

Review

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[Jochen Greiner](#)^{*}, Eithar Mohamed, [Daniel M Fletcher](#), [Patrick J. Schuler](#), Hubert Schrezenmeier, Marlies Götz, [Barbara-ann Guinn](#)^{*}

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Review

Immunotherapeutic Potential of Mutated NPM1 for the Treatment of Acute Myeloid Leukemia

Jochen Greiner ^{1,2,*}, Eithar Mohamed ^{3,†}, Daniel M. Fletcher ^{3,†}, Patrick J. Schuler ⁴, Hubert Schrezenmeier ^{5,6}, Marlies Götz ^{1,2} and Barbara-ann Guinn ^{3,*}

¹ Department of Internal Medicine III, University Hospital Ulm, 89081 Ulm, Germany; jochen.greiner@uni-ulm.de; marlies.goetz@alumni.uni-ulm.de

² Department of Internal Medicine, Diakonie Hospital Stuttgart, 70176 Stuttgart, Germany

³ Centre for Biomedicine, Hull York Medical School, University of Hull, Hull, HU6 7RX, UK. E.A.Mohamed-2019@hull.ac.uk; D.M.FLETCHER-2018@hull.ac.uk; Barbara.Guinn@hymns.ac.uk

⁴ Department of Otorhinolaryngology, University Hospital Ulm, 89075 Ulm, Germany; patrick.schuler@uniklinik-ulm.de

⁵ Institute of Transfusion Medicine, University of Ulm, 89073 Ulm, Germany; h.schrezenmeier@blutspende.de

⁶ Institute of Clinical Transfusion Medicine and Immunogenetics Ulm, German Red Cross Blood Transfusion Service Baden-Württemberg-Hessen and University Hospital Ulm, 89081 Ulm, Germany

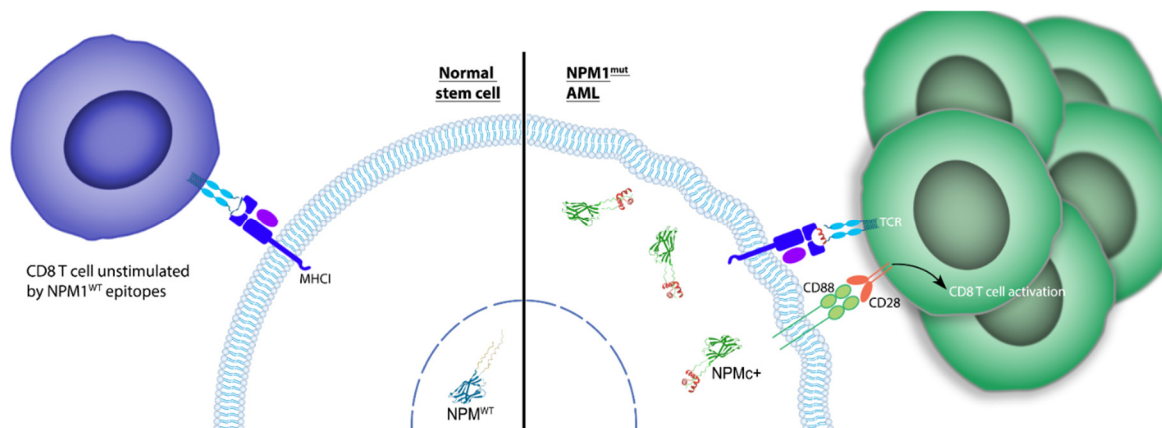
* Correspondence: barbara.guinn@hymns.ac.uk; Tel: +44 1482 466543 or greiner@diak-stuttgart.de; Tel.: +49 731-150 6786

† These authors contributed equally to this review and would like to be considered as joint 2nd authors.

Abstract: Acute myeloid leukemia (AML) is a malignant disease of the blood and bone marrow that is characterized by uncontrolled clonal proliferation of abnormal myeloid progenitor cells. Nucleophosmin 1 (NPM1) gene mutations are the most common genetic abnormality in AML, detectable in blast cells from about one third of adults with AML. AML NPM1mut is recognized as a separate entity in the World Health Organization (WHO) classification of AML. Clinical and survival data suggest that patients with this form of AML often have a more favorable prognosis, which may be due to the immunogenicity created by the mutations in the NPM1 protein. Consequently, AML with NPM1mut can be considered an immunogenic subtype of AML. However, the underlying mechanisms of this immunogenicity and associated favorable survival outcomes need to be further investigated. Immune checkpoint molecules, such as the programmed cell death-1 (PD-1) protein and its ligand, PD-L1, play important roles in leukemogenesis through their maintenance of an immunosuppressive tumor microenvironment. Preclinical trials have shown that the use of PD-1/PD-L1 (PDx) checkpoint inhibitors in solid tumors and lymphoma work best in novel therapy combinations. Patients with NPM1mut AML may be better suited to immunogenic strategies that are based on the inhibition of the immune checkpoint pathway of PDx than patients without this mutation, suggesting the genetic landscape of patients may also inform best practice for the use of PDx inhibitors.

Keywords: acute myeloid leukemia; NPM1 mutation; immunogenic subtype; Programmed Death Ligand 1; immunotherapy

Graphical abstract



1. Introduction

Nucleophosmin 1 (NPM1.1 or NPM1) is a mainly nucleolar protein that shuttles between nucleoli, nucleoplasm and cytoplasm chaperoning nucleic acids and proteins. It is expressed abundantly in all tissues [1] and plays a crucial role in cell cycle control, centrosome duplication, ribosome maturation and export, as well as cellular responses to a variety of stress signals. NPM1 is altered by overexpression, chromosomal translocations and mutations in solid and hematological cancers (reviewed in [2]). In hematological malignancies the NPM1 gene is frequently combined with other genes to give fusion proteins that retain the oligomerization domain in the N-terminal of NPM1 including NPM1-RAR α in a subset of acute promyelocytic leukemia patients [3] and the rare NPM1-MLF1 [4] translocation which is associated with the progression of myelodysplastic syndrome (MDS) to acute myeloid leukemia (AML). 99% of the point mutations in NPM occur in exon 12 and mutation A, a duplication of TCTG, is the most common followed by mutation D, an insertion of CCTG, between nucleotides 960 and 961. These point mutations, although different, almost always lead to the same outcome, a frameshift that creates a C-terminus sequence with a disrupted nucleolar localization signal (NoLS) and a third nuclear export sequence (NES). As a consequence, NPM1 no longer predominantly resides in the nucleolus but relocates to the cytoplasm [5–8] where it is referred to as NPMc+. NPMc+ accumulation in the cytoplasm can be detected by immunocytochemistry [9] and qPCR which allows the monitoring of molecular minimal residual disease (MRD) in patients [10]. NPM1c+ interacts with a number of other proteins (Figure 1), including itself, mostly using its N-terminal domain (NTD)(reviewed in [11]).

