NAM-NAD+-ADO metabolic reprogramming is a key factor for DNMT3A mutation to promote leukemia development through regulating cell cycle and immune microenvironment

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Abstract

DNMT3A-R882H mutation is a frequent epigenetic mutation in acute myeloid leukemia (AML)-M4 and M5, participating in the regulation of cell growth and differentiation by blocking the binding of transcription factor complexes to DNA. However, effective approaches that directly target this inactivating mutation for therapy remain lacking. For the first time, we show that the DNMT3A-R882H mutation can switch leukemia cells to undergo nicotinamide (NAM) and nicotinamide adenine dinucleotide (NAD+) metabolism and influence the formation of cyclin-CDK complexes by affecting the deacetylation function of SIRT6. Inhibition of nicotinamide phosphoribosyltransferase (NAMPT) can reduce the NAD + required for SIRT6 function, degrade CDKN1A/CDKN1B, and effectively induce cell cycle arrest and apoptosis. The NAMPT inhibitor FK866 can optimize the immune-infiltrating microenvironment by reducing the production of the downstream metabolite adenosine (ADO). Animal experiments have shown that NAMPT inhibition or knockdown can significantly inhibit tumor cell growth, reduce spleen infiltration of tumor cells, and prolong mouse lifespan. Our findings provide a potential direction for a targeted therapy at the metabolic level in AML with DNMT3A mutations.

Introduction

Numerous studies have shown that metabolic reprogramming plays a role in the occurrence and development of tumors as one of the typical malignant features of tumors [1–4]. During tumor evolution, metabolic pathways are reprogrammed to meet the material and energy demands of tumor growth. Meanwhile, a close relationship exists between gene abnormality and metabolic reprogramming. For instance, the activation of oncogenes and the inactivation of tumor suppressor genes can promote metabolic reprogramming [5]. Thus, the metabolic reprogramming pathways caused by causative genes can be targeted.

In a previous study, we found that 20.5% of acute myeloid leukemia (AML)-M5 and 13.6% of AML-M4 patients have DNMT3A mutations, with a mutation hotspot at position 882 of arginine (R882), which leads to lower methyltransferase activity [6]. DNMT3A gene polymorphism can act as an independent predictor of chemotherapy sensitivity and prognosis of patients with AML in southern China [7]. Tumor cells that carry this mutation are prone to immune escape or long-term incubation in preleukemia stem cells, which leads to relapse and poor prognosis [8]. However, the mechanism behind this phenomenon has not been fully understood to date. We suspect that DNMT3A R882 mutation can cause metabolic reprogramming to play a role in the malignant progression and immune escape of AML.

In this study, we found that nicotinate and nicotinamide (NAM) metabolism was the predominant altered metabolic pattern in a DNMT3A-R878H (homologous sequence to human R882H) mutant AML mouse model. Changes in the related gene expression were also detected in mouse models, patient samples, and cell lines. The metabolic change of this kind was attributed to changes in the expression profiles of NAM-metabolizing enzymes, mainly nicotinamide phosphoribosyltransferase (NAMPT) and CD38, as a result of mutations in DNMT3A.

Therefore, we determined the possible effects of altering NAM levels on tumors. We found the potential efficacy of inhibiting NAMPT based on the metabolic profile and related biological functions of AML with the DNMT3A mutation. The NAMPT inhibitor FK866 can induce cell differentiation, apoptosis, and cell cycle arrest through the SIRT6-cyclin-CDKs pathway. Moreover, FK866 can significantly prolong the survival time of tumor-bearing mice and reduce the splenic infiltration of tumor cells.

Finally, through immune infiltration analysis of patient samples and single-cell sequencing data of a mouse AML model with mutated DNMT3A, we found that the abnormal metabolic enzyme profile of NAM-ADO likely affects the immune infiltration of NK and $CD8^+$ T cells in AML patients as well as in the mouse model with DNMT3A mutation. Comprehensive analysis of the TCGA database and immune infiltration data showed that the aberrant expression of NAMPT in leukemia may also affect the expression of NK-related checkpoint molecules. In addition, we propose that adenosine, one of the final metabolites of NAM, can further promote the expression of key metabolic enzymes of the NAM-ADO axis in tumor cells.

