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ORIGINAL PAPER

Enhanced stimulation of antigen-specific immune responses against nucleophosmin 1 mutated acute myeloid leukaemia by an anti-programmed death 1 antibody

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Summary

Nucleophosmin1 (NPM1) is one of the most commonly mutated genes in AML and is often associated with a favourable prognosis. Immune responses play an increasing role in AML treatment decisions; however, the role of immune checkpoint inhibition is still not clear. To address this, we investigated specific immune responses against NPM1, and three other leukaemia-associated antigens (LAA), PRAME, Wilms' tumour 1 and RHAMM in AML patients. We investigated T cell responses against leukaemic progenitor/stem cells (LPC/LSC) using colony-forming immunoassays and flow cytometry. We examined whether immune checkpoint inhibition with the anti-programmed death 1 antibody increases the immune response against stem cell-like cells, comparing cells from NPM1 mutated and NPM1 wild-type AML patients. We found that the anti-PD-1 antibody, nivolumab, increases LAA stimulated cytotoxic T lymphocytes and the cytotoxic effect against LPC/LSC. The effect was strongest against $NPM1^{mut}$ cells when the immunogenic epitope was derived from the mutated region of NPM1 and these effects were enhanced through the addition of anti-PD-1. The data suggest that patients with NPM1 mutated AML could be treated with the immune checkpoint inhibitor anti-PD-1 and that this treatment combined with NPM1-mutation specific directed immunotherapy could be even more effective for this unique group of patients.

KEYWORDS
acute myeloid leukaemia, anti-PD-1, NPM1 mutation, nucleophosmin 1

INTRODUCTION

Acute myeloid leukaemia (AML) is a genetically heterogeneous malignancy characterized by clonal expansion of immature and abnormal myeloid blasts leading to bone marrow failure; thus, it is a disease originating from malignant clonal stem cells in the bone marrow.[1]

In recent years, new strategies for the treatment of AML have been established.[2–5] Cellular approaches such as allogeneic haematopoietic stem cell transplantation and donor

lymphocyte infusions have long been part of routine clinical practice.[6] The approach is based on the graft-versus-leukaemia effect, in which allogeneic T cells recognize target antigens presented on malignant cells and this remains one of the best treatment options for patients who can tolerate the rigours of the treatment. However, there are a high number of patients who relapse after intensive treatment[7] and immunotherapeutic approaches, especially those targeting leukaemic progenitor/stem cells (LPC/LSC), could be an option to prevent disease relapse. In this context, the potency of immunotherapeutic approaches are becoming increasingly clear[8,9] with a variety, including monoclonal antibodies, cytokines, immunomodulatory agents, as well as cellular immunotherapies such as vaccination, dendritic cell treatment, T cell activating antibodies such as immune checkpoint inhibitors, bispecific antibodies and chimeric antigen receptor modified T cells (CARs) being explored (recently reviewed in[10]). However, to date there has been a lack of a frequently expressed surface antigen that could act as a target for antibody and CAR-T cell treatments suitable for adult AML patients. Redirected T-cell receptor T cells could target intracellular antigens presented in the context of MHC (recently reviewed in[11]). Indeed LPC/LSC are postulated to be the source population of relapsing AML[12] and could also provide targets for immunotherapeutic strategies.

We have previously demonstrated the potential of several leukaemia-associated antigens (LAA; preferentially expressed antigen in melanoma [PRAME - P300], Wilms' tumour 1 [WT1], receptor for hyaluronan-mediated motility (RHAMM - R3) and the mutation specific nucleophosmin1 [NPM1]), which is found in nearly one-third of newly diagnosed patients,[13] can generate T cell-specific immunogenic responses against leukaemic progenitor and stem cells.[14] NPM1 is the most common genetic alteration in adults with AML,[15] causing distinct biological and clinical features. NPM1 is a high conserved nucleo-cytoplasmic shuttling protein, mutations in which cause aberrant cytoplasmic dislocation which led the World Health Organization (WHO) to recognize AML patients with a NPM1-mutation as a distinct entity.[16] Generally, the presence of the NPM1 mutation, a four-base insertion in exon 12 of the NPM1 gene, and a normal karyotype, is associated with a favourable outcome for patient survival following induction and consolidation chemotherapy.[17,18] In this study we compared anti-LAA activity by cytotoxic T lymphocyte (CTL) from $NPM1^{mut}$ and $NPM1^{wt}$ AML patients. Furthermore, we investigated whether these immune responses could be additionally stimulated by a programmed cell death protein 1 (PD-1) targeting antibody.

METHODS

Sample preparation, isolation and freezing

Peripheral blood samples were evaluated from an extended cohort of 30 AML (15 AML *NPM1*^{mut} and 15 AML *NPM1*^{wt}) patients following informed consent and in accordance with the Declaration of Helsinki. The local ethics committee (no. 334/09 and no. 221/14) approved the study protocol. Peripheral blood mononuclear cells (PBMC) from healthy donors (HD) were separated by Bicol (Pan Biotech) density gradient centrifugation, cryopreserved and stored in liquid nitrogen. All patient samples consisted of more than 90% leukaemic blasts. Healthy volunteer samples were obtained from the German Red Cross Ulm.

