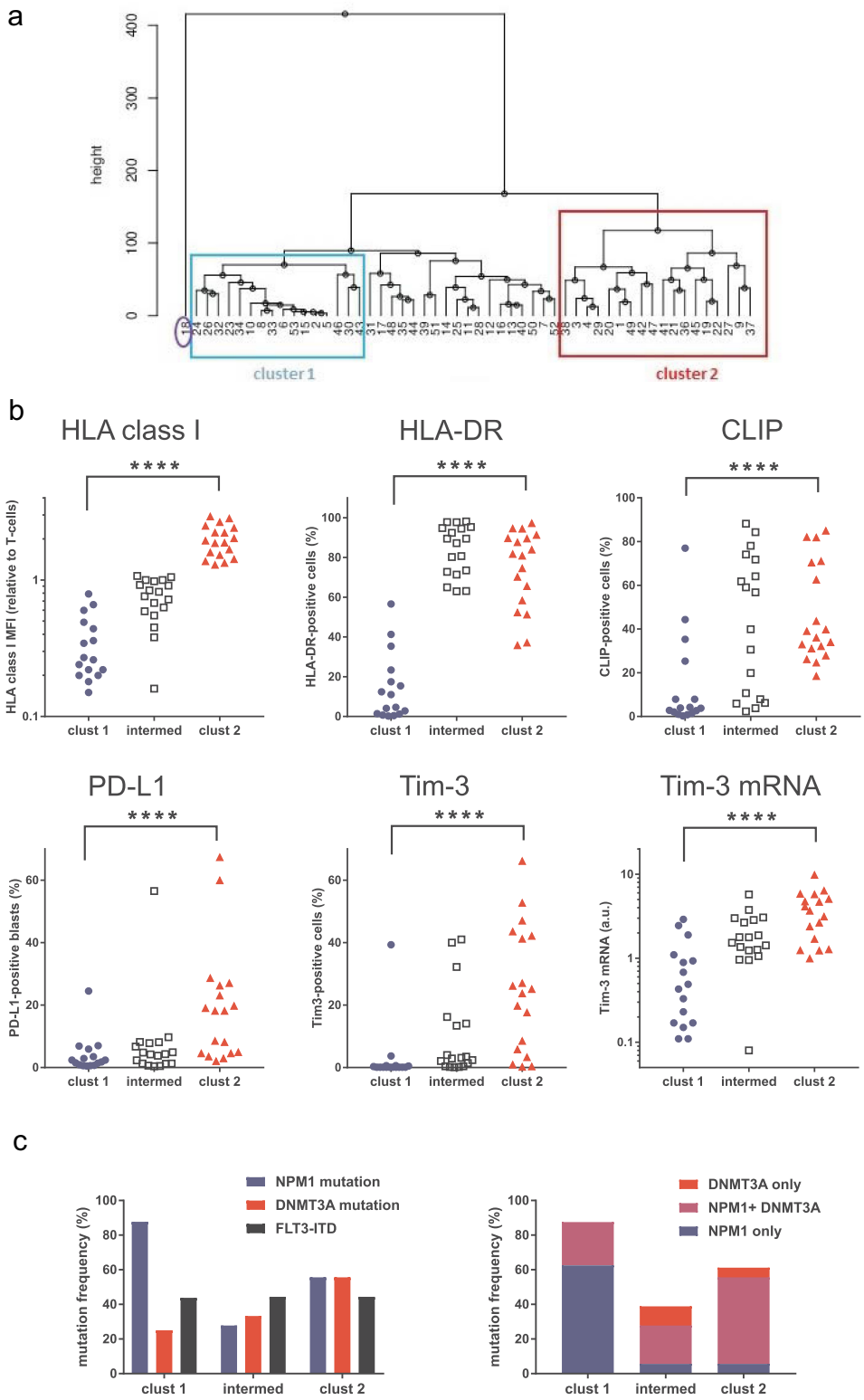

Figure 1. Results of hierarchical clustering of AML samples according to immune escape-related markers. (A) Surface amounts of HLA class I, HLA-DR, CLIP, PD-L1, and TIM-3 were measured using flow cytometry and used as the input for clustering analysis. (B) The graphs show the individual values in samples from the two clusters (cluster 1, cluster 2) defined in the tree diagram (A). The group denoted as "intermed" includes all the remaining samples except for the outlying ID 18. TIM-3 mRNA values were not used for hierarchical clustering, but are shown for comparison. Mann-Whitney test was performed to evaluate differences between cluster 1 and cluster 2, and p-values were found to be less than 0.0001 for all of the parameters. (C) Relative incidence of recurrent mutations in the above defined groups (N = 16, 18, and 18 for cluster 1, intermediate group, and cluster 2, respectively).