Our data indicate that knockdown or inhibition of NAMPT can induce cell cycle arrest, apoptosis, and cell differentiation in AML with high NAMPT expression and that it can ameliorate the immunosuppressive tumor microenvironment by reducing adenosine production.

Materials And Methods

Flow cytometry

Cells were harvested and stained using antibodies for the cell markers CD38, Annexin-V, Propidium Iodide, CFSE, and CD3. The mixture was incubated in 100 µl PBS supplemented with 2% FBS in darkness for suitable length of time at 4°C before detection. An isotype-matched antibody served as a negative control. Flow cytometry was performed on eight-laser cytometers (BD). Dada were analyzed using FlowJo software.

Statistical analysis

Part of the data was analyzed with R, and the specific methods will be described later. Other data analysis was performed with Graphpad prism 8.0, and data was expressed as a mean ± SD. The statistical significance between groups was assessed using a Student's T-test. For all in vivo survival experiments, Kaplan–Meier estimates of the survival function were calculated for each group and differences assessed using the log-rank test. P value < 0.05 was regarded as statistically significant.

Cell Culture

Cell lines including OCI-AML3, U937, NB4 and THP-1 were cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 µg/ml streptomycin, and 100 IU/ml penicillin. All cell

lines were maintained at 37°C under 5% CO2. Moreover, cell samples were ruled out mycoplasma contamination.

Apoptosis Assay

The cells were stained using the APC Annexin V Apoptosis Detection Kit with PI (Biolegend) according to the manufacturer's instructions and then detected by flow cytometry analysis. All data were analyzed using FlowJo software.

Cell Cycle Analysis

Cell cycle distribution was analyzed using cell cycle staining solution. The cell samples were washed with pre-cooled PBS, and then gently dropped into 75% cold ethanol to fix the samples. Finally, the cells were washed with PBS and resuspended. After adding RNaseA (50 μg/ml) and incubated at 37°C for half an hour, the PI (50 ug/ml) was added and incubated at room temperature under darkness for 15 minutes before flow cytometry analysis. Data were analyzed using FlowJo software (Version 10.4).

Cell Viability Assay

For cytotoxicity analysis, 10,000 cells of each group were seeded in a 96-well plate. After different time and concentration drug treatments, 10 μl CCK-8 (YiSheng) was added to each well and cultured in a cell incubator for 1 h. Finally, the absorbance was measured at 450 nm by a micro-plate reader (Tecan Infinite 200). There were 4 replicate wells in each group and three replicates were performed.

Metabolite Detection

Serum and cell metabolites were detected by coupling Ultimate 3000 Ultra-High Performance Liquid Chromatograph (UHPLC) and Q Exative Quadrupole-Electrostatic Field Orbitrap Mass Spectrometer from Thermo Fisher. The raw data were normalized for principal component analysis (PCA). Orthogonal Partial Least Squares-Discriminant Analysis was selected to characterize the differences between groups. Intracellular NAD+/NADH levels were detected using NAD+/NADH detection Kit (WST-8 method) (Beyotime). Intracellular adenosine levels were detected using adenosine assay Kit (Abcam).

Western Blot Analysis

Western blot analysis
Western blot assays were performed to detect protein expression. Primary antibodies were used at a dilution of 1:1000 and incubated overnight at 4°C. The anti-NAMPT, anti-CD38, anti-DNMT3A, anti-Caspase8, anti-Caspase9, anti-Sirt1, and anti-Sirt6 antibodies were purchased from Abcam. The anti-

Caspase3 and anti-Caspase7 antibodies were purchased from Santa Cruz (USA). The anti-CD39 and anti-CD73 antibodies were purchased from Abclona. Other antibodies were purchased from Cell Signaling Technology. Goat anti-rabbit or mouse IgG-HRP was used as a secondary antibody at 1:3000 dilution. Detection was performed by using ECL western blotting substrate.

Quantitative Reverse Transcription-polymerase Chain Reaction (Q-rt-pcr)

Total cellular RNA was extracted using the spin-column-based EZ-Press RNA purification kit and reverse transcribed to obtain cDNA according to the instructions. Q-RT-PCR was performed using an ABI 7500 real-time PCR machine (Applied Biosystems) based on Real Master Mix (YiSheng). β-Actin was used as the endogenous control gene. The 2-△△Ct method was used to obtain the relative expression levels of genes in the form of fold variation. Supplementary Table 1 lists the primer sequences.