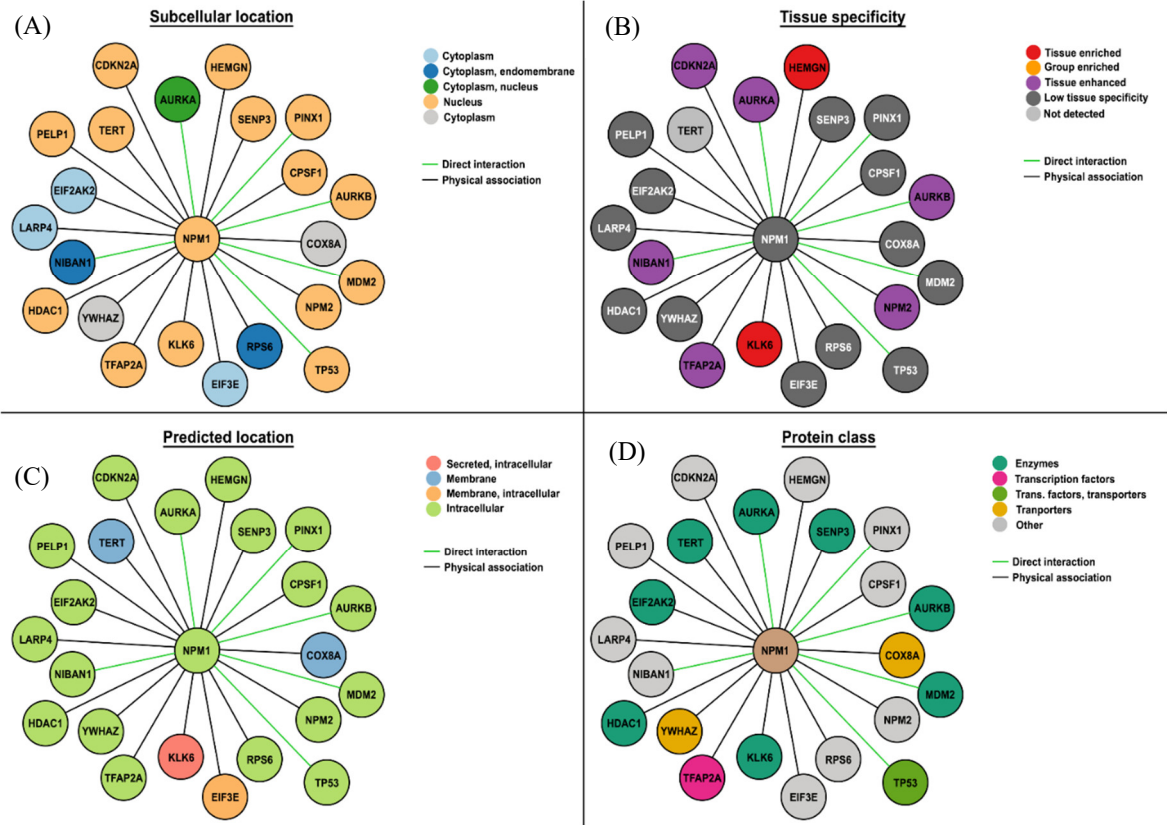


Figure 1. Direct interactions between NPM1^{WT} and proteins. The (A) subcellular localization; (B) tissue specificity; (C) predicted subcellular localization and (D) protein class are indicated for each protein that NPM1^{WT} interacts with. Data taken from Protein Atlas (<https://www.proteinatlas.org/>; accessed 18 Jun 2024) and drawn in photoshop. As expected most interactions are with nuclear proteins, most proteins are expressed in multiple tissues, intracellular and enzymes based on direct interactions and proximity.

Alterations in NPM1 are considered a gate-keeper mutation, one of the first hits in the process of leukemogenesis (reviewed in [12]). AML patients with a nucleophosmin-1 mutation (AML NPM1^{mut}) are recognized as a separate entity in the World Health Organization (WHO) classification of AML [13,14], since NPM1^{mut} are a founder genetic event, that is rare in MDS [15] with distinct genetic, pathological, immunophenotypic and clinical characteristics (Table 1). In this review we will discuss NPM1^{mut} AML in the context of immunotherapeutic approaches that have been developed to treat it.

Table 1. Notable associated features of AML patients with NPM1^{mut} (based on [17,19]).

AML NPM1 ^{mut}	AML NPM1 ^{WT}
Gatekeeper mutation, association with AML, own WHO subgroup, found in all AML cells, stable (seen again at relapse)	
Associated with older AML patients >35 years old, <i>de novo</i> AML and increased frequency in female	
Good response to induction therapy, better prognosis in older but not younger AML patients	
High WCC	
Normal Karyotype	t(8;21), inv(16), t(15;17)
FAB M1-M6, more often M4 and M5	FAB M0
Diseased cells are CD33 ⁺ and >90% are CD34 ⁻	LSCs are CD34 ⁺ CD38 ⁻

FLT3-ITD (2 x more common)	Biallelic CEPBA mutations occur
NPM1 ^{mut} /FLT3-ITD ⁻ patients have a better prognosis than NPM1 ^{mut} FLT3-ITD ⁺ patients	
Upregulated HOX genes (A4, A5, A6, A7, A9, A10, B2, B3, B5, B6)	
Upregulated HOX-related genes - PBX3 and MEIS1	
LSC express CD96, IL12RB1	

LSC: leukemic stem cells.