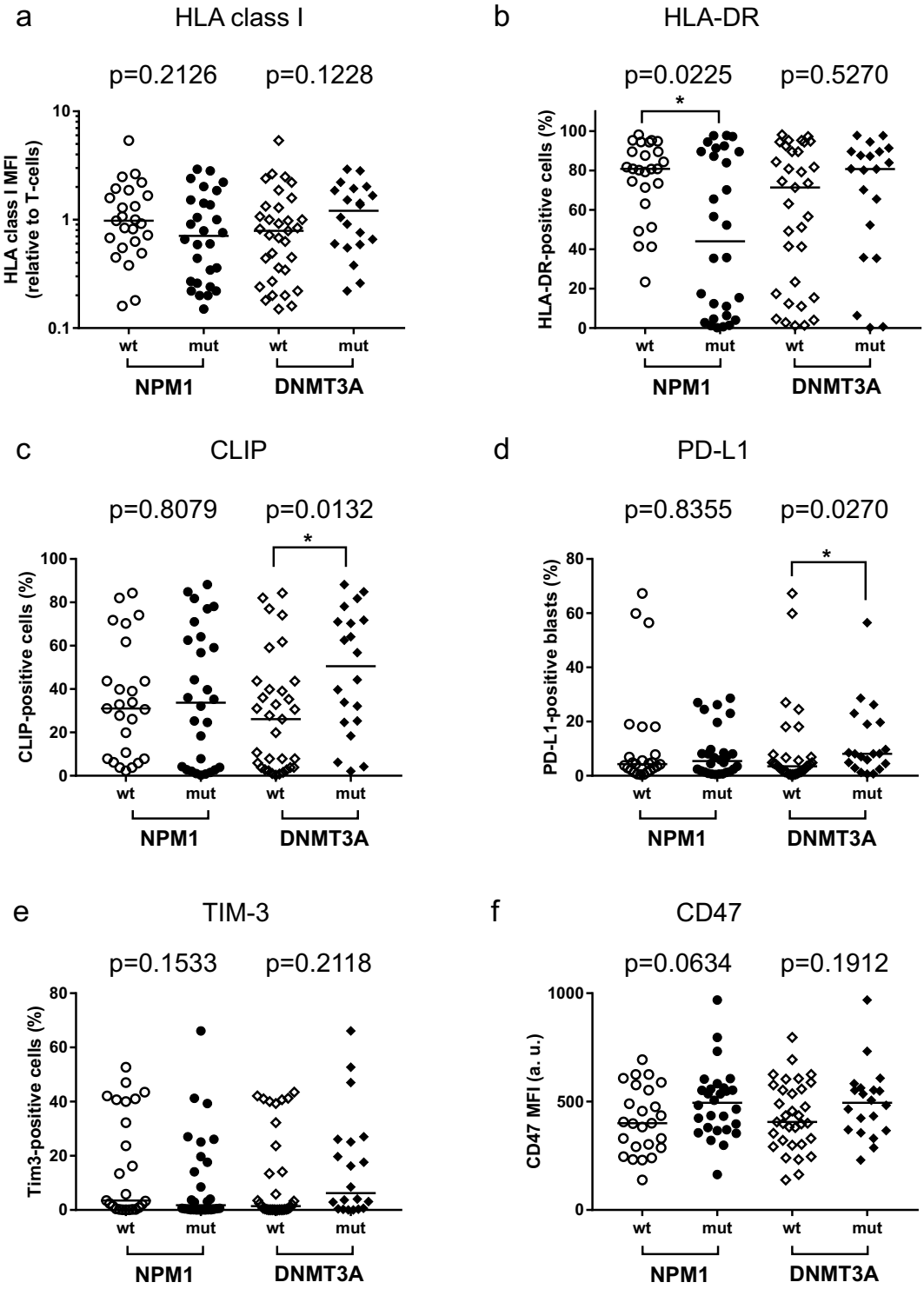

Figure 2. Comparison of the measured values in groups according to NPM1 or DNMT3A mutation status. The cohort was subdivided according to NPM1/DNMT3A mutation, and the values of the indicated surface marker were compared using the non-parametric Mann-Whitney test. CD47 values (panel F) were compared using two-tailed t-test. The resulting p-values are indicated on top of the corresponding graphs. The horizontal bars show the medians, and the star symbol denotes a statistically significant difference between groups.