Oncomine

Analysis of the data in Oncomine (www.oncomine.org) validated the mRNA levels of NAMPT in different types of AML. Oncomine provides powerful genome-wide expression analysis and cancer microarray information which are publicly available. In this study, p-value < 0.0001, fold change > 1 and the gene level in the top 10% were set as the significance threshold. Student's t-test is used to analyze the difference of NAMPT expression in AML.

Human Protein Atlas (Hpa)

The HPA database (https://www.proteinatlas.org/) is based on proteomics, transcriptomics and systems biology data, which can map tissues, cells, organs, etc. HPA was used to collect the corresponding RNA expression levels of NAMPT in different types of immune cells from the HPA immune cell dataset.

R Programming Language (Version 3.6.3)

The survival package (version 3.2–10) and the survminer package (version 0.4.9) were used for statistical analysis and visualization the combining survival and prognosis data from previous research [9] to draw single gene KM curves. The prognosis type is overall survival with 0–50 vs 50–100 as the grouping criteria.

The ggplot2 package (version 3.3.3) was used to analyze and visualize correlationship between genes and immunosuppressive molecules as well as their correlation with key clinical variables (FAB subtypes). The ssGSEA algorithm of the GSVA package [1.34.0 version][10] was used to perform immune infiltration analysis. The procedures include converting RNA-seq data in FPKM format into TPM format and log2

conversion, data filtering. The 24 immune cell markers were obtained from the research previously described [11] Significance mark: ns, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Animal Studies

The NOG mice were purchased from Charles River. The mice were randomly divided into 3 groups for the following experiments (n = 6 for each group). On the first day, two groups of mice were injected with OCI-AML3 and the remaining group was injected with shNAMPT-OCI-AML3 (3×10^6 cells per mouse). After 7 days, tumor proliferation in mice was observed by fluorescence imaging. For the experimental group, mice were treated daily with the NAMPT inhibitor FK866 (30 mg/kg/day) starting from day 8. The IVIS spectral imaging system was used to measure the bioluminescence signal twice a week. All animal studies were performed in accordance with ethical standards and were approved by the Committee on Animal Care and Use for Research at Shanghai Jiao Tong University School of Medicine, which are consistent with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care international.

Results

DNMT3A mutation leads to aberrant metabolism of NAM-NAD + in the AML mouse model.

We performed UHPLC-HRMS/MS detection on the serum metabolites of DNMT3A wild-type ($DNMT3A^{WT}$) and DNMT3A-R878H mutation ($DNMT3A^{MUT}$) mice and found 55 different metabolites (Fig. 1A). KEGG enrichment revealed that the most notable changes belonged to the metabolism of nicotinate and NAM, as reflected in the decreased levels of NAM and 1-methylnicotinamide in the serum of diseased mice (Fig. 1B, C). Unlike serum metabolites, the downstream metabolites of NAM, 1-methylnicotinamide, and adenosine 3'-monophosphate (AMP), were elevated in the tumor cells (Fig. 1D).

GOKEGG analyses of RNA-Seq data from mouse models, human samples (GSE27187), and U937 cells harboring the DNMT3A R882H mutation (GSE90931) indicated significant changes in the regulation of nicotinamide mononucleotide (NMN) and NAD + metabolic pathways (Fig. 1E, F). The dependence of tumors on different NAD + pathways differs according to gene amplification and epigenetic remodeling of tissue lineage [12–13]. These data demonstrate that in acute myelomonocytic leukemia caused by mutations in DNMT3A, the metabolism of nicotinate and NAM is abnormal and may play a role in disease progression and prognosis through the synthesis and catabolism of NMN and NAD+.

Mammals primarily use NAM as a precursor for NAD + biosynthesis. NAD + is cleaved by NAD+-consuming enzymes to generate NAM, which is then converted to NMN by NAMPT and finally to NAD + by nicotinamide mononucleotide adenylyltransferase. This cycle produces a stable pool of NAD+. Thus, we analyzed the changes in vital enzymes in the catabolic and anabolic processes of NAM and NMN, including NAMPT and CD38, in tumor cells. CD38 can metabolize the NAD + produced by NAMPT into ADPR for further metabolism [14]. An analysis of leukemic cells showed that NAMPT and CD38 were

upregulated remarkably in both the mRNA and protein levels of leukemic cells in DNMT3A^{MUT} mice (Fig. 2A, B).

