Acute myeloid leukemia with mutated nucleophosmin 1: an immunogenic acute myeloid leukemia subtype and potential candidate for immune checkpoint inhibition

Clinical and preclinical data suggest that acute myeloid leukemia (AML) with mutated *nucleophosmin 1* (*NPM1*^{mut}) might constitute an immunogenic leukemia subtype. In general, AML with *NPM1*^{mut} correlates with better prognosis, but the underlying mechanisms still remain to be elucidated. Our group previously described specific immune responses against epitopes derived from the mutational region of *NPM1*.[1] Furthermore, in a smaller cohort of AML patients we found a significantly better overall survival for patients with specific cytotoxic T-lymphocyte (CTL) response against *NPM1*^{mut}-derived immunogenic epitopes,[2] when compared to *NPM1*^{wt} AML patients. This suggests that immune responses against *NPM1*^{mut} might in part contribute to the more favorable outcome in AML patients. Recently, checkpoint inhibition targeting targeting Programmed cell death protein 1 (PD-1)/Programmed cell death 1 ligand 1 (PD-L1) has been proven to be an effective novel immunotherapeutic approach in cancer, including hematological malignancies.[3,4] Therefore, we questioned whether *NPM1*^{mut} AML patients might be candidates for PD-1/PD-L1-directed immune checkpoint inhibition. Thus, we performed flow cytometry and microarray analyses to assess PD-L1 (CD274) expression in leukemic cells, including leukemic progenitor/stem cell compartments, of AML patients with mutant *versus* wild-type (WT) NPM1.

In total 30 AML patient samples (15 AML NPM1^{mut} and 15 AML *NPM1*^{wt}) were evaluated, with the informed consent of the patients involved. The patient characteristics are described in Table 1A. At diagnosis, peripheral blood mononuclear cells (PBMC) were separated from blood samples by Ficoll (Pan Biotech, Germany) density gradient centrifugation. Samples were stained with CD34/CD38/CD274 and cell-surface expression was evaluated by flow cytometry using a fluorescence-activated cell sorting (FACS) Aria flow cytometer (BD Biosciences). An *NPM1*^{mut} specific antibody for intracellular immunostaining (*NPM1* hu fluorescein isothiocyanate (FITC), specific for the mutant form of nucleophosmin; Bio-Techne) was used to demonstrate the presence of *NPM1*^{mut} in the leukemic progenitor/stem cell compartment of *NPM1*^{mut} AML cases. Moreover, for gene expression analysis of the CD34^{+}CD38^{-} compartment enriched for leukemia stem cells (LSC) and progenitor cells, patient samples were sorted using a FACS Aria flow cytometer based on four quadrants (CD34^{+}CD38^{-}, CD34^{+}CD38^{+}, CD34^{-}CD38^{+} and CD34^{-}CD38^{-}). PD-L1 expression in CD34^{+}CD38^{-} cells was then assessed by Affymetrix U133plus2.0 microarray analysis in comparison to the other cell compartments, designated "AML bulk cells".

Via FACS analysis, we observed that many AML cases had relevant expression of PD-L1, and that bulk AML cells of *NPM1*^{mut} AML showed a significantly higher PD-L1 expression in comparison to *NPM1*^{wt} AML patients (median of 1.4% positive cells, range 0.0-8.5%, *versus* median of 0.3% positive cells, range 0.1-1.1%, respectively; $P<0.0001$; Table 1B). Importantly, PD-L1 expression was detected at a higher percentage of leukemic progenitor/stem cells (CD34^{+}CD38^{-}) in NPM1^{mut} than in that of *NPM1*^{wt} AML (median of 3.6% positive cells, range 0.0-17.2%, *versus* median of 0.3% positive cells, range 0.0-3.0%, respectively; $P<0.0001$; Table 1B and Figure 1A). Results for non-LSC are shown in Figure 1B. In general, the LSC fraction showed a higher PD-L1 expression than the non-LSC bulk AML cells ($P=0.0013$; Figure 1C). Figures 1D-F display the FACS analysis data of representative patient samples. In Figure 1D, the data of an *NPM1*^{mut} AML case with a relatively high percentage of PD-L1 expressing cells (3.98%) in the LSC fraction is shown. In comparison, Figure 1E shows data for an *NPM1*^{mut} AML patient with lower expression (0.76%), and Figure 1F an *NPM1*^{wt} AML patient with no PD-L1 expression. In order to demonstrate that in *NPM1*^{mut} AML cases the LSC enriched compartment cells also carry the *NPM1*^{mut}, intracellular immunostaining was performed with an *NPM1*^{mut} specific antibody. This analysis showed that PD-L1 positive progenitor/leukemic stem cells of *NPM1*^{mut} AML cases showed significant expression of mutant NPM1, thereby indicating that the PD-L1 positive cells are part of the malignant clone (Figure 1G). By microarray analysis, we detected CD274 expression in both AML bulk and in leukemic progenitor/stem cells, however, expression was not significantly different with