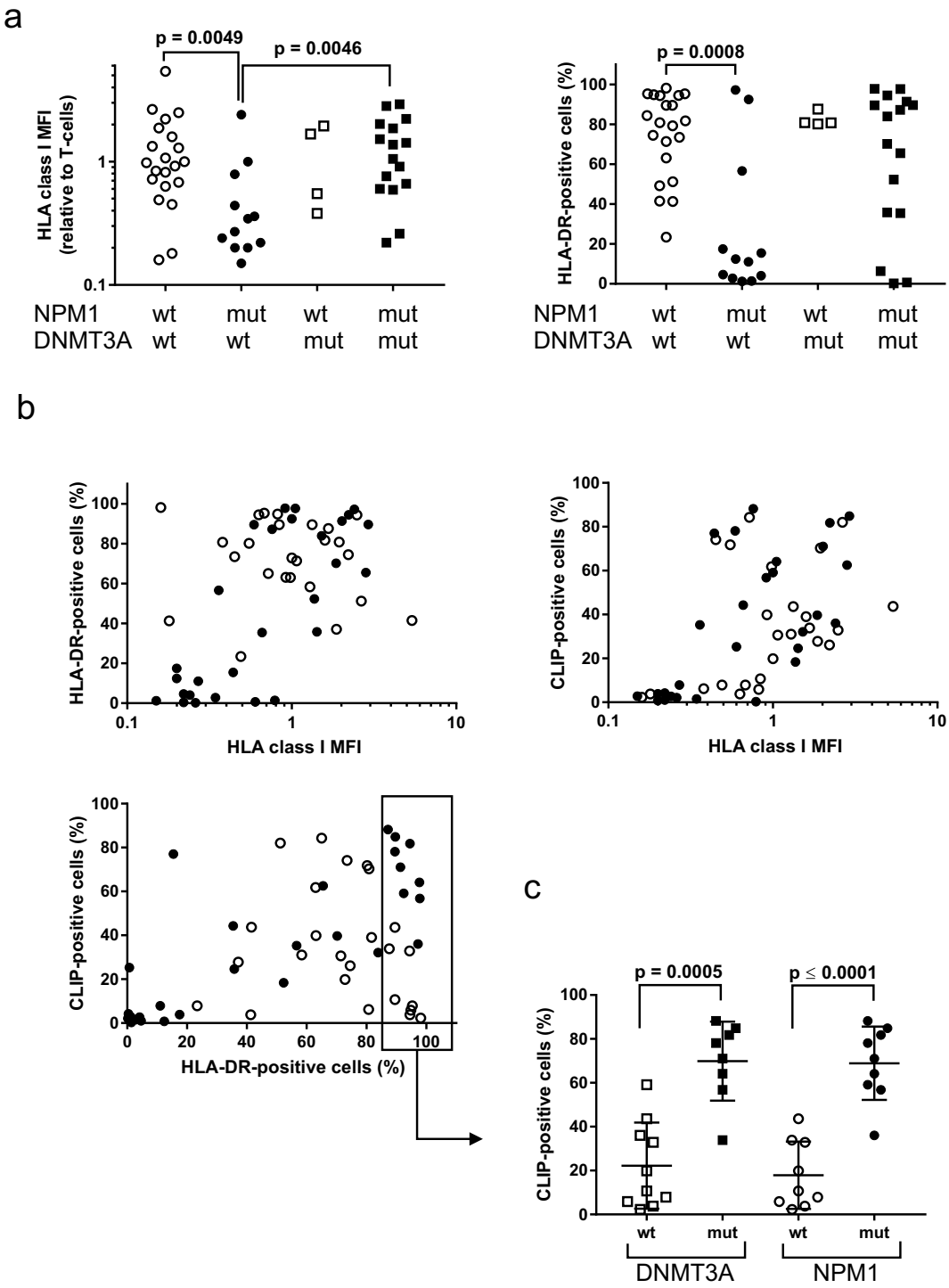

Figure 3. HLA-related markers in NPM1/DNMT3A groups. (A) HLA class I and HLA-DR surface expression in patient subgroups according to NPM1 and DNMT3A mutations. Difference between groups were assessed using Mann-Whitney test, the resulting p-values are given in the graphs. (B) Correlations between HLA class I, HLA-DR, and CLIP. Open symbols: samples with wild-type NPM1, closed symbols: samples with mutated NPM1. (C) Samples with more than 85% HLA-DR-positive blasts were divided according to NPM1 or DNMT3A mutation and CLIP amounts were compared using the Mann-Whitney test. The resulting p-values are indicated in the graph.

database), which showed lower levels of HLA transcripts in samples with *NPM1* mutation (Figure S4a). Importantly, the validation cohort was not limited to patients with hyperleukocytosis.

The effect of *NPM1* mutation was prevented by concurrent *DNMT3A* mutation (Figure 3a), which usually results in decreased *DNMT3A* activity. This is in agreement with a previous study describing enhanced HLA class I expression in neuronal cells with *DNMT3A* and *DNMT1* knockout.[47] Interestingly, the two patients with low HLA expression in the double-mutated group in Figure 3a did not have the prevalent DNMT3A mutation type, i.e. R882 substitution, but a mutation with unknown impact on DNMT3A activity (Supplementary Table, ID15 and ID53).