Viral- and leukaemia-associated antigens

Cytomegalovirus (CMV [NLVPMVATV]) peptide served as a positive control, no peptide in CFI served as a negative/growth control. The following LAA were chosen according to previous analyses,[12] PRAME (P300 [ALYVDSLFFL]), RHAMM (R3 [ILSLELMKL]), WT1 [RMFPNAPYL]) and NPM1 [AIQDLCVAV] only for AML $NPM1^{mut}$ patients). We used this NPM1 peptide, because it induced a higher frequency of T cell responses in all AML patients.[19] All peptides were HLA-A2 restricted.

Patient characteristics and selection of LAA

We screened more than 150 different AML primary patient samples to check their potential to form CFU. Of these, 30 AML patient samples could be evaluated in CFI assays as to the growth and inhibition of stem and progenitor cells in connection with CTL and/or the checkpoint inhibitor anti-PD-1. To define the potential of specific CTL, allogeneic T cells were stimulated against various LAA in mixed lymphocyte peptide culture (MLPC). Only HLA-A2 positive patient samples and HDs were used, since all LAA were HLA-A2-restricted.

Mixed lymphocyte peptide culture (MLPC)

In MLPC, peptide specific CD8+ allogeneic T cells were generated from HD samples, providing effector cells (E) for further assays. Briefly, samples were thawed, counted and divided in two portions. One fraction, employed as antigen presenting cells (APC), were irradiated with 30 Gy and pulsed with the respective peptides for 1.5 h at 37°C. Thereafter APC were mixed with the second fraction denominated effector cells (E) at a ratio of 1:1. On day two IL-2 (2.5 ng/ml) and IL-7 (20 ng/ml) were added and incubated for 7–9 days and then used for functional assays.

Addition of nivolumab to cell culture

In line with the results of a former titration,[14] 5 µg of the anti-PD-1 antibody, nivolumab, was added to the MLPC on day 0 to the respective wells containing the E fraction for 1 h then the irradiated and stimulated APC fraction was added.

In this way, the direct effect of anti-PD-1 on CD8+ T cells was measured. CD8+ T cells were stimulated with CMV or the respective LAA only as control.

Colony-forming immunoassays

Allogeneic T cells from MLPC were used as effectors and the ratio of effector (E): target (T) was 5:1 or 10:1, depending on the number of effectors gained. Primary patient cells served as T and T stimulated with no peptide served as a growth control. E and T were incubated together at 37°C for 4 h and resuspended in IMDM-medium 2% FCS and added to 3 ml HSC-CFU complete medium (Miltenyi Biotech), then drawn into a syringe. Then, 1.1 ml medium was placed into each cell culture dish (Thermo Scientific). Colonies were analysed after a 14-day incubation time, the difference between control and sample in percent was calculated and displayed. For individual representative CFI assay images with or without CTL conditioning please see Figure S1.

Enzyme-linked-immuno-spot (ELISPOT)

Membrane bottom 96 well plates were coated with a solid antibody phase. Subsequently, the membranes were incubated with allogeneic prestimulated peripheral blood lymphocytes from MLPC and APC from leukaemia patients at a ratio of 5:1. The cytokines bound to the solid antibody phase were visualized by specific, biotin-coupled antibodies, alkaline phosphatase and by the appropriate substrate. The evaluation was carried out by an ELISPOT reader. IFNγ (Mabtech) and granzyme B (BD) ELISPOTs were performed according to the manufacturer's instructions.

Statistical analysis

Statistical tests were performed using GraphPad PRISM version 8. The program was also used to evaluate assays, for comprehensive analysis, for organizing data and for graphing. As a statistical analysis we used the ordinary one-way Anova test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

RESULTS

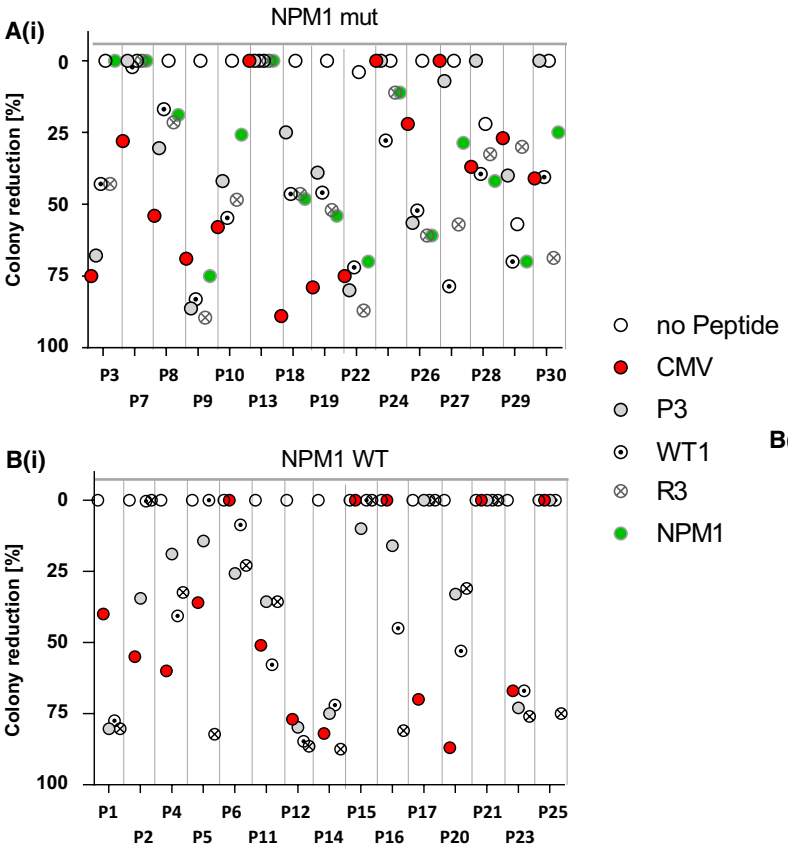
There was a reduction in the number of colonies (colony forming units; CFU) in colony forming immunoassays (CFI) when using cells from AML $NPM1^{mut}$ patients (Figure 1A) and AML $NPM1^{WT}$ patients (Figure 1B). Samples from 13/15 (87%) $NPM1^{mut}$ AML patients showed immune responses against at least one LAA and 9/15 (60%) against all four LAA. Mean colony reductions for LAA were as followed: 35% for $NPM1^{mut}$, 32% for P300, 45% for WT1 and 43% for R3. In $NPM1^{WT}$ AML patient samples the inhibitory potential of all four LAA were detected in 8/15 (53%) samples. Mean colony reduction for LAA were 33% for P300, 34% for WT1 and 46% for R3. As expected, in $NPM1^{WT}$ patients no responses were found against the NPM1 epitope, and responses against the other antigens were comparable to those of $NPM1^{mut}$ patients, although immune responses varied slightly depending on the antigen and the patient. All data was generated from three independent assays.