To explore the mechanism for this metabolic profile change, we stably transfected the DNMT3A-R882H mutant (DNMT3A^{MUT}) or DNMT3A^{WT} into the U937 cell lines and found that both the transcriptional and translational levels of NAMPT were significantly elevated in cells that harbored DNMT3A^{MUT}. However, changes in CD38 expression levels were not obvious. The levels of the rate-limiting enzyme NAPRT in the Preiss–Handler NAD + synthesis pathway did not change significantly, implying that the DNMT3A^{MUT}-carrying tumor was mainly dependent on NAMPT for survival (Fig. 2C, D, E). Meanwhile, compared with the DNMT3A^{WT}-carrying U937, the U937 cells with DNMT3A^{MUT} had more NAD+/NADH (Fig. 2F). The abnormal expression of NAMPT caused by this mutation may have caused the unusual levels of extracellular NAM and intracellular NAD+/NADH as its corresponding downstream products. These results suggest that DNMT3A R882H mutation modulates the metabolic state of tumors by altering the expression of metabolic enzyme NAMPT along the NAM-NAD + axis.

NAMPT expression is significantly elevated in FAB AML-M4/M5 leukemia.

Studies have shown that NAMPT is overexpressed in colorectal cancer, gastric cancer, and glioma and is used as an anticancer target [15–19]; however, it has not become a widespread target in AML treatment, mainly due to the genetic heterogeneity of AML. Therefore, the selection of AML with high sensitivity to the NAMPT inhibitor has become a key premise for choosing NAMPT as a therapeutic target for leukemia.

Oncomine database analysis showed that the NAMPT expression in AML-M4/M5 was higher than that in normal tissues (Table 1); this result is consistent with TCGA data (Fig. 3A). At the same time, no difference was observed in the expression of NAPRT in different FAB types (Fig. 3B).

Extracellular NAD+ (eNAD+) and intracellular NAD+ (iNAD+) levels are highly correlated because of the intramembrane transport of NAD precursors, metabolic intermediates, and NAD itself [20]. eNAD is degraded by three major classes of specific exoenzymes: the NADases CD38 and CD157, ADP-ribosyl transferases, and ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1) and NT5E [21–23]. CD38, CD157, ENPP1, and NT5E can promote the formation of adenosine (ADO), a potent immunosuppressive factor [24–25]. However, FAB AML subtype analysis of TCGA found no significant differences in the expression levels of these NADases (Fig. S1), except for CD157 (Fig. 3C).

AML-M4/M5 leukemias include acute myelomonocytic leukemia and acute monocytic leukemia, which are characterized by neutrophilic precursor cells or monocyte precursor cells that make up 20% of the bone marrow. According to immune cell data in the Human Protein Atlas (HPA, https://www.proteinatlas.org/), NAMPT expresses much higher in neutrophils, nonclassical/classical monocytes, and intermediate monocytes than in other cells (Fig. 3D). This result suggests that in AMLM4/5 leukemias, an overall increase in NAMPT expression is plausible.

These data suggest that DNMT3A-R882H, a high-frequency mutation in acute myelomonocytic/monocytic leukemia, may promote the malignant proliferation of immature myelomonocytic/monocytic leukemia cells, resulting in increased expression of NAMPT as well as metabolic reprogramming of cells, which ultimately promote the malignant progression of the tumor.

Inhibition of NAMPT induces cell cycle arrest, apoptosis and differentiation.

In a knockout mouse model, DNMT3A gene silencing results in the accelerated proliferation and impaired differentiation capacity of hematopoietic stem cells [26]. In a xenograft model, hematopoietic stem cells that carry DNMT3A mutations have a survival advantage cover wild-type hematopoietic stem cells [27].

We performed GSEA analysis of RNA-seq data from human samples (GSE27187), mouse model, and U937 cells transfected with the DNMT3A-R882H mutation (GSE90931) and found that the cell cycle and mitotic signaling pathways were significantly enriched in the mutant group (Fig. 4A, B, C).

NAMPT converts NAM to NMN and further to NAD + by promoting the reversible binding of components in PRPP to NAM. NAMPT content and its enzyme activity affect NAD + involved metabolism and NAD+-dependent enzyme through the synthesis of NAD+, thereby regulating tumor cell metabolism and proliferation.