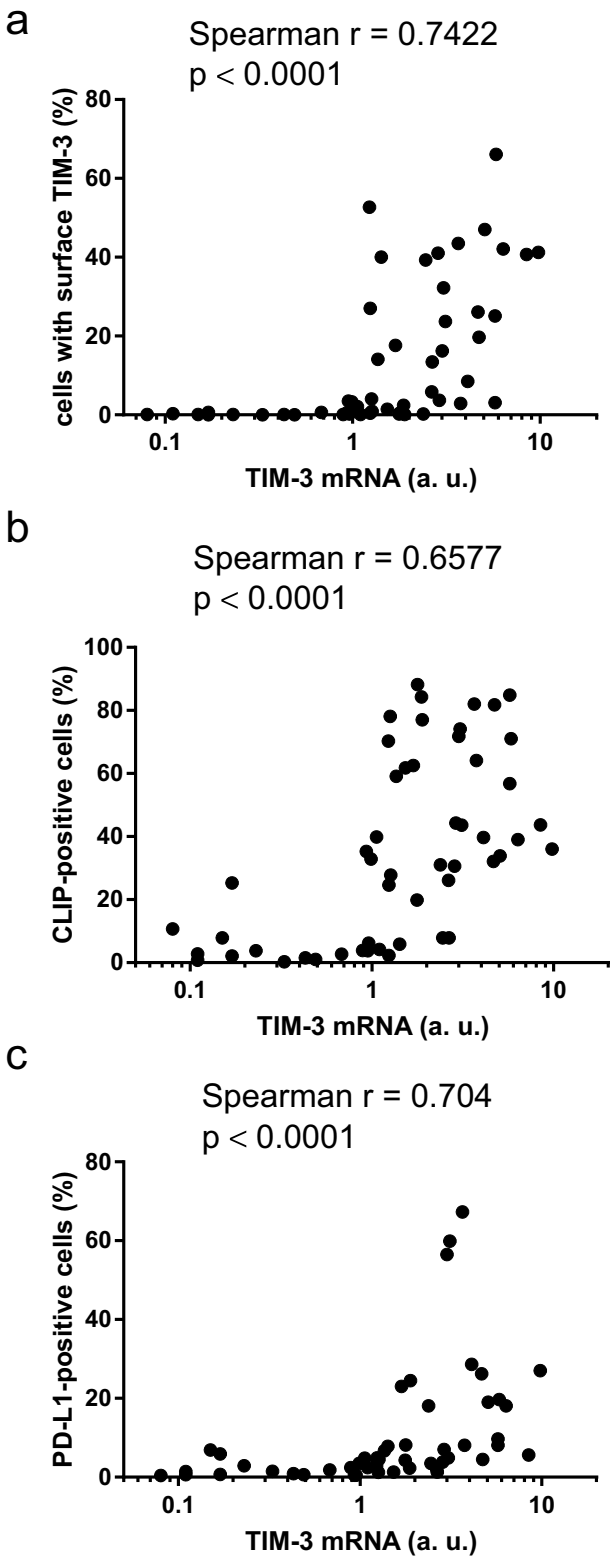

Figure 4. Correlation of TIM-3 mRNA with TIM-3 protein, PD-L1, and CLIP surface positivity of AML blasts. (A) Correlation of TIM-3 trancript amount with the fraction of TIM-3-positive cells determined by flow cytometry in mononuclear cell preparations. (B) The fraction of cells expressing the invariant CLIP peptide versus TIM-3 mRNA. (C) The fraction of cells expressing the inhibitory receptor PD-L1 versus TIM-3 mRNA. Spearman correlation test was performed to assess the correlation, the obtained correlation coefficients (r) and p-values are given in the graphs.

Lower HLA class I expression could help leukemia cell escape from elimination by cytotoxic T-cells. NPM1-derived antigens are recognized by cytotoxic T-cells, which could create a selection pressure in favor of cells with low HLA expression in patients with *NPM1* mutation. Cells lacking HLA class I should be eliminated by NK cells due to the loss of inhibitory interaction between KIR and HLA-C. However, HLA class I expression on AML blasts was always partially preserved (at least 20% compared to the autologous T-lymphocytes, Figure 2), and no significant increase in cell lysis by donor NK cells was