Table 1A. Summary of patient characteristics and PD-L1 (CD274) positive cells.

No. of AML patients	30
NPM1 mut	15
NPM1 WT	15
Sex	
Male	12
Female	18
Age	
Mean (years)	60.3
Range (years)	31-77
Sample Type	
PBMC	30
Genetic finding	
normal karyotype	17
complex karyotype	5
FLT3 ITD pos	4
FLT3 TKD mut	3
t(8;21)	1
t(6;11)	1
t(5;14)	1
inv16	2
CEBPA mut	2
46,XY,t(5;14)	1
46,XX,del(9)	1
46,XX,+8p	1
46,XX,der(10)	1

Table 1B

	LSC	Bulk
AML *NPM1* mut	3.6 (0.0-17.2)	1.4 (0.0-8.5)
AML *NPM1* WT	0.3 (0.0-3.0)	0.3 (0.1-1.1)

(A) Clinical characteristics of AML patients included into the clinical trial. MUT: mutated; WT: wild-type; pos: positive; AML: acute myeloid leukemia; PBMC: peripheral blood mononuclear cells. (B) Median number of the PD-L1 expressing cells. MUT: mutated; WT: wild-type; LSC: leukemia stem cells.

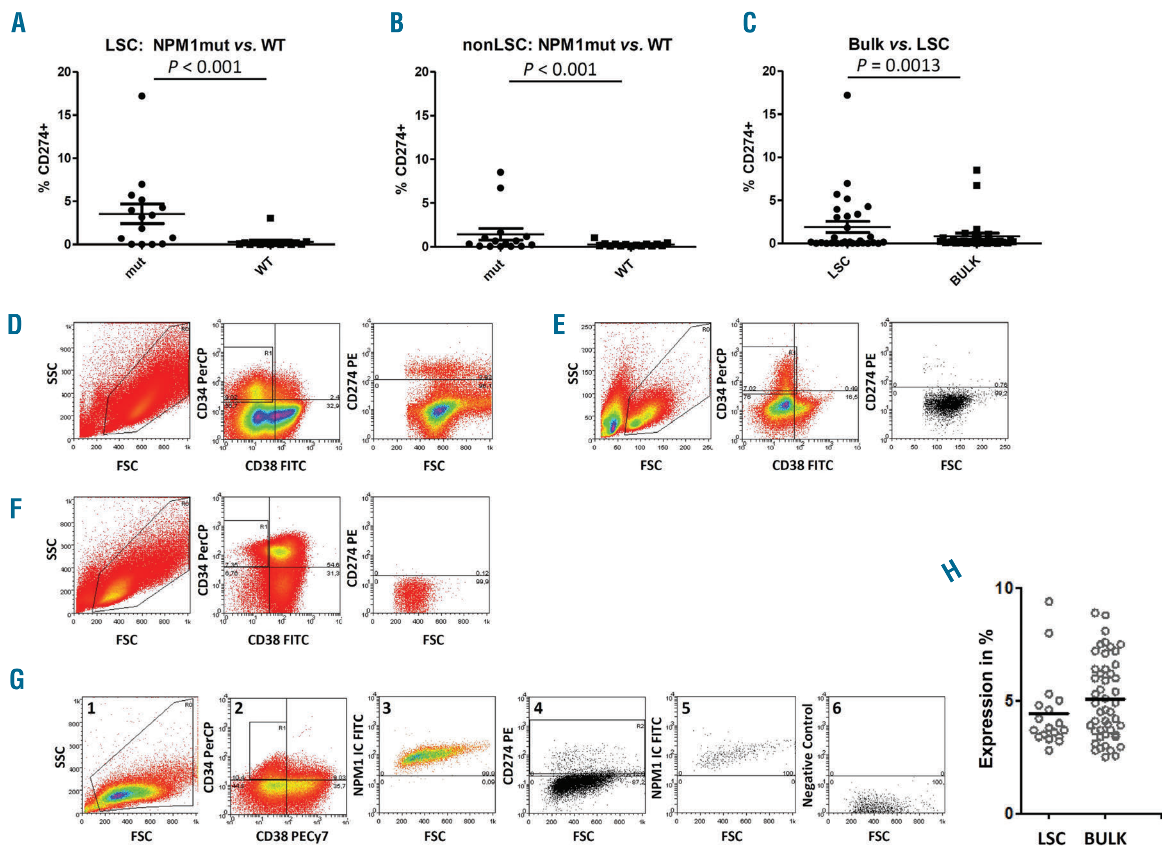

Figure 1. Flow cytometry and microarray based analysis of CD274 expression in AML. (A) More LSC compartment cells of $NPM1^{mut}$ AML patients showed PD-L1 expression compared to $NPM1^{wt}$ cases (P<0.0001). (B) Similarly, more non-LSC cells of $NPM1^{mut}$ AML patients showed PD-L1 expression compared to $NPM1^{wt}$ cases (P<0.0001). (C) The expression analysis (PD-L1 in AML bulk cells (all cells) *versus* LSC demonstrated that more LSCs express PD-L1 than bulk AML cells) (P=0.0013). (D) - (F) Typical examples of FACS analysis of PD-L1 expression in LSC-enriched cells of two $NPM1^{mut}$ patients and one $NPM1^{wt}$ AML patient. (D) Shows an $NPM1^{mut}$ AML patient with a relatively high percentage (3.98%) of PD-L1 expressing cells in the LSC fraction compared to (E) another $NPM1^{wt}$ AML patient with lower expression (0.76%) in LSC. (F) Demonstrates an exemplary $NPM1^{wt}$ AML patient without relevant PD-L1 expression. (G) FACS staining of LSC ($CD34^+CD38^-$) separated