Clinically, a higher NAMPT expression is associated with poor prognosis correlated with tumor growth, metastasis, and cellular dedifferentiation in melanomas [28, 29]. In gliomas, NAMPT acts as an important dedifferentiation inducer to help glioma tumor stem-like cells (GSCs) maintain stemness and self-renewal properties [30]. High levels of NAMPT in lymphomas are associated with higher aggressiveness [31].

Elevated NAM metabolism by NAMPT in the LSCs of relapsed AML is also the mechanistic basis of ven/aza resistance as well as of the metabolic vulnerability of relapsed/refractory LSCs [32]. As a new generation of NAMPT inhibitor, FK866 is characterized by its low toxicity; drug toxicity occurs only at high doses, mainly manifested as thrombocytopenia, anemia, or neutrophil toxicity. We aimed to determine whether or not high expression of NAMPT caused by DNMT3A-R882H mutation confers tumor sensitivity to FK866 and lower toxicity after treatment.

First, we treated OCI-AML3, THP1, and NB4 with FK866 and found that OCI-AML3 carrying the DNMT3A mutation was the most sensitive to FK866. We then treated the U937 cell line carrying the $DNMT3A^{MUT}$ or $DNMT3A^{WT}$ with FK866 and found that U937 cells that harbored $DNMT3A^{MUT}$ were more sensitive to FK866 than those harboring $DNMT3A^{WT}$ and are even more sensitive to FK866 than OCI-AML3 (Fig. 4D). The THP1 cell line showed negligible change in its cell viability after treatment with a high concentration of FK866.

Furthermore, the cells underwent apoptosis and cell cycle arrest after FK866 treatment or NAMPT knockdown (Fig. 4E, F, G, H). Western blot experiments showed that CCNE2 and CCND3 were affected by

low concentrations of FK866, and the apoptosis-initiating protein caspase8/9 also changed accordingly (Fig. 4I).

Impaired myeloid precursor differentiation and malignant clonal expansion are two major hallmarks of AML malignancy. Myeloid differentiation markers CD11b and CD14 were elevated in shNAMPT-OCI-AML3 cells (Fig. 4J). Morphologically, shNAMPT-OCI-AML3 cells displayed more characteristics of mature myeloid cells than OCI-AML3 cells (Fig. 4K), indicating that NAMPT depletion promotes myeloid differentiation.

Therefore, our data suggest that NAMPT inhibition or knockdown induced tumor cell apoptosis, cycle arrest, and differentiation in AML that harbored DNMT3A mutation.

Inhibition of NAMPT induces depolymerization of the cyclin-CDK complexes via SIRT6.

FK866 mainly induced apoptosis and significant cell cycle arrest in DNMT3A^{MUT} cells in a short time (less than 24 h) without too much ATP loss, which greatly reduced the cell expansion rate. We speculate that this is related to NAD+-dependent enzymes.

String showed that NAMPT mainly interacted with the Sir2 family, of which the most credible interaction was SIRT6 (Fig. 5A). NAM inhibits SIRT6 activity by competing with NAD + and binding to the sirtuin protein [33]. We propose that the decreased NAM caused by the DNMT3A^{MUT} may lead to the enhanced enzymatic activity of SIRT6. Analysis of acetylation sites of histone lysine showed that SIRT6 acetyltransferase activity can be elevated by DNMT3A-R882H mutation (Fig. 2C) and is inhibited in a concentration-dependent manner by FK866 (Fig. 4I).

Therefore, we examined the possible role of NAMPT in the regulation of the malignant cell cycle through NAD+-dependent enzymes. The correlation of SIRT6, cyclin and CDKs expression in leukemia from the TCGA database showed that the expressions of SIRT6 and CCND3 and their kinases CDK4 and CDK2 were significantly positively correlated (Fig. 5B). In addition, both SIRT6 and CCND3 were highly expressed in FAB M5 leukemia, leading to poor prognosis in AML (Fig. 5C, D). SIRT6 may affect the cyclin-CDK complexes through CDKN1A/B, according to the prediction result of String (Fig. 5E). Immunoblotting showed that the deacetylation function of SIRT6 was affected by NAMPT inhibition or NAMPT knockdown, accompanied by a marked increase in CDKN1A/B protein levels (Fig. 5F, G).