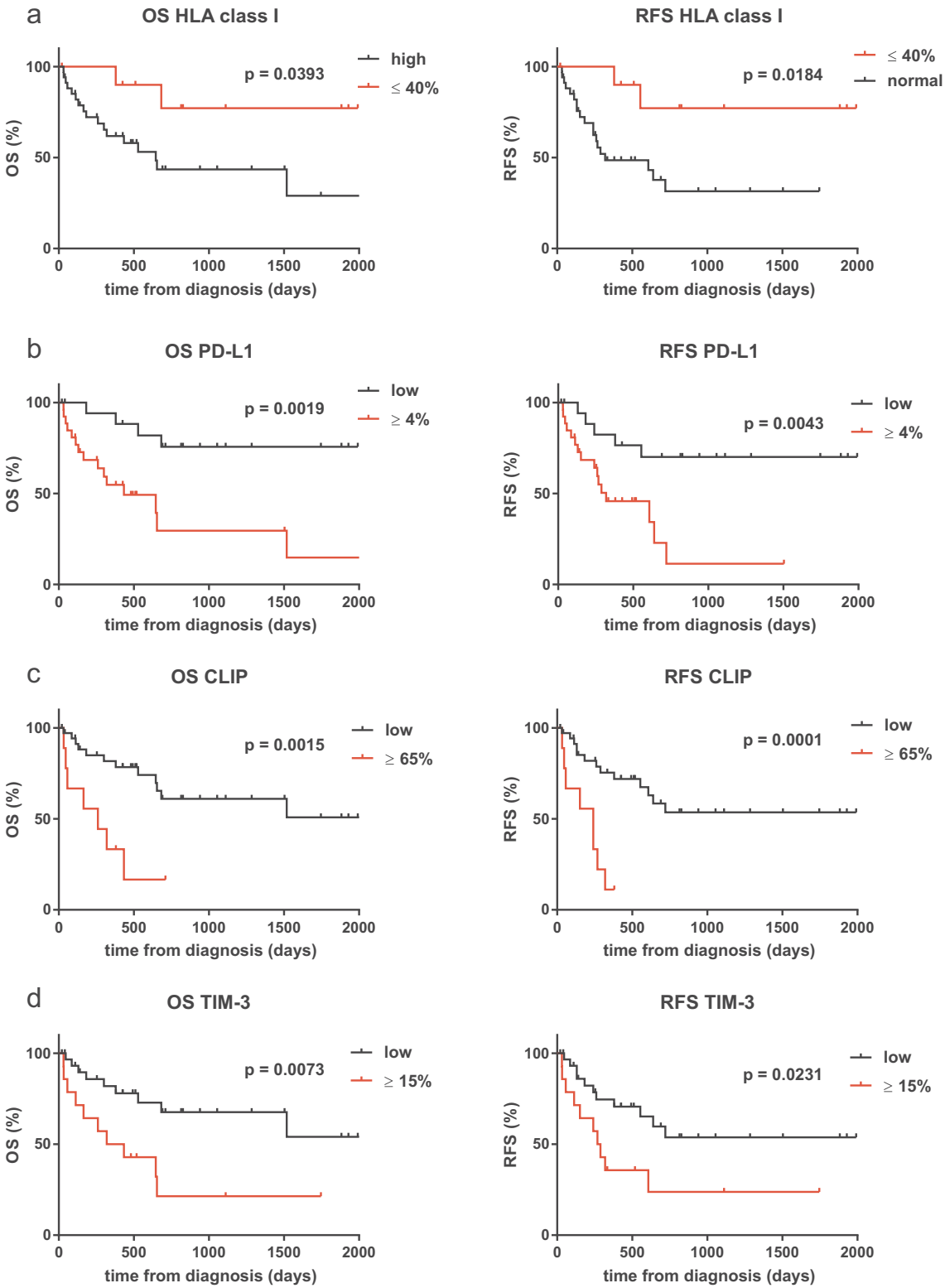

Figure 5. Survival analysis in groups according to selected immunophenotype markers. The analyses of the overall survival (OS, left column) and of the relapse-free survival (RFS, right column) in groups with low versus high levels of the indicated marker were performed using GraphPad Prism software, the obtained p-values for survival difference between groups are given in the graphs. The cutoff values were optimized with regard to the resulting p-values for OS.

found in the group with low HLA levels (Figure 6). The observed HLA class I decrease thus might represent a compromise between reduced sensitivity to T-cell attack and increased risk of lysis by NK cells, which would be reached during leukemia immunoediting. This phenotype was not associated with activation of other mechanisms of immune escape (Figure 1b, cluster 1). Similar observation was made in a study describing gene expression changes in AML patients who relapsed after allogenous hematopoietic cell transplantation: two major subgroups of relapsing patients were characterized by reduced HLA

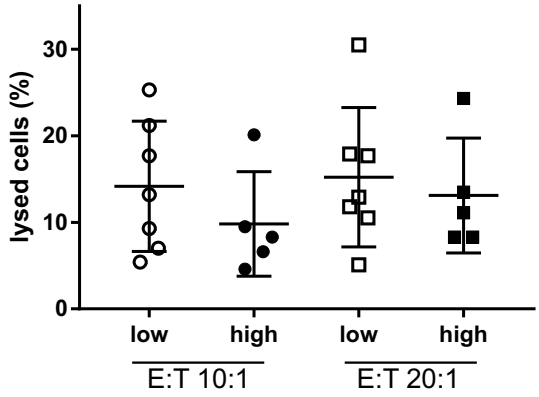