cells in $NPM1^{mut}$ AML samples. These LSC enriched cells harbor cytoplasmic NPM1, thus indicating that these cells belong to the leukemic clone. (G1) Shows a representative dot plot of one $NPM1^{mut}$ patient, gate R0 represents the blast population. In (G2) the LSC population in gate R1 (R1: 9.73%) is displayed. (G3) Shows the percentage of the $NPM1^{mut}$ population (99.9%) within the LSC gate R1. In (G4) the percentage of the PD-L1 (CD274) positive cells in gates R0 and R1 (6.19%) is exhibited and (G5) shows the percentage of the $NPM1^{mut}$ population within the PD-L1 (CD274) positive cells (using gates $R0^+R1^+R2$ consecutively, 100%). (G6) Represents the negative control (CD34/CD38/CD274/ no $NPM1^{mut}$ antibody). (H) Affymetrix gene expression analysis of CD274 expression in LSC (CD34+CD38- enriched cells) *versus* AML patient blasts. No significant difference in PD-L1 expression was detected in LSC compared to non-LSC cells. LSC: leukemia stem cells; FSC: forward-scattered light; FITC: fluorescein isothiocyanate.

regard to the ribonucleic acid (RNA) level (Figure 1H), in contrast to FACS analysis. Nevertheless, microarray analysis comparing enriched LSC populations of $NPM1^{wt}$ and $NPM1^{mut}$ AML patients underlined the potential relevance of the immune system in $NPM1^{mut}$ AML patients, as highly immunological regulatory pathways are differentially regulated in $NPM1^{mut}$ compared to $NPM1^{wt}$ samples.[5] However, aside from the PD-L1 expression in this patient population, a high number of other relevant factors e.g., other molecular markers, different characteristics of subclones, the microenvironment and niche of leukemic and normal stem cells, and other immune checkpoint molecules may influence immune reactions against $NPM1^{mut}$ AML cells and hence, the outcome of the patients.