2. AML NPM1^{mut} and Prognosis

Survival rates for patients with AML are unfortunately still low. The 7+3 regimen achieves an estimated 5-year survival rate of 30% to 35% in younger adult patients (age <60) and 10-15% in older patients (age >60) [16]. Therefore, exploring new therapeutic options, especially for older patients, is crucial to improve survival rates and address risk factors. NPM1^{mut} are found more commonly in older adult AML patients (over 35 years of age)(Table 1) and correlate with high white counts and normal karyotypes [17]. They are present in the M1-M6 subtypes (French American British (FAB) classification of AML), but absent in AML M0 and patients with t(8;21), inv(16), t(15;17), or CCAAT/enhancer-binding protein- α (CEBPA) mutations [17]. Homeobox (HOX) genes (A4, A5, A6, A7, A9, A10, B2, B3, B5, B6) as well as the HOX-related genes, PBX3 and MEIS1, are upregulated in AML NPM1^{mut} samples while CD34 mRNA levels are low/absent in AML patients with NPM1^{mut}.

There are distinct patterns of co-mutations in NPM1^{mut} patients and there are gene–gene interactions that are particularly pronounced, causing patterns of co-incidental mutation defined groups with a favorable, or conversely, adverse prognosis [20]. The concurrence of NPM1^{mut} is twice as frequent in patients with fms-related receptor tyrosine kinase 3-internal tandem duplication (FLT3-ITD) than those with wild-type NPM1 (NPM1^{WT}). This may be explained, by both being caused by replication slippage errors [21] with the belief that NPM1^{mut} precedes FLT3-ITD [8,22]. FLT3-ITD is associated with poor survival in younger (<60 years) but not in older (60-74 years) AML patients, while NPM1^{mut} is associated with good survival in older, but not younger patients [23]. However, patients with NPM1^{mut} but without a FLT3-ITD translocation often have a favorable prognosis [17] including a better overall survival (OS) rate [24].

Angenendt and colleagues [25] conducted a study on a group of over 2,400 AML patients with NPM1^{mut}/FLT3-ITD^{neg/low}, who had varying karyotypes and chromosomal abnormalities. The results showed that in patients with NPM1^{mut}/FLT3-ITD^{neg/low} AML, unfavorable cytogenetics were linked to a lower 5-year OS rate (52.4%, 44.8%, 19.5% for normal, aberrant intermediate, and adverse karyotype, respectively). This highlights the significant impact of karyotype abnormalities on the survival outcome of NPM1^{mut}/FLT3-ITD^{neg/low} AML patients. In patients who have adverse-risk cytogenetics, those with NPM1^{mut} have a similarly poor prognosis as those with NPM1^{WT} and should receive treatment accordingly.

AML NPM1^{mut} were not found to be associated with event-free survival, OS or probability of relapse at 60 months post-diagnosis but because NPM1^{mut} were significantly associated with normal karyotypes and the presence of FLT3-ITD (Table 2) there appears to be a trend towards improved survival in patients in the intermediate risk group without FLT3-ITD but with NPM1^{mut} ($p=0.05$) [17].

Clinical data showed that AML NPM1^{mut} generally correlated with a better prognosis for patients (reviewed in [26]); that may be explained by more robust immune responses against leukemic cells in general and the leukemia stem cell (LSC) fraction in particular. In 2014, Schneider et al. [27] compared LSC-enriched populations in AML patients with and without NPM1 gene alterations. The CD34+CD38⁻ cell fraction’s existence within the LSC pool indicated a connection between the features of LSCs and the presence of NPM1^{mut}. Most diseased cells in NPM1^{mut} AML patients are CD33⁺ and CD34⁻; LSC are usually seen in the CD34⁺CD38⁻ cell fraction. This finding raises important issues about the role of immune surveillance in leukemia progression and underscores the potential

therapeutic implications of targeting immune-related pathways. However, additional validation and characterization are required to understand the underlying mechanisms.

Table 2. Definition of risk groups as defined by clinical features, molecular characteristics and cytogenetics indicators.

Risk	Molecular and cytogenetics indicators
Poor	FLT3-ITD [28,29]; partial tandem duplication (PTD) [30,31] of the mixed lineage leukemia gene (<i>MLL</i>), and increased expression of the transcription factor ecotropic virus integration site 1 (EVI1) [32]; DNMT3A, MN1, BAALC, EGR-1, AF1q [33]
Intermediate	IDH1 (isocitrate dehydrogenase 1 (NADP ⁺), soluble), IDH2 (isocitrate dehydrogenase 2 (NADP ⁺), mitochondrial), and TET2 (tet methylcytosine dioxygenase 2) [34]; t(9;11)(p21.3;q23.3); MLLT3-KMT2A [35]
Favourable	Mutations in the transcription factor CEBPA - favorable response to therapy [36,37]; t(8;21), inv(16)/t(16;16), t(15;17) and NPM1 ^{mut} [35]

3. AML NPM1^{mut} Treatment Strategies

While certain immunotherapeutic methods, such as allogeneic stem cell transplantation (SCT) and donor lymphocyte infusion, are established treatments for AML patients, there are also other potential treatments in development or waiting to be implemented in clinical practice. Many mechanisms and properties are still unclear, suggesting significant potential for immunotherapy in NPM1^{mut} AML.

Given that NPM1^{mut} has a high association with AML and has already been shown to be associated with improved patient survival, it is a desirable target structure for customized immunotherapy approaches especially for patients with chronic MRD or NPM1^{mut} receiving maintenance treatment. In an AML NPM1^{mut} patient in molecular relapse, pre-emptive donor lymphocyte infusion resulted in poly-specific cytotoxic T-lymphocyte (CTL) responses against multiple identified LAAs, including NPM1 #3 [38].

Jäger et al. [39] examined 64 patients with AML NPM1^{mut} who needed an allogeneic hematopoietic SCT (alloHSCT) due to additional adverse prognostic factors (1st line), and inadequate response to or relapse during or after 2nd line CT were retrospectively analysed. They showed that MRD-negative patients in CR achieve better 2-year progression free survival (PFS) and 2-year OS compared to patients with MRD-positive patients in CR (Table 3). The best results were obtained by patients who underwent alloHSCT because of a recurrence or chronic illness when relapse was identified molecularly, following CT completion. Compared to patients who relapsed while receiving CT, those who encountered a molecular or hematologic relapse after finishing CT responded well to salvage CT. Following alloHSCT, patients with a second MRD-CR had a high probability of long-term remission. During CT, patients who experienced hematologic relapse had poor survival outcomes [40]. Therefore, alloHSCT in NPM1 AML patients depends on the disease burden, relapse type, and response to CT. AlloHSCT transplantation does not seem to benefit NPM1^{mut}/FLT3-ITD-negative patients when used as a first-line treatment; nevertheless, further clinical trials are being conducted, and MRD needs to be considered [39].