Figure 6. NK cell-mediated lysis of primary AML cells with low or high HLA class I expression. The cytotoxic assay with NK cells from a healthy donor was performed in triplicates at two different effector to target (E:T) ratios as indicated. The means of the triplicates are given in the Figure for samples with low HLA levels (N = 7, open symbols) or with normal HLA levels (N = 5, closed symbols). The bars indicate means and s.d. of the values shown in each group. In the positive control (K562 cells, not shown in the graph), the percentage of lysed cells reached 94% under the same experimental conditions at E:T ratio 10:1.

class II expression or upregulation of multiple inhibitory receptors including PD-L1, respectively.[48] In our cohort, the cases with decreased HLA expression had better outcome (Figure 5a).

The invariant peptide CLIP is produced as a part of HLA class II and is replaced by antigens in the endoplasmic reticulum. In case of defective antigen presentation, CLIP is not removed from HLA molecules and is exposed on the cell surface. As expected, samples with low HLA expression usually also had lower surface amounts of CLIP (Figure 3b). However, high CLIP levels were significantly associated with *NPM1* and/or *DNMT3A* mutations in samples with high HLA-DR expression (Figure 3c). Reduced antigen presentation is thus a relevant immune escape mechanism in AML, and seems to be specifically associated with *NPM1* and/or *DNMT3A* mutation.