LSC might be the source of leukemic disease relapse following treatment, making them a critical target for further therapeutic options. Immunotherapy in cancer treatment has experienced a breakthrough in recent years. Consequently, in addition to allogenic stem cell transplantation, T-cell activating immunotherapeutic approaches like immune checkpoint inhibition, chimeric antigen receptor T cells (CARs) or bi-specific T-cell activating antibodies are becoming increasingly important treatment strategies.[3,6,7] Genetic instability of tumors increases the neoantigen load and might therefore be a prerequisite for a broader spectrum of anti-tumor immune responses.[8] However, mechanisms of immune responses and responsible antigen structures have to be further investigated. In AML, the mutational load is lower compared to most solid tumor entities,[8] but genetic instability and neoantigen load might depend on the AML subtype. The expression of PD-L1 (CD274) seems to be a predictive marker for the response to PD-1/PD-L1-directed immunotherapies in different solid and hematological malignancies,[9] however, there are further challenges to face and overcome in order to clarify the role of PD-L1 expression for immune checkpoint inhibition. In accordance, our findings underline that $NPM1^{mut}$ patients might be better candidates for immune checkpoint PD-1/PD-L1-driven immunogenic approaches than other AML subtypes. For example, immune checkpoint inhibition utilized as a maintenance therapy following chemotherapy might be able to prevent relapse, especial-

ly as the immune response against leukemic cells is stronger in *NPM1*^{mut} compared to *NPM1*^{wt} AML patients.[2] Thus, PD-1/PD-L1 inhibition in *NPM1*^{mut} AML might further boost immune responses, which are possibly responsible for the high cure rate in this AML cohort.

Moreover, immune responses induced by immune checkpoint molecules may result in the activated effector T cells being able to lyse not only NPM1-positive, but also NPM1-negative subclones, independent from their PD-1/PD-L1 status, due to a broader stimulation of these T cells. Markedly, genes with immunological functions seem to play an important role in the *NPM1*^{mut} AML subtype,[5] and as such could also induce immune responses against subclones without *NPM1* mutation. These facts might explain why the existence of *NPM1*^{mut} clones could mirror the overall immunogenicity of a genetically unstable underlying disease.

In patients relapsing after allogeneic hematopoietic stem cell transplantation, reactivating the immune response *via* immune checkpoint has been demonstrated to produce a response,[10] as has treatment with hypomethylating agents which have the ability to enhance the expression of PD-1/PD-L1.[11] Thus, the combination of immunotherapies holds great promise to further improve the efficacy of T cells against cancer cells.[7,12] A high number of immunotherapies are currently under investigation as part of combination therapies, e.g., in combination with vaccination strategies. As mutated NPM1 is an immunogenic neoantigen with epitopes derived from the mutational region of NPM1 inducing specific immune responses in over 75% of patients,[1] it is an interesting immunogenic target structure, and *NPM1*^{mut}-derived peptides might be used in combination with immune checkpoint inhibition to prevent AML relapse. Moreover, polyvalent immune responses of CTL against *NPM1*^{mut} specific and known leukemia-associated antigens in *NPM1*^{mut} AML might also be further exploited to achieve negativity for *NPM1*^{mut} MRD.[13]

Today, due to the good prognosis of most *NPM1*^{mut} AML cases, the majority of *NPM1*^{mut} patients do not receive an allogeneic stem cell transplantation in first complete remission. However, Röllig and colleagues demonstrated a very favorable clinical outcome for *NPM1*^{mut} patients undergoing upfront allogeneic stem cell transplantation.[14] This approach further supports the positive effects of the immune system in *NPM1*^{mut} leukemia eradication. In addition, Kuželová and colleagues noted that AML patients expressing certain groups of human leukocyte antigen alleles are predisposed to develop an efficient anti-AML immune response against the cytoplasmatic located NPM1^{mut} protein.[15]

In summary, we detected higher PD-L1 expression in *NPM1*^{mut} patients, especially in the leukemic progenitor/stem cell compartment of *NPM1*^{mut} AML patients. This observation further supports the hypothesis that NPM1-directed immune responses might play an important role in tumor cell rejection, which tumor cells try to escape *via* the expression of PD-L1. Therefore, in *NPM1*^{mut} AML cases the immunogenicity of neoantigens derived from the NPM1 mutation and the higher CD274 expression constitute promising target structures for individualized immunotherapeutic approaches. PD-1/PD-L1-directed immune checkpoint inhibition approaches to enhance *NPM1*^{mut} specific T-cell responses might be combined with antigen-directed immunotherapies e.g., peptide vaccination against immunogenic NPM1 epitopes, in order to eradicate persisting MRD following conventional chemotherapy.