A further inhibition in CFU was apparent following the addition of anti-PD-1 (Nivolumab) to CTL for several days before starting CFI. CTL from HD led to an inhibition in CFI of AML $NPM1^{mut}$ patients when the target LAA was P300 (range 0%–81%; mean: 32%), WT1 (range 0%–74%; mean: 22%) or R3 (range 0%–75%; mean: 21%) (Figure 2A). When CTL from AML $NPM1^{WT}$ patients were used to target LAA in the presence of anti-PD-1, the number of CFUs ranged from 0% to 59%; mean: 25% for P300, ranged from 0% to 1%; mean: 34% for WT1 and 0%–60%; mean: 23% for R3 (Figure 2B). When CTL from AML $NPM1^{mut}$ and $NPM1^{WT}$ patients were used to target LAA in the presence of anti-PD-1, the number of CFUs ranged from 0% to 81%; mean: 28% for P300, ranged from 0% to 81%; mean: 28% for WT1, 0%–75% range; mean: 22% for R3 and 0%–82% range; mean: 47% for NPM1 (Figure 2C). The inhibition in CFI was calculated for each antigen separately, thus the anti-PD-1 effect was shown to be antigen-dependent. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Immune effects increased considerably in $NPM1^{mut}$ patients when incubated with an LAA or NPM1 and this was furthered when anti-PD-1 was added to CTL to be used in CFI with a mean reduction of 47%. 12 of 15 $NPM1^{mut}$ patients (80%) showed a reduction in CFU with the LAA NPM1 and all 15 showed a reduction with anti-PD-1. A reduction in CFI of 50% or more was observed in 7/15 $NPM1^{mut}$ AML patient samples. Four of 15 patients showed a slightly lower additional reduction in the presence of anti-PD-1 in the range of 44%–28% (Figure 3A). Figure 3B shows an overview of all 15 NPM1mut AML patients. The reduction due to the addition of anti-PD-1 was very significant. The ELISPOT results for IFNγ (Figure 3C) and granzyme B (Figure 3D) of NPM1mut patients nos. 9, 10 and 22 with the mutation-specific peptide NPM1 showed an average fold change of 1.5 for IFNγ and 3.4 for granzyme B. Data was generated from three independent assays.

Fluorescence-activated cell scanning (FACS) analyses of 30 $NPM1^{mut}$ and 30 $NPM1^{WT}$ AML patient samples (Figure 4) showed that progenitor and stem cells from $NPM1^{mut}$ patients in particular have significantly higher PD-L1 expression than those from $NPM1^{WT}$ patients. The expression levels in $NPM1^{mut}$ stem-like cells ranged from 0% to 48% (mean: 7%) and in bulk cells from 0% to 11% (mean 1.43%), whereas in $NPM1^{WT}$ AML the expression levels were considerably lower. In stem cell-like cells expression ranged from 0% to 3% (mean: 3.3%) and in bulk cells from 0% to 1% (mean 0.24%).

FACS analyses of the detached cell mixture from CFI after 20 days of incubation showed that there was still a number of LPC/LSC in CFI detectable and that numbers were comparable with the addition of NPM1-specific T cells and with



A(ii)

mut Pts.	P300	WT1	R3	NPM1
Pt. 3	68	43	43	0
Pt. 7	14	14	30	37
Pt. 8	31	17	22	19
Pt. 9	86	83	90	75
Pt. 10	42	55	48	26
Pt. 13	0	0	0	0
Pt. 18	29	0	0	23
Pt. 19	39	46	52	54
Pt. 22	80	72	87	70
Pt. 24	0	0	75	0
Pt. 26	57	52	61	61
Pt. 27	7	79	57	29
Pt. 28	0	40	33	42
Pt. 29	40	70	30	70
Pt. 30	0	41	69	25
mean	**33**	**41**	**46**	**35**

B(ii)