Table 3. Outcomes of AML NPM1^{mut} patients (data from [39]).

Treatment	Pre-transplant status	2-year PFS	2-year OS
1st line CT: AlloHSCT indicated due to intermediate or poor-risk genetic features (n=27)	MRD ⁻ CR	77%	81%
	MRD ⁺ CR	41%	71%
	Active Disease	20%	52%

2nd line CT: AlloHSCT indicated as salvage therapy in case of molecular persistence, relapse or hematological relapse (n=37)	MRD ⁻ CR	86%	86%
	MRD ⁺ CR	47%	68%
	Active Disease	24%	52%

DiNardo et al. [41] showed that the BCL2 inhibitor, venetoclax, had high response rates and durable remissions associated with NPM1 or IDH2 mutations in older AML patients. Subsequently, Jäger et al. [39] showed in a small number of patients, that hypomethylating agents combined with Venetoclax may be an effective alternative treatment for NPM1^{mut} AML especially where an isocitrate dehydrogenase (NADP⁺ 2) IDH2-mutation is present. Effective relapse methods are offered, with a particular emphasis on the incidence of mild or moderate graft versus host disease (GvHD), which is most likely caused by an immunological graft versus leukemia (GvL) effect.

Forghieri et al. [42] recently wrote about a promising therapeutic approach involving the use of neoantigen-specific T cells, i.e., genetically engineered T cells, such as CAR-T or TCR-transduced T cells directed against NPM1^{mut} peptides bound to HLA. In patients with full-blown leukemia, adoptive or vaccine-based immunotherapies may not be very effective, but these strategies, possibly in combination with immune checkpoint inhibitors (ICIs), may be promising for maintaining remission or pre-emptively eradicating persistent measurable residual disease (discussed further in Section 5.3). This also applies to patients who are not eligible for alloHSCT.

4. Immunogenic Mutation Related Targets

Different CD4⁺ and CD8⁺ T-cell responses to epitopes originating from mutant NPM1 regions have been identified [43] including two HLA-A2-restricted peptides of NPM1^{mut}, #1 and #3, induced distinct T-cell responses (33% and 44%, respectively). Compared to healthy individuals, NPM1^{mut} AML patients exhibited significantly higher frequencies of CTL responses against peptide #3 ($p=0.046$). Schneider et al. [27] identified specific T-cell responses in AML against epitopes originating from the mutated region of NPM1. In a survival analysis of 25 patients with AML NPM1^{mut}, we observed that those exhibiting specific CD8⁺ cytotoxic T-cell immune responses against one or two immunogenic peptides had a better OS compared to those without such immune responses ($p=0.004$) [44].

Epitope-based differences in immune responses seem to exist ($p=0.026$); however, given the limited sample size, caution should be exercised when interpreting this result. Due to the inclusion of older patients (7 of 25 were over 60 years old) and other genetic alterations (FLT3-ITD in 11 of 25 NPM1^{mut} patients), the survival rate of all NPM1^{mut} patients was lower. Subsequently, Schneider et al. [27] isolated LSC-enriched population from both NPM1^{mut} and WT patients, and noted that the frequencies of CD34⁺CD38⁻ cells in AML NPM1^{mut} samples were significantly lower. They found a number of differentially expressed genes of immunological relevance in these populations, including immunoglobulin superfamily member 10 ($p=0.00034$), CD96 ($p=0.00052$) and IL-12 receptor beta 1 (IL12RB1, $p=0.000834$). These markers are involved in immune mechanisms and antigen presentation, highlighting the potential of immune-based therapies that target this subtype of AML patients to be effective.

Kuželová et al. [45] showed that certain HLA-I alleles were also associated with better OS in AML NPM1^{mut}, indicating a predisposition for an effective anti-AML immune response against NPM1^{mut} based on the MHC presentation of the epitopes. The group found that in a cohort of 63 AML patients with NPM1^{mut}, compared to 94 patients with NPM1^{WT}, there was a significant decrease in HLA-B*07, B*18 and B*40 expression. Kuželová et al. also demonstrated a notable OS advantage for patients with NPM1^{mut} who expressed one of these depleted haplotypes ($p=0.02$) suggesting HLA-B*07, B*18 and B*40 presentation of NPM1^{mut} epitopes are more effective at generating an immune response than other haplotypes, and hence their absence.

Among the most prevalent leukemia-associated antigens (LAAs) in AML are Wilms' tumor antigen 1 (WT-1), Proteinase 3, and Receptor for hyaluronan-mediated motility (RHAMM; reviewed in [46]). Cancer-testis antigens (CTAs), such as HAGE and PASD1, are expressed less frequently (23%

and 35% respectively) but have disease-specific expression [47,48] with little to no expression in healthy tissues, except in immunologically protected sites. All have been shown to generate antigen-specific CD8⁺ T cell responses from AML patients [49] and peptide vaccination studies investigating these antigens [50–54] revealed immunological and clinically-relevant responses. Greiner et al. [55] examined anti-LAA responses using T-cells from AML NPM1^{mut} and NPM1^{WT} patients. Samples from 13/15 (87%) NPM1^{mut} AML patients showed immune responses against at least one LAA and 9/15 (60%) against all four LAAs (NPM1^{mut}, Preferentially expressed Antigen in Melanoma (PRAME) P300, RHAMM R3 and WT1). In NPM1^{WT} patients no responses were found against the NPM1^{mut} epitope, but responses against the other LAAs were comparable to those of NPM1^{mut} patients, although immune responses varied slightly depending on the antigen and the patient.

5. Monoclonal Antibody Therapies

The standard treatment for AML has remained largely unchanged during the past few decades, which is the standard “7 + 3” regimen and includes a seven-day infusion of cytarabine and a three-day treatment with an anthracycline. It is commonly used to achieve complete remission (CR) and is particularly successful in patients aged 65 years and under. In recent years significant advances in our understanding of the pathogenesis of AML, the development of diagnostic assays and approval of new treatments has led to updates in the standard care for AML patients.