Jochen Greiner,[1,2] Susanne Hofmann,[1,3] Michael Schmitt,[3] Marlies Götz,[1] Markus Wiesneth,[4] Hubert Schrezenmeier,[4] Donald Bunjes,[1] Hartmut Döhner[1] and Lars Bullinger[1]

[1]Department of Internal Medicine III, University of Ulm; [2]Department of Internal Medicine, Diakonie Hospital Stuttgart; [3]Department of Internal Medicine V, University of Heidelberg and [4]Institute of Clinical Transfusion Medicine and Immunogenetics Ulm, German Red Cross Blood Transfusion Service Baden-Württemberg-Hessen and Institute of Transfusion Medicine, Ulm, Germany

Funding: this work was supported in part by research funding from BMS (Bristol-Myers Squibb) to JG, and funds from the Deutsche Forschungsgemeinschaft (Heisenberg-Professur BU 1339/8-1 and SFB 1074 project B03) to LB.

Correspondence: greiner@diak-stuttgart.de
doi:10.3324/haematol.2017.176461

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Greiner J, Ono Y, Hofmann S, et al. Mutated regions of nucleophosmin 1 elicit both CD4(+) and CD8(+) T-cell responses in patients with acute myeloid leukemia. Blood. 2012;120(6):1282-1289.
2. Greiner J, Schneider V, Schmitt M, et al. Immune responses against the mutated region of cytoplasmatic NPM1 might contribute to the favorable clinical outcome of AML patients with NPM1 mutations (NPM1mut). Blood. 2013;122(6):1087-1088.
3. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015;27(4):450-461.
4. Hude I, Sasse S, Engert A, Brockelmann PJ. The emerging role of immune checkpoint inhibition in malignant lymphoma. Haematologica. 2017;102(1):30-42.
5. Schneider V, Zhang L, Bullinger L, et al. Leukemic stem cells of acute myeloid leukemia patients carrying NPM1 mutation are candidates for targeted immunotherapy. Leukemia. 2014;28(8):1759-1762.
6. Fesnak AD, June CH, Levine BL. Engineered T cells: the promise and challenges of cancer immunotherapy. Nat Rev Cancer. 2016; 16(9):566-581.
7. Ott PA, Hodi FS, Kaufman HL, Wigginton JM, Wolchok JD. Combination immunotherapy: a road map. J Immunother Cancer. 2017;5:16.
8. Lawrence MS, Stojanov P, Polak P, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013; 499(7457):214-218.
9. Spencer KR, Wang J, Silk AW, Ganesan S, Kaufman HL, Mehnert JM. Biomarkers for immunotherapy: current developments and challenges. Am Soc Clin Oncol Educ Book. 2016;35:e493-503.
10. Albring JC, Inselmann S, Sauer T, et al. PD-1 checkpoint blockade in patients with relapsed AML after allogeneic stem cell transplantation. Bone Marrow Transplant. 2017;52(2):317-320.
11. Yang H, Bueso-Ramos C, DiNardo C, et al. Expression of PD-L1, PD-L2, PD-1 and CTLA4 in myelodysplastic syndromes is enhanced by treatment with hypomethylating agents. Leukemia. 2014;28(6):1280-1288.
12. Martin-Liberal J, Ochoa de Olza M, Hierro C, Gros A, Rodon J, Tabernero J. The expanding role of immunotherapy. Cancer Treat Rev. 2017;54:74-86.
13. Hofmann S, Gotz M, Schneider V, et al. Donor lymphocyte infusion induces polyspecific CD8(+) T-cell responses with concurrent molecular remission in acute myeloid leukemia with NPM1 mutation. J Clin Oncol. 2013;31(3):e44-47.
14. Rollig C, Bornhauser M, Kramer M, et al. Allogeneic stem-cell transplantation in patients with NPM1-mutated acute myeloid leukemia: results from a prospective donor versus no-donor analysis of patients after upfront HLA typing within the SAL-AML 2003 trial. J Clin Oncol. 2015;33(5):403-410.
15. Kuzelova K, Brodska B, Fuchs O, Dobrovolna M, Soukup P, Cetkovsky P. Altered HLA class I profile associated with type A/D nucleophosmin mutation points to possible anti-nucleophosmin immune response in acute myeloid leukemia. PloS One. 2015; 10(5):e0127637.