WT Pts.	P300	WT1	R3
Pt. 1	80	78	80
Pt. 2	35	0	0
Pt. 4	19	41	32
Pt. 5	14	0	82
Pt. 6	26	9	23
Pt. 11	36	58	36
Pt. 12	80	85	87
Pt. 14	75	72	88
Pt. 15	10	0	0
Pt. 16	16	45	81
Pt. 17	0	0	0
Pt. 20	33	53	31
Pt. 21	0	0	0
Pt. 23	73	67	76
Pt. 25	0	0	75
mean	**33**	**34**	**46**

FIGURE 1 T cells derived from HDs were stimulated with LAA in mixed lymphocyte peptide culture (MLPC) and shown to inhibit CFU formation. Allogeneic CTL from HD (A) showed an average inhibition in 15 $NPM1^{mut}$ AML patients for P300 of 32%, for WT1 of 45%, for R3 of 43% and for NPM1 of 35%. The inhibitory potential ranged from 0% to 90% and there was a great variety in immune responses. CTL responses in (B) 15 $NPM1^{WT}$ patients led to an average inhibition of colony growth for P300 of 33%, for WT1 of 34% and for R3 of 46%. The inhibitory potential for the LAA were comparable between $NPM1^{mut}$ and $NPM1^{WT}$ patients. In 13 of 15 $NPM1^{mut}$ and 12 of 15 $NPM1^{WT}$ patients, T cells were activated against at least one LAA and successfully decreased the colony number in CFI assays. $N = 3$ number of different assays. [Colour figure can be viewed at wileyonlinelibrary.com]

the addition of NPM1 and anti-PD-1 to CFI (Figure 5A). The control contained unstimulated T cells. The effect of the presence of CD8+ T cells was demonstrated in CFI through the use of NPM1 stimulated T cells and NPM1 stimulated T cells in addition to anti-PD-1 (Figure 5B). The control contained patient cells only (NPM1 vs. control $p = 0.0003$; NPM1 PD-1 vs. control $p = 0.0007$). We examined CD33 expression in $NPM1^{mut}$ patient cells after 20 days of CFU culture (Figure 5C). These findings represent patients with AML arising from predominantly committed myeloid precursors. CD33, a maturation marker, is slightly decreasing compared to the control, however this is hard to interpret in the detached CFU, since all cells in culture are detached, not only the colonies. We detected a $CD33^{+}$ cell population in FACS analysis, which we expected in $NPM1^{mut}$ CFI assays, but we could see a difference if cultures were stimulated with the NPM1 peptide or when anti-PD-1 was included. The control contained unstimulated T cells. $n = 5$ number of patients. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Individual representative CFI assay images of three patients (nos. 8, 9 and 19) with or without CTL conditioning are shown in Figure S1.

DISCUSSION

We have previously shown that the immune checkpoint inhibitor PD-1 can work in combination with peptide-directed approaches in AML to inhibit LPC/LSC.[14] In this study we showed that 87% of $NPM1^{mut}$ AML patients had immune responses against at least one LAA and 60% of patients against all four LAA ($NPM1^{mut}$, PRAME P300, RHAMM R3, WT1) studied. When examining colony forming immunoassays, $NPM1^{WT}$ patients showed no response against the $NPM1^{mut}$ epitope as expected but their responses against the other three antigens were comparable to those of $NPM1^{mut}$ patients, with variations depending on the patient and the antigen. The reduction in CFUs was enhanced when anti-PD-1 was added to CTLs for several days before starting CFI. For $NPM1^{mut}$ patients the largest reduction of CFU in CFIs occurred when PRAME was the target, while for $NPM1^{wt}$ it was WT1. However, the ranges in CFU were from 0% to 81% in each and ≥0% to 59% for the other LAA. Immune effects increased considerably in $NPM1^{mut}$ patients when the LAA NPM1 was a target. CTLs from 12 of 15 $NPM1^{mut}$ patients led to a reduction in CFU when NPM1 was the target. In