5.1. α CD33

CD33 is primarily a myeloid differentiation antigen with initial expression at the very early stages of myeloid cell development and it is not expressed outside the hematopoietic system or on pluripotent HSCs. FLT3-ITD and NPM1^{mut} were found to have increased CD33 expression in patients. Both antigens are expressed on proliferative blasts and leukemia stem cells (LSC) and dual targeting of CD33 and CD123 may enhance treatment efficacy for AML [56]. Several approaches such as monoclonal antibodies and CAR-T cells have been suggested to target overexpression of CD33 and CD123 on AML blasts. Lintuzumab is humanized IgG1 against CD33 that has shown only modest activity in clinical trials [57–59]. Due to toxicity in the liver and normal hematopoietic cells, the phase 3 trial was terminated [60] reflecting the fact that CD33 is largely expressed on myeloid lineage cells, including progenitor cells as well as on neutrophils, NK cells, a subset of B cells, and Kupffer cells in the liver [61].

Due to the short-term effect of α CD33, caused by its' rapid internalization when it binds, bivalent antibodies conjugated to toxins have been developed to enhance CD33 efficacy [60]. In 2017, after a decade of controversy, the FDA authorized Gemtuzumab-Ozogamicin (GO), a humanized monoclonal α CD33 antibody coupled with the cytotoxic chemical calicheamicin. When GO binds to CD33, it is internalized and calicheamicin is released, triggering DNA double-strand breaks that cause cell death.

Several trials have shown that augmenting the standard treatment with GO, a conjugate of CD33 antibody and cytotoxin calicheamicin has had promising results. It is recommended that GO is added to the first cycle of standard 7+3 induction therapy, especially for CD33-positive NPM1^{mut} AML [13,62,63]. In the randomized AMLSG 09-09 study for NPM1^{mut} patients, CR rates were similar with and without GO, but relapse-free survival was prolonged to a clinically relevant extent in those patients who achieved a CR [64]. The inclusion of GO in intensive chemotherapy (CT) for NPM1^{mut} AML patients led to a notable decrease in NPM1^{mut} transcript levels throughout all treatment cycles, explaining this significant reduction in relapse rates [65,66].

GO has been approved in conjunction with aggressive treatment for newly diagnosed AML patients [67]. GO was initially designed as a monotherapy with a single 9 mg/m² dose for the first recurrence of CD33⁺ AML. The CR recovery rate is 13%, with a median recurrence-free survival of 6.4 months. The most common consequences were grade 3 and 4 neutropenia (98%), thrombopenia (99%), and a few veno-occlusive disorders (VOD, 0.9%). In up to 25-35% of patients with newly diagnosed or relapsed/refractory (R/R) AML, GO induced CR or CR with incomplete platelet recovery especially in AML patients with a favorable prognosis [68]. GO also eliminated MRD in

extensively treated individuals with a reduced response [69]. GO enhanced the 5-year OS and significantly reduced relapse rates in AML patients with NPM1^{mut} ($p=0.0028$) [70]. However, combining GO with intensive CT for NPM1 AML patients in randomized AMLSG 09-09 trial (NCT00893399) resulted in a high mortality rate [71]. Patient-administered GO had lower associated relapse rates compared to the standard treatment [71].

5.2. α CD123

Targeting CD123 has been suggested as an AML treatment; however, its efficacy was unsatisfactory except in NPM1^{mut} AML/or patients with a FLT3-ITD mutation. This is because these patients have an LSC population enriched in CD34⁺/CD38⁻ cells [72]. Using a naked antibody that targeted CD123 (CSL362) caused lysis of the AML cells that expressed CD123 due to the activation of the innate immune system [73]. However in a clinical trial of 40 patients with R/R AML, only two patients had a clinical response [73].

Tagraxofusp (SL-401) is a recombinant α CD123 (IL-3 receptor alpha chain) antibody based on the diphtheria toxin. Following its attachment to CD123 and inhibition of eukaryotic elongation factor 2 (eEF2), tagraxofusp was internalized. In patients with a blastic plasmacytoid dendritic cell neoplasm, a myeloid malignancy with high CD123 expression, SL-401 has led to clinical improvement [74]. To treat CD123-positive AML and MDS, SL-401 has also been used with azacitidine (AZA) or venetoclax/AZA. Three of the four previously untreated MDS patients and eight of the nine AML patients who had not received treatment before achieved CR. It is noteworthy that TP53 mutations were present in three responding MDS patients and two responding AML patients. When paired with venetoclax/AZA in CD123-positive R/R AML, pivekimab sunirine, another α CD123 antibody-drug combination that causes DNA alkylation, produced objective response rates (ORRs) of 51% with promising efficacy [75].

Using a mouse model that targeted both CD33 and CD123 via a bispecific conjugate, LSCs were eliminated through a T-cell dependent mechanism both in-vivo and in-vitro [76]. This model suggests that NPM1^{mut} AML is initiated by CD123⁺ LSCs [76].

5.3. The immune Checkpoint Inhibitors - α PD-1 and α PD-L1

Checkpoint inhibition targeting the Programmed cell death protein 1 (PD-1)/Programmed cell death 1 ligand 1 (PD-L1), PDx axis have successfully achieved responses in a number of tumor types, including hematopoietic malignancies (reviewed in [77]). However, responses in AML have shown fewer promising results perhaps reflecting their lower mutation burden. This may explain why recent ex vivo studies may provide evidence that support the use of immunogenic strategies for AML NPM1^{mut} in the presence of an ICI.

A systematic review that compared 19 randomized clinical trials involving 11,379 patients with solid tumors including non-small cell lung cancer, renal cell carcinoma and gastric urothelial carcinoma [78] showed that α PD-1 treatments (such as nivolumab and pembrolizumab) achieved better OS and PFS compared to α PD-L1 therapeutic antibodies, with no significant difference in safety profiles. The reason is thought to be that α PD-1 can bind PD-1 on T cells, blocking binding of PD-1 to both PD-L1 and PD-L2 ligands on antigen presenting cells (APC; cancer cells) at the same time. However the PD-L1 antibody can only bind PD-L1, so T cells may still be inhibited by the interaction between PD-1 and PD-L2, even when PD-L1 signaling is blocked (reviewed in [79]). The level of PD-L1 on APCs may affect the response of patients to α PD-L1 treatment with PD-L1^{low/negative} patients having a compensatory increase in PD-L2 levels making the difference in treatment efficacy between α PD-1 and α PD-L1 exacerbated.