High surface PD-L1 positivity was associated with worse prognosis in our cohort (Figure 5b). In agreement with our previous results,[36] PD-L1 surface protein levels correlated with v1/v2 PD-L1 mRNA (Figure S2). In the previous study, the negative impact of high v1/v2 PD-L1 mRNA was restricted to patients with *FLT3-ITD*.[36] This could be due to the fact that the correlation between protein levels and v1/v2 transcript is better in *FLT3-ITD*-positive cells (Figure S2).

NPM1 was reported to be required for PD-L1 expression and *NPM1* mutation thus could result in lower PD-L1 amounts. The average PD-L1 positivity in the group with mutated *NPM1* was indeed slightly lower (Figure 2), but the difference was not statistically significant. Instead, analysis of the dataset from Vizome/BeatAML database showed a statistically significant decrease in TIM-3 transcript levels in association with *NPM1* mutation (Figure S4b).

Virtually all the studied markers were more frequent in samples with *DNMT3A* mutation (Figure 2), and this fact may contribute to the adverse prognostic impact of DNMT3A R882 substitutions.[49] Correlation between lower expression/activity of DNMT3A and higher expression of PD-L1 was also observed in other tumor types.[32–34] Consistently, high methylation of PD-L1 promotor was associated with a reduced risk for relapse and prolonged overall survival in AML patients.[50]

The functionality of TIM-3 is highly context-dependent. TIM-3 positivity was associated with T-cell exhaustion, but also with increased cytotoxic capacity of NK cells.[51,52] In AML, higher numbers of TIM-3+ NK cells at diagnosis predicted better outcome.[53] TIM-3 is also present on the surface of AML blasts,[54] but its role in leukemogenesis or in leukemia maintenance is still not clear. Experiments with mouse xenografts showed that AML-initiating cells are usually TIM-3--positive.[55] In its secreted form, TIM-3 might inhibit the cytotoxic T-cells.[15] Correlation of PD-L1, and also of CLIP with TIM-3 mRNA (Figure 4b,c) indicates that TIM-3 can be a marker of immune resistance and that different mechanisms of immune escape may be activated in parallel. It seems likely that the regulation of different mechanisms enabling leukemia cells to escape from the immune system is to some extent shared, so that a resistant cell acquires a complex immunoresistant phenotype including increased expression of inhibitory receptors, reduced antigen presentation, and secretion of inhibitory molecules like TIM-3. TIM-3 transcript could be used as a prognostic factor reflecting this complex immune resistance associated with worse outcome (Figure S5). Consistently with our findings, AML patients with high TIM-3 transcript levels had lower rates of complete remission and lower overall survival rates after 1-year follow-up.[56] High TIM-3 levels were also associated with significantly shorter overall survival in solid tumors.[57]

CD47 binds to an inhibitory receptor on macrophages. Its expression is transiently elevated during hematopoetic stem cell mobilization and release into the peripheral blood.[58] CD47 was detected in all AML samples, the mean amount being slightly higher in those with *NPM1* mutation (Figure 2). This is in agreement with a previous study showing that CD47 positivity of AML blasts in the bone marrow correlated with *NPM1* mutation.[58]

Hierarchical clustering revealed one outlying sample (ID 18) with high TIM-3 and HLA class I expression (Figure 1a). The *TIM-3* gene is located on the chromosome 5 and the aberrant mRNA TIM-3 level could thus be due to chromosome 5 duplication, which was found in the karyotype of this patient (Supplementary Table).

Conclusions

We identified several potential immune escape markers in AML patients with hyperleukocytosis. In the absence of *DNMT3A* mutation, samples with mutated *NPM1* displayed decreased HLA expression, while they were usually negative in the other markers. In contrast, *DNMT3A* mutation was associated with higher levels of all the tested markers. CLIP, PD-L1, and TIM-3 were often co-expressed, suggesting parallel activation of different immune escape mechanisms. In our cohort, reduced HLA expression was associated with better outcome, whereas high PD-L1/CLIP/TIM-3 expression had adverse prognostic meaning. Inherently activated immune escape pathways might partly explain the very poor prognosis of patients with AML and hyperleukocytosis in a mechanistic way. Restoring immunosurveillance in combination with cytoreductive treatment could reduce the relapse rate and improve patient outcome.

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Data availability statement

All the data are available in the article or in Supplementary Table.

Disclosure statement

The authors report there are no competing interests to declare.

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