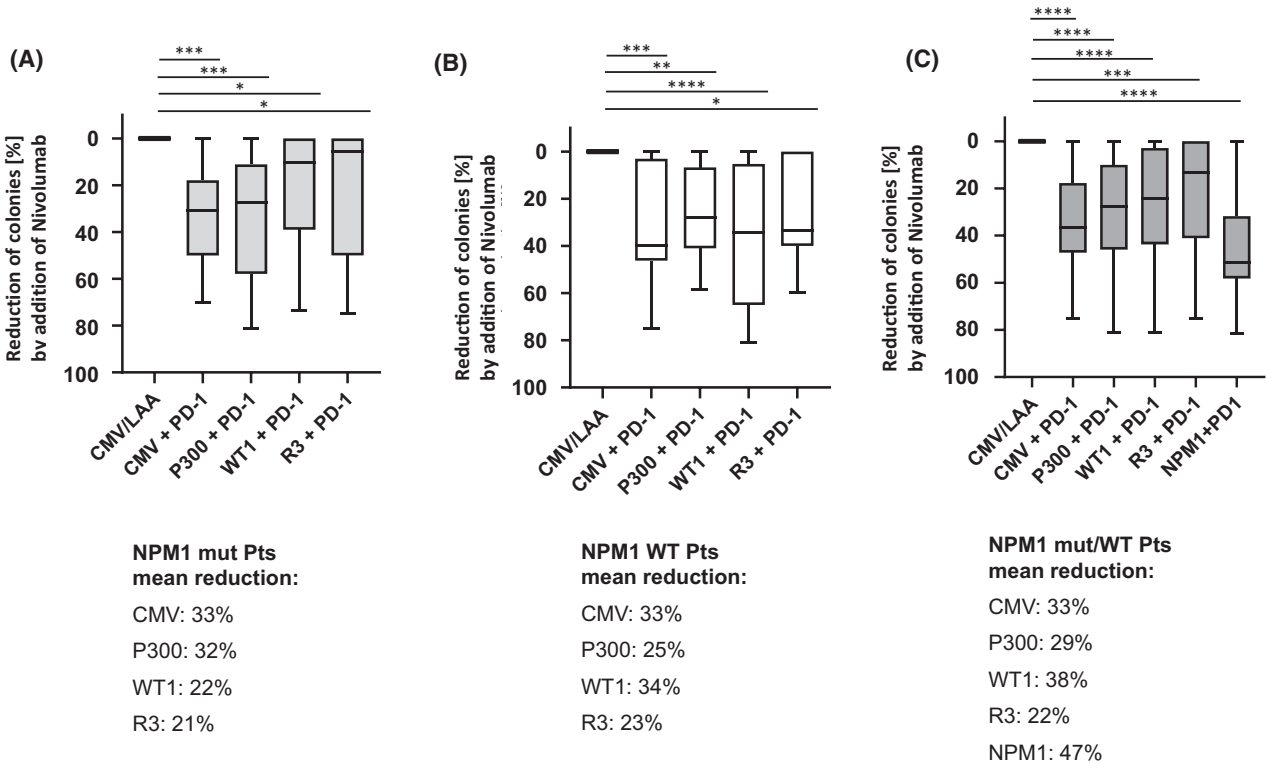


FIGURE 2 NPM1^{mut} versus NPM1^{WT} patients functional CFI assays were used to analyse the immunogenicity of LAA and PD-1 stimulated CD8+ allogeneic T cells on LPC/LSC. The number of colonies for CMV/LAA without anti-PD-1 antibodies served as a control. All CMV/LAA combined with anti-PD-1 antibodies showed significant inhibitory capacity in both cohorts. Mean reduction in colonies following the addition of nivolumab for (A) NPM1^{mut} AML (grey; CMV $p = 0.0005$, P300 $p = 0.0008$, WT1 $p = 0.0317$, R3 $p = 0.0348$; (B) NPM^{WT} AML (white; CMV $p = 0.0001$, P300 $p = 0.0047$, WT1 $p = < 0.0001$, R3 $p = 0.0116$). Both cohorts were comparable; (C) for 15 NPM1^{mut} and 15 NPM^{WT} AML (dark grey; CMV $p = < 0.0001$, P300 $p = < 0.0001$, WT1 $p = < 0.0001$, R3 $p = 0.0004$ and NPM1 $p = < 0.0001$). $N = 3$ number of different assays. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

addition, all 15 AML patients showed a reduction in CFU that was enhanced when anti-PD-1 was added to CTL prior to their use in CFI.

Progenitor and stem cells from NPM1^{mut} patients in particular were shown to have significantly higher PD-L1 protein expression (by flow cytometry) than those from NPM1^{WT} patients. Analysis of the CFU showed that the majority of cells were CD34^{+} and CD38^{-} and this was maintained in the presence of NPM1 stimulated and NPM1 stimulated/anti-PD-1 T cells. In CFUs the percentage of CD8+/CD4- cells increased when NPM1 peptide or NPM1 peptide and anti-PD-1 were added. CD33^{+} numbers decreased somewhat in the presence of NPM1, and in the presence of NPM1 and anti-PD-1.

However, even within the NPM1 mutated patient group there was a heterogeneity of genetic backgrounds[20] that can drive AML with different outcomes (recently reviewed in[21]). Indeed, Mer et al.[20] recently identified "primitive" LSC rich and "committed" subgroups within the NPM1^{mut} AML patients with a significantly worse survival being associated with the former group (log-rank test $p = 0.002$) as well as increased sensitivity for kinase inhibitors (sorafenib, sunitinib and ruxolitinib) being demonstrated.

In this study we were able to show that immunologically, T cells from AML patients with NPM1 mutations as well as those without could be stimulated to respond to LAA. There were, as expected, no responses by T cells from NPM1^{WT} patients against the NPM1 epitope. However, the T cells, indifferent of the NPM1 status of the AML patients, responded very similarly to the other antigens under investigation (PRAME, RHAMM and WT1) although there were slight variations between immune responses depending on the antigen and patient. We choose to examine anti-PRAME, RHAMM and WT1 responses due to their elevated frequency of expression in AML (30%, 60% and 12.6%, respectively).[22–24] Other frequently expressed genetic aberrations in AML such as FLT3-ITD (30%)[25] or cancer-testis antigens such as PASD1 (33%)[26] and HAGE (23%)[27] could have been examined but the focus on PRAME, RHAMM and WT1 provided the necessary proof-of-principal as to whether T cells from NPM1^{mut} or NPM1^{WT} patients differed in their responses to other LAA, in the presence or absence of anti-PD-1, as determined by CFI and FACs-based phenotyping of targets and effectors.