Expression of CD34/CD38/CD274 surface markers for LPC/LSCs were evaluated in 20/20 NPM1^{mut}/NPM1^{wt} AML patient samples via flow cytometry analyses. The LSC fraction showed a higher level of PD-L1 expression than the non-LSC fraction. The influence of α PD-1 antibody on these antigen-specific immune responses and the formation of stem-like colonies were assessed as well. It is noteworthy that high PD-L1 expression in NPM1^{mut} patients was detected, especially in the leukemic progenitor compartment. 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 expression of PD-L1. Immunogenicity of neoantigens derived from NPM1^{mut} cells with higher PD-L1 expression constitute promising target structures for individualized immunotherapeutic approaches.

In addition, we have previously shown that CD8⁺-specific LAA-immune responses against PRAME, WT-1, RHAMM or NPM1^{mut} expressing LPC/LSC from AML patients with mutated or wild type NPM1 were similar except for their response to NPM1^{mut} epitopes which were only seen in samples from NPM1^{mut} patients (vidé *suprá*). We also found that T cell-mediated anti-tumor responses from AML patients were enhanced by the presence of α PD-1 blocking antibody [55]. Along with the expression of PD-1 on LSCs isolated from NPM1^{mut} patients, this suggests that treatment with α PD-1 antibodies combined with immunotherapeutic vaccine approaches could represent new treatment options for this biologically distinct group of patients [55,80].

The immune-modulating drug lenalidomide has been shown to promote anti-tumor immunity including anti-inflammatory, anti-proliferative, pro-apoptotic and anti-angiogenicity [81]. The effects of lenalidomide are distinct from CT, hypomethylating agents, or kinase inhibitors, making lenalidomide an attractive agent for use in AML treatment including in combination with existing active agents. Combinations of immunotherapeutic approaches are believed to increase antigen-specific immune responses against leukemic cells including LPC/LSCs. In this study, we used a combination of LAA-peptides with the ICI, α PD-1 (nivolumab), and lenalidomide to enhance the killing of LSC/LPCs *ex vivo* [82]. We used primary cells from AML patients and observed the T-cell responses of all patients *in vitro*. Results using the combination of ICI α PD-1 and the immune modulatory drug, lenalidomide, showed enhanced LPC/LSC killing. The effect was greatest against NPM1^{mut} cells when the immunogenic epitope was derived from the mutated region of NPM1 [24]. These studies show that antigen-specific immune responses against leukemic cells as well as LPC/LSC, are enhanced by combinations of immunotherapeutic approaches especially when using a combination of LAA-peptides, the α PD-1 antibody, and one further immunomodulating drug, providing an interesting opportunity to enhance the outcomes in future clinical studies.

We have recently examined whether all-trans retinoic acid, AZA, α CTLA4 or Lenalidomide, in combination with α PD-1, to determine whether this could further improve the destruction of leukemic cells and also LPC/LSC from AML patients [83](Figure 2). We identified which LAA (WT-1, PRAME or NPM1) generated the strongest immune response (as measured by colony-forming immunoassays) and using samples from 20 AML patients with more than 90% leukemic blasts. AZA, in combination with α PD-1, had a pronounced effect on T-cell activation and inhibition of LPC/LSC growth.

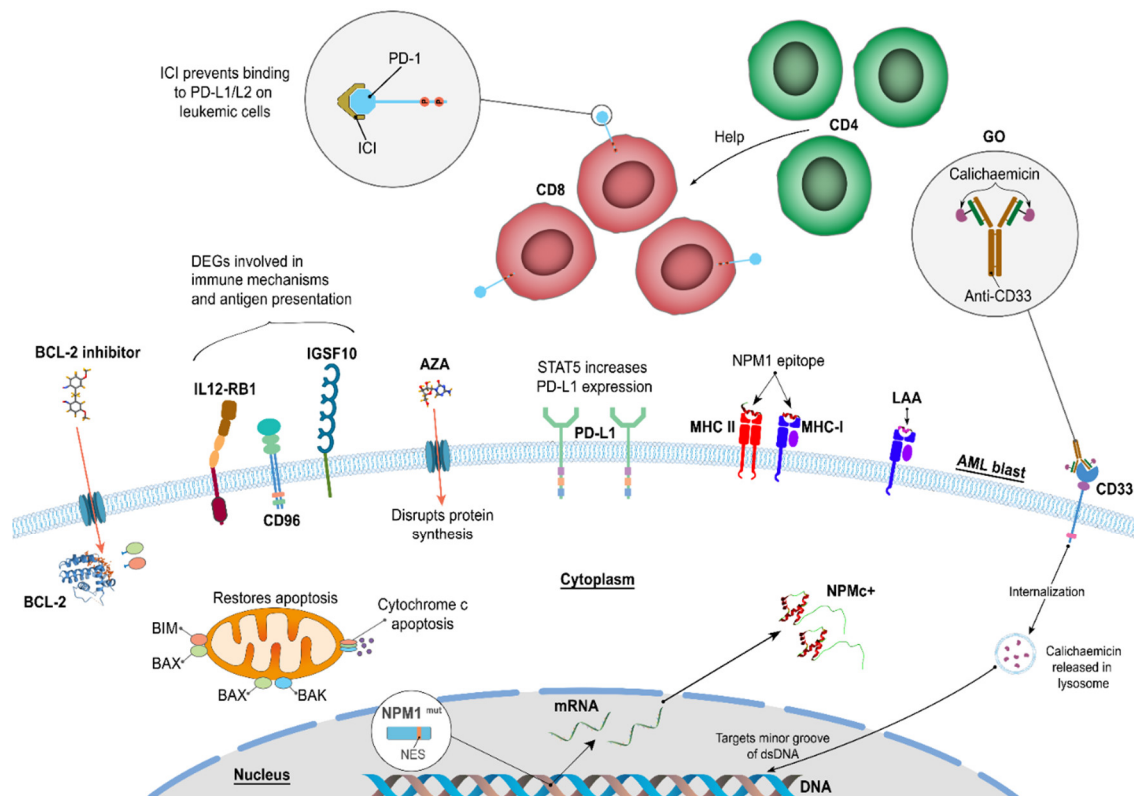


Figure 2. Combinations of immunotherapeutic treatments can use complementary strategies to enhance anti-leukemic responses. We have shown that these combinations can increase antigen-specific immune responses against leukemic cells as well as LPC/LSC [82,83]. Image generated using information from the following articles [5–8,27,39,41,47,55,56,64–71,80,83–88].