PD-L1 (also known as B7-H1) binds to the PD-1 receptor on activated T cells, suppressing anti-tumour immunity through the inhibition of T cell activation and cytokine production. In solid tumours PD-L1 overexpression is common and associated with poor clinical outcomes[28] and PD-1 expressing Tregs have been shown to play a crucial role in regulating the peripheral immune response.[29] The expression of PD-L1 on AML cells is somewhat controversial, being both present[30] and elevated in the periphery and bone marrow of

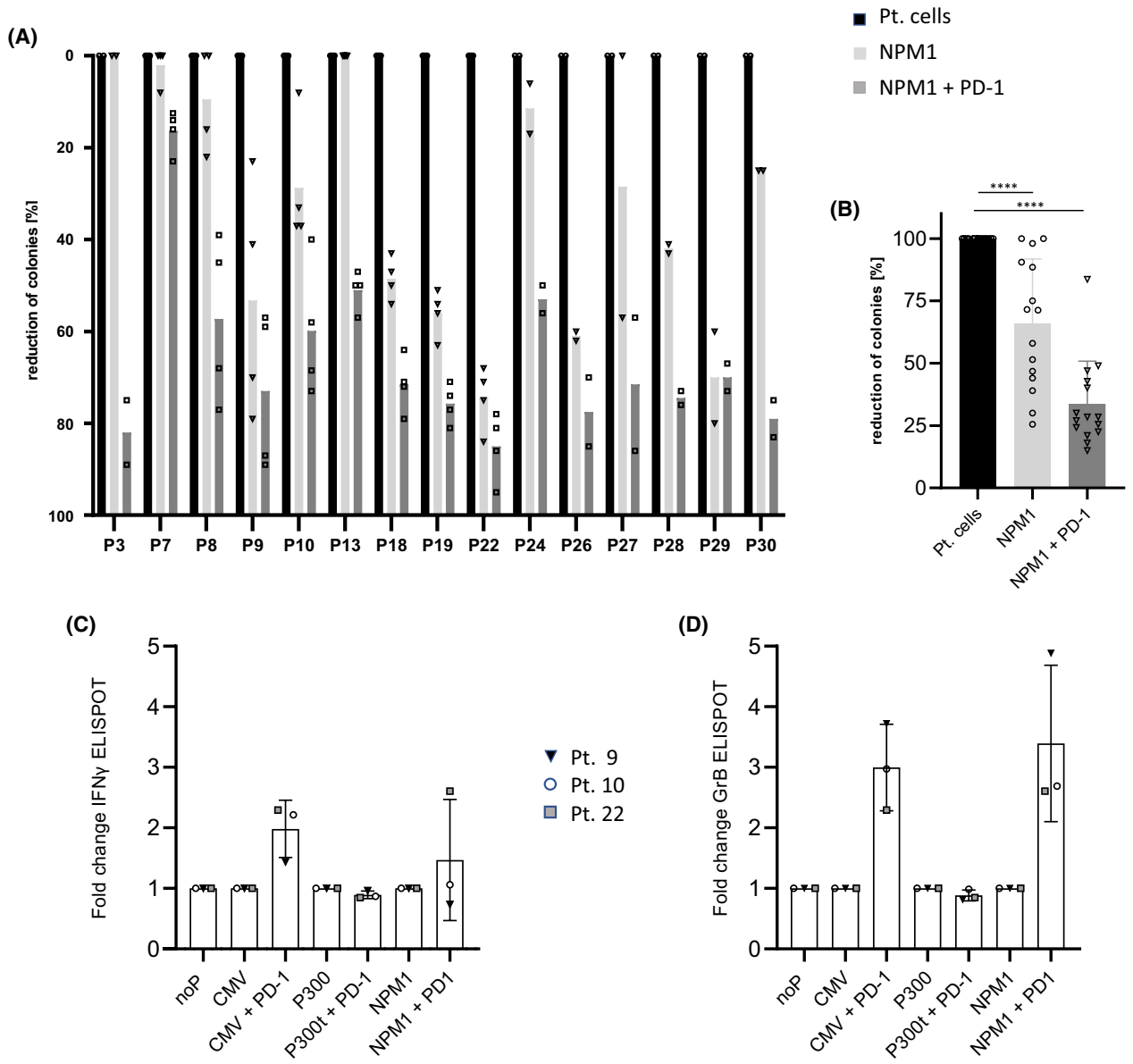


FIGURE 3 NPM1^{mut} patients functional CFI assays showed immunogenicity of the mutation related LAA NPM1 with/without PD-1. (A) Patient cells alone served as growth controls (black bars). NPM1^{mut} stimulated CTL induced immune responses in 12/15 (80%) patient samples (light grey bars), whereas immune responses against the LAA NPM1^{mut} were detectable in 15/15 patient samples when anti-PD antibodies were also added (dark grey bars). By addition of anti-PD-1 antibodies, in 11/12 (92%) patient samples with NPM1^{mut} mediated immune responses showed additional colony reduction which ranged from 29% to 71% with an average of 49%. Particularly strong additional reduction with PD-1 in CFI of 50% or more was observed in 7/15 (47%) NPM1^{mut} AML patient samples; (B) summary of all 15 NPM1 mutated AML patients. Patient (Pt) cells alone served as growth control (black bars), patient cells with NPM1^{mut} stimulated CTL (light grey bars; $^{****}p < 0.0001$) and patient cells withNPM1^{mut} stimulated CTL with addition of anti-PD-1 (dark grey bars; $^{****}p < 0.0001$); $n = 3$ number of assays; (C) IFNγ ELISPOT and (D) granzyme B ELISPOT of 3 exemplary NPM1 mutated AML patients (patient 9, 10 and 22). The fold change is shown in relation to no peptide/the respective LAA. [Colour figure can be viewed at wileyonlinelibrary.com]

AML patients.[31] Despite earlier studies that indicated that PD-L1 expression in AML cells failed to directly influence T cell activation, proliferation nor cytokine production,[32] Dong et al.[33] recently showed that PD-L1 expression on AML cells promoted Treg expansion and that PD-1 signalling blockage by anti-PD-L1 impaired Treg production and slowed tumour development in a mouse model of AML. In addition, Dong et al. showed that the frequency of intratumour PD-1+ Tregs was able to predict AML patient survival.

The PD-1 signal blocking antibody, nivolumab has been used to treat high risk AML patients (3 mg/kg iv every 2 weeks, after cycle 6 every 4 weeks and after cycle 12 every 3 months until disease relapse) in a single-arm, open label, phase II study[34] and demonstrated the safety and feasibility of maintenance Nivolumab. In the study of Reville et al.[34] 15 high-risk acute myeloid leukaemia patients were enrolled three of these had an NPM1 mutation. The low number of NPM1 patients enrolled in this trial may be attributed to the

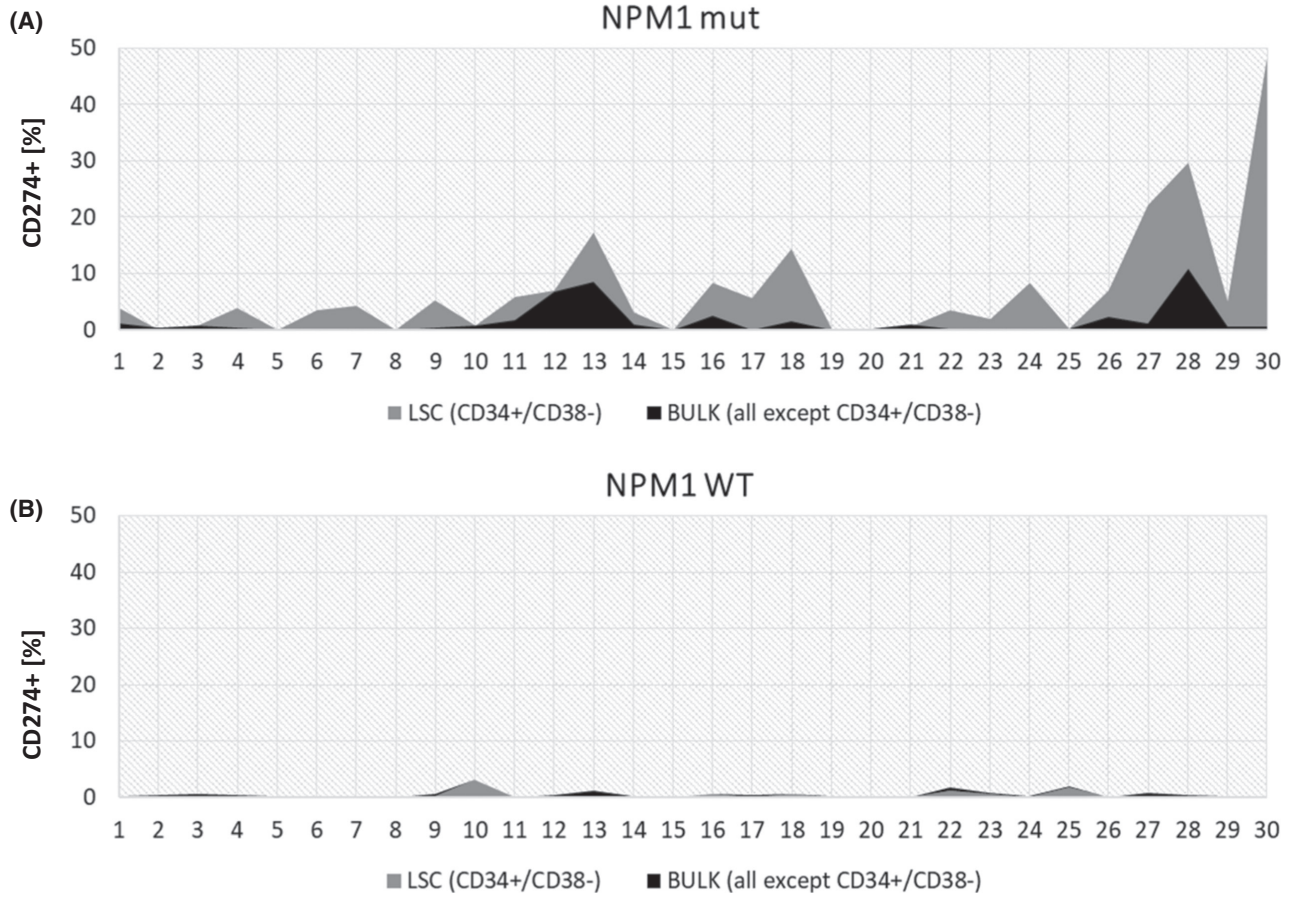


FIGURE 4 Comparing bulk versus LPC/LSC PD-L1 (CD274) expression in 30 NPM1^{mut} and 30 NPM1 WT AML patients. FACS analysis demonstrated that LPC/LSC showed higher PD-L1 expression levels than bulk cells (all cells except CD34^{+}/CD38^{-}). Progenitor and stem cells from NPM1 mutated patients (A) in particular had significantly higher PD-L1 expression than those from NPM1 wild-type patients (B). The expression levels in NPM1^{mut} stem-like cells ranged from 0% to 48.4% (mean: 7.01%) and in bulk cells from 0.03% to 10.8% (mean 1.43%), whereas in NPM1 WT AML the expression levels were considerably lower. In stem-like cells expression ranged from 0% to 3.04% (mean: 3.31%) and in bulk cells from 0% to 1.05% (mean 0.24%).

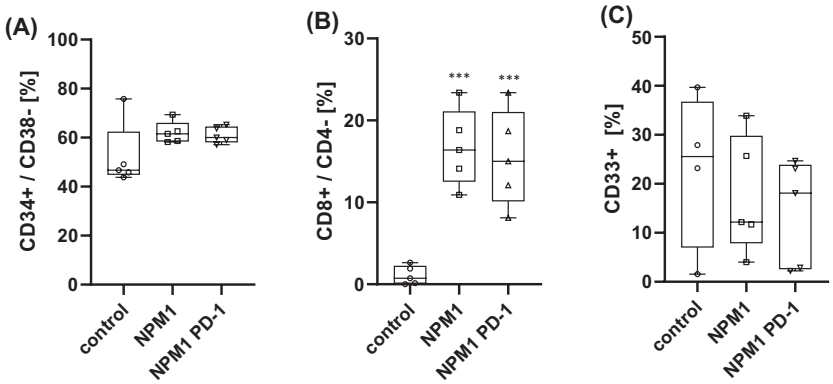


FIGURE 5 Flow cytometry of extracted colonies from CFI of NPM1^{mut} patients. A population of (A) CD34^{+}/CD38^{-} stem-like cells could be detected in CFI cultures. These numbers were consistent between test groups. There was (B) a significant increase in the frequency of CD8+ T cells in populations cultured with the NPM1^{mut} peptide in CFIs, and even more so following the addition of NPM1^{mut} peptide and anti-PD-1. The control contained patient cells without CTL. In (C) we detected a CD33^{+} cell population, which was slightly decreased in the presence of NPM1^{mut} and more so NPM^{mut} peptide and anti-PD-1. The results are from five representative NPM1^{mut} patients.

fact that NPM1 mutated patients are usually a favourable patient group and not a high-risk group. Although allo-stem cell transplantation remains the most effective post-remission therapy[35] it is not universally available. Maintenance oral azacytidine has been shown to improve relapse-free (p = 0.0001) and overall survival (p = 0.0009).[36] Our data