Other strategies for immunotherapy, that can be used in combination with PDx need to be considered. One approach is the signal transducer and activator of transcription 5 (STAT5), which promotes PD-L1 expression by facilitating histone lactylation and driving immunosuppression in AML and thus may benefit from PD-1/PD-L1 immunotherapy. ICI could block the interactions of PD-1 with PD-L1 and thereby enhance the reactivity of CD8⁺ T cells in the microenvironment when co-culture with STAT5 activated AML cells occurs [84].

Daver et al. found that AZA in combination with α PD-1 resulted in improved OS and an encouraging response rate, particularly in hypomethylating agent (HMA)-naïve and salvage-1 patients [85]. A randomized phase III study and a randomized phase II study of AZA with or without PD-1 inhibitor in first-line elderly AML patients and a randomized trial of PD-1 inhibitor for the eradication of MRD in high-risk AML in remission has started. Clinical and immune biomarker-enriched trials are likely to yield further improved outcomes with HMA in combination with ICI therapies in AML, but this remains to be seen as more study results are made available, and especially for NPM1^{mut} AML patients.

The results suggest that PD-1/PD-L1 in combination with NPM1^{mut} related approaches or other strategies such as STAT5 or HMA may have notable impact on immunotherapeutic outcomes when used for the treatment of AML.

6. Venetoclax and Hypomethylating Agents

The aberration of DNA methylation is a common early event in the pathogenesis of AML, caused by genetic alterations in DNA methyltransferases (DNMTs) and Ten-Eleven-Translocation (TET) dioxygenases and leading to transcription dysregulation (reviewed in [86]). The standard treatment protocol for newly diagnosed NPM1^{mut} AML patients who are unfit and/or elderly (>60 years), is a combination of venetoclax and HMAs such as AZA which inhibits DNMTs by its incorporation into

DNA or low-dose cytarabine (LDAC). The Food and Drug Administration (FDA)-approved molecule, venetoclax, targets the NPM1 apoptotic pathway by inhibiting B-cell leukemia/lymphoma-2 (BCL-2) [41,87,88]. The increased sensitivity to venetoclax may be due to an NPM1 mutant-primed impairment in mitochondrial function [89]. Venetoclax-based regimens are especially beneficial for NPM1^{mut} AML patients at first diagnosis and in the case of patients who are R/R to treatment. Regarding the effectiveness of venetoclax in NPM1^{mut} AML, the human monocytic leukemia cell line THP-1 which harbors a NPM1^{mut} exhibits increased sensitivity to chemotherapies through the reduction of NF-κB activity and BCL-2/BAX expression [90]. High BCL2 protein levels are associated with improved outcomes in patients with R/R AML or untreated AML, according to predictive indicators for venetoclax sensitivity [91]. However, not every patient has experienced this. A plausible rationale could be that a FLT3-ITD or TP53 deletion in NPM1^{mut} AML patients leads to a mitochondrial malfunction. Moreover, as NPM1^{mut} AML patients age, IDH1/2 mutations develop, which impacts how well venetoclax-based therapy can work [92].

Single-agent HMA or LDAC regimens are often better tolerated and have lower treatment-related mortality rates than conventional CT [93]. With a median survival of 6–10 months, response rates to HMAs alone are modest (10%–50%) [93,94]. Despite its efficacy, relapse within 18 months is a major obstacle of HMA due to drug resistance. One suggested mechanism is that HMA interferes with DNA methylation and in the long term causes a failure of cells to undergo cell cycle arrest [95].

The combination of HMA and venetoclax is suggested to maximize the efficacy of treatment, evading the resistance created through the use of a single agent. In a phase 3 clinical trial (NCT02993523) involving venetoclax plus AZA [41], 66.7% of NPM1^{mut} AML patients experienced CR and incomplete remission with the majority of the patients testing negative for detectable residual disease (MRD). With a predicted two-year survival rate of 70%, the one-year survival rate for elderly individuals with NPM1^{mut} AML surpassed 80%. Usually, 1-2 cycles were required to generate the desired response with a favorable safety profile [41]. Similarly, venetoclax plus LDAC achieved CR and an incomplete CR of 89% and 78%, respectively, in NPM1^{mut} AML patients in trials NCT02287233 and NCT03069352 [96,97]. The combination of venetoclax and HMA is considered to be advanced in AML treatment, dose scheduling and further understanding of the mechanism of action and resistance remain to be explored [41].

7. Discussion

Our review has described treatments that are of particular relevance to AML NPM1^{mut} patients. These patients are unique with a tendency to be over 35 years of age, have a high WCC, have FLT3-ITD, a normal karyotype and upregulated HOX and HOX associated genes and low to absent CD34⁺ expression on their blasts. Representing one-third of all AML patients, AML NPM1^{mut} has become a separate entity in the World Health Organization (WHO) classification of AML, reflecting the patients' ability to benefit from a targeted treatment strategy. Although treatment of AML patients has changed little until recently [35], the AMLSG09-09 clinical trial showed that the addition of GO can extend relapse-free survival to a clinically relevant extent in CD33⁺ NPM1^{mut} AML patients who achieve a CR [64,65]. It is notable that most of the diseased cells in NPM1^{mut} AML patients are CD33⁺ and CD34⁺ even though LSCs are usually seen in the CD34⁺ and CD38⁺ fraction from AML patients [98]. This suggests that if NPM1^{mut} occurs in the LSC population they are readily depleted by the immune system or that NPM1^{mut} tend to occur in non-LSCs which are more easily seen by the immune response leading to their destruction and the associated improved patient survival.

This is reflected by the association between NPM1^{mut} and improved survival in older (>70 years old) who have normal cytogenetics, independent of other molecular and clinical prognostic indicators, and in contrast to younger patients [99]. The haplotypes HLA-B*07, B*18 and B*40 are depleted in patients with NPM1^{mut} but when patients with NPM1^{mut} express one of these haplotypes OS is increased [45]. The association of NPM1^{mut} with a better prognosis for patients (reviewed in [26]); may be explained by more robust immune responses against leukemic cells in general and the leukemia stem cell (LSC) fraction in particular. Indeed Schneider et al. showed that AML NPM1^{mut}

patients harboring CD8⁺ responses against one of two predicted NPM1^{mut} peptides had better OS than those without [44].

Recently hypomethylating agents and the Bcl-2 inhibitor, venetoclax, combinations gave impressive results in older patients with NPM1^{mut} AML compared to traditional standard-of-care regimens [100]. However, NPM1^{mut} patients with adverse cytogenetics should be treated the same as NPM1^{WT} patients with unfavorable cytogenetics which were linked to a lower 5-year OS rate [25].

Greiner et al. showed that CD4⁺ and CD8⁺ T cells from AML patients could respond to peptides from the mutated regions of NPM1 [43]. They found that AML NPM1^{mut} patients with CTL responses against NPM1^{mut} peptide had better overall survival [45] and used LAAs as a specific method of stimulating T-cell immune responses against LSCs and determining which combinations of immunotherapeutics could further enhance AML cell killing [82].

Elevated PD-L1 expression on AML NPM1^{mut} cells (ratio of v1/v2 as determined by qPCR) is associated with poorer survival [101] suggesting that anti-PD-1 treatment could block this signaling pathway, enhance T cell killing of leukemic cells and cause an associated improvement in patient survival. In vitro we showed that the immune checkpoint inhibitor, α PD-1, can enhance killing by LAA-stimulated CTLs [27,55]. Notably, the effect was greatest when T cells from NPM1^{mut} patients were stimulated with NPM1^{mut} peptide suggesting anti-PD-1 antibodies, such as nivolumab, could be used to treat AML NPM1^{mut}. This may reflect the features of AML NPM1^{mut} that are especially associated with successful α PD-1 treatment of cancers including disease stability and a targetable mutation.

We recently examined whether α PD-1, ATRA, AZA, α CTLA4 and/or Lenalidomide were the best treatments to facilitate the destruction of autologous AML colonies in NPM1^{mut} and NPM1^{WT} patients [83]. Results were enhanced in patients with NPM1^{mut} compared with NPM1^{WT} with α PD-1 and AZA providing the most effective T-cell mediated reduction in AML cells in CFI assays. This concurs with our understanding of PDx treatments and when they work best. When CTLs recognize LAAs, their response includes the production of inflammatory cytokines such as IFN γ and the upregulation of PD-1 on their surface. This prevents excessive T-cell responses and limits the immune response. The tumor cells concurrently upregulate PD-L1, inhibiting T-cell responses against them [104]. The blockage of this PD-1/PD-L1 interaction can lead to enhanced tumor killing and improved patient survival rates (reviewed in [103]). However results in the treatment of AML have been mixed, reflecting the features of NPM1^{WT} that make AML a poor target for PDx therapies. These include rapidly progressing disease that generates a high tumor, but low mutational burden. However PD-1 is regulated by DNA methylation [104], demonstrating a benefit for HMA and α PD-1 treatment in AML patients. The combination of venetoclax and HMAs has been shown to improve OS and CR compared with AZA alone in patients >75 years of age with previously untreated AML who are ineligible for intensive chemotherapy [41].

It is clear that the development and progression of AML is closely linked to an impaired immune response. Leukemic cells manipulate the tumor microenvironment by creating a niche that directly promotes their survival and makes them resistant to immunotherapeutic strategies. Therefore, innovative strategies for immunotherapies need to be further developed and adapted to overcome the obstacles in the treatment of AML and especially NPM1^{mut} AML [105]. However AML NPM1^{mut} offer several advantages over AML NPM1^{WT}, by virtue of their predominance of CD33⁺ diseased cells and an absence of CD34⁺ suggesting the target population are not the more difficult to treat CD34⁺ LSC cells. The presence of a NPM1^{mut} provides a unique target for treatment, whose immunogenicity can be enhanced by treatment strategies that are likely to be enhanced by α PD-1, HMAs and immune modulators.

8. Conclusions and Future Developments

In the hunt for additional NPM1^{mut} peptide targets for future treatment strategies, and ideally the identification of those that are naturally processed and presented, we could use mass spectrometry technologies that have been previously used to identify LAA-associated peptides processed and presented on MHC on CML and AML cells [106,107]. Similar strategies could be used

to isolate peptides presented by MHC class I and II molecules on CD34-CD33⁺ cells from AML NPM1^{mut} patients, especially those associated with improved survival. This would provide naturally occurring epitope(s) for future studies that could be used to enhance NPM1^{mut}-directed immune responses. It may even lend credence to the development of NPM1^{mut} analog peptides, shown previously to stimulate enhanced immune responses against naturally occurring epitopes presented on HLA-A*0201⁺ AML cells [50].

We have discussed the association between NPM1^{mut} AML and the unique features associated with it. These include normal cytogenetics and enhanced survival in patients >70 years of age. This phenomenon appears to be associated with the capacity of NPM1^{mut} cells to stimulate a CD8⁺ T cells response against AML cells and lead to relapse-free survival in patients who achieve a CR. More recent studies have built on the standard of care treatment to show that the additional use of hypomethylating agents and Bcl-2 inhibitors can cause improved OS and CR in patients >75 years of age who are not eligible for intensive chemotherapy. Future studies should examine other available treatment options and their efficacy in combination and the clinical trials that provide the proof of principle for a group of elderly AML patients who now have meaningful treatment options.

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Abbreviations

alloHSCT	allogeneic hematopoietic stem cell transplant	LDAC	low-dose cytarabine
AML	Acute myeloid leukemia	LPC/LSC	leukemic progenitor/stem cells
APC	Antigen presenting cells	MDS	Myelodysplastic syndrome
AZA	5'-azacitidine	MRD	Minimal residual disease
BCL-2	B-cell leukemia/lymphoma-2	NPM1	Nucleophosmin 1
CEBPA	CCAAT/enhancer-binding protein-α	OS	overall survival
CR	Complete remission	PD-1	programmed cell death-1
CT	Chemotherapy	PD-L1	Programmed cell death 1 ligand 1
FAB	French American British	PDx	PD-1/PD-L1 axis
FDA	Food and Drug Administration	PFS	Progression-free survival
FKT3-ITD	fms related receptor tyrosine kinase 3-internal tandem duplication	PRAME	Preferentially expressed Antigen in Melanoma
GO	Gemtuzumab-Ozogamicin	RHAMM	Receptor for hyaluronan-mediated motility
HMA	hypomethylating agents	R/R	Relapsed/refractory
ICI	immune checkpoint inhibitor	STAT5	Signal transducer and activator of transcription
LAA	Leukemia associated antigens	WHO	World Health Organization

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