suggest that patients harbouring a NPM1 mutation would particularly benefit from PD-1 addition to their treatment regimen.

In our study we showed that Nivolumab could enhance target killing by expanded effectors in CFI and that indifferent of their NPM1 status T cells can be stimulated to kill CD33+ LPC/LSC and in doing so may prevent relapse. A targeted immunotherapeutic treatment has the benefit of removing residual disease in first remission delaying or preventing relapse. The detection of PD-L1 expression in $NPM1^{mut}$ patients, rather than $NPM1^{WT}$ patients, especially in the leukaemic progenitor compartment defines AML patients with the NPM1 mutation as a potential subgroup for PD-1/PD-L1 directed immunotherapy.

In summary, we have shown that T cells from AML patients with and without the $NPM1^{mut}$ can respond to LAA after stimulation, with T cells from $NPM1^{mut}$ showing addition responses against $NPM1^{mut}$ peptide through the use of proliferation assays. In addition, all T cell mediated antitumour responses were enhanced by the presence of anti-PD-1 blocking antibody. Along with the expression of PD-1 on LSCs from $NPM1^{mut}$ patients this suggests that treatment with anti PD-1 antibodies combined with immunotherapeutic vaccine approaches could represent new treatment options for this biologically distinct group of patients.

AUTHOR CONTRIBUTION

Conceptualization, J.G.; methodology; C.B.; validation, H.S., C.B. and V.S.; formal analysis, C.B., S.H., V.S. and M.G.; investigation, H.D and M.G.; resources, P.J.S. and H.D.; data curation, S.H. and V.S.; writing - original draft preparation, J.G.; writing - review and editing, J.G., P.J.S., H.D., V.S. and B.G.; supervision, J.G.; funding acquisition, J.G. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

J.G. received funds from BMS (Bristol-Myers Squibb); V.S. completed this study while a graduate student at the University of Ulm and is currently an AbbVie employee, but AbbVie did not fund or participate in the study or development of the publication. The other authors do not have any potential conflicts of interest with regard to this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author or from marlies.goetz@alumni.uni-ulm.de upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the University of Ulm, date of approval 20.10.2014, approval file number 221/14 and by the Ethics Committee Landesärztekammer Baden-Württemberg, date of approval 21.11.2014, approval file number B-F-2014-105 221/14. Informed consent was obtained from all subjects involved in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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