Next, we treated OCI-AML3 with the SIRT6 agonist UBCS039 or overexpressed SIRT6 in OCI-AML3 and found that agonism or overexpression of SIRT6 reduced the protein levels of CDKN1A/B (Fig. 5H). Compared with the acetylated state, the deacetylated CDKN1A/B protein were more labile [34]; this result may be part of the effect of SIRT6 on the cyclin-CDK complexes.

Co-immunoprecipitation experiments showed that after NAMPT inhibition or knockdown, the formation ability of the CDK1-CCNB1, CDK2-CCNE2, and CDK4-CCND3 complexes deteriorated, whereas the binding ability between CDKN1A/B and the corresponding cyclin-CDKs were enhanced (Fig. 5I, J, K).

These results indicate that DNMT3A-R882H mutation promoted the formation of cyclin-CDK complexes through NAMPT-SIRT6-CDKN1A/CDKN1B, thereby accelerating the cell cycle cycling. FK866 can stabilize CDKN1A/CDKN1B and impede the formation of cyclin-CDK complexes to interrupt the cell cycle progression and greatly inhibit tumor cell proliferation.

To further verify the role of FK866 in animals, we selected NOG mice for the animal experiments. Compared with controls, Tumor growth decreased after FK866 treatment (Fig. 5L, M), mice injected with shNAMPT tumor cells and tumor-bearing mice treated with FK866 had longer overall survival times (Fig. 5N). Moreover, inhibition or knockdown of NAMPT significantly reduced the splenic infiltration of tumor cells (Fig. 5O). FK866 showed low toxicity in the animal experiments; that is, it had no apparent effects on animal organs and body weight. These results indicate that FK866 can be used as a possible drug for the treatment of AML that is characterized by high NAMPT expression.

AML with high NAMPT expression mediates immune suppression mainly based on aberrant NK infiltration and its suppressed function.

To explore the effect of NAM-metabolizing enzymes on the immune microenvironment of AML, we divided AML samples from TCGA into two groups based on the expression of NAM-metabolizing enzymes and calculated their immune infiltration enrichment scores. Results show that infiltration of total NK cells and NK CD56 dim/bright cells in the immune microenvironment was greatly affected when the tumor cells highly expressed the NAM-metabolizing enzymes NAMPT, CD38.In addition, correlation analysis of the TCGA-AML expression profile found that multiple NK immune checkpoints, including PD-L2, PD-L1, CD86, and SIRPA, were positively correlated with the expression of the NAM-metabolizing enzymes (Fig. 6A, B).

To further investigate the role that NAMPT elevation caused by $DNMT3A^{MUT}$ plays in immune infiltration, we analyzed immune infiltration in 10 AML-M5 patients, of which 6 cases carried $DNMT3A^{MUT}$, and found a number of differences in the immune infiltration of the two groups (Fig. S2). Differences in immune infiltration were mainly manifested in the NK CD56dim cells, cytotoxic cells, and CD8 + T cells (Fig. 6C).

Single-cell sequencing of mouse leukemic cells also displayed a reduction in NK cell immune infiltration (Fig. 6D). GO analysis of immune cell populations showed that compared with $DNMT3A^{WT}$ mice, $DNMT3A^{MUT}$ mice exhibited apparent changes in the immune response-related pathways of NK cells and the apoptosis-related signaling pathways of $CD8^{+}$ cells (Fig. 6E, F).

We then evaluated the level of adenosine after NAMPT or CD38 inhibition. The experimental results showed that inhibiting NAMPT or CD38 greatly suppressed the production of adenosine in the cells, whereas adding NMN (a NAD precursor) greatly increased the amount of cell adenosine (Fig. 6G). The addition of CD38 inhibitor to the OA3 and PBMC co-culture system significantly improved the killing ability of PBMC on tumor cells, whereas adenosine weakened the killing capacity (Fig. 6H). In addition, the addition of adenosine reduced the numbers of NK cells during PBMC culture (Fig. 6I).

These results imply that the tumor cells with the DNMT3A^{MUT} may damage the immune microenvironment by enhancing NAM-NAD+-ADO. On the one hand, this metabolic reprogramming can reduce the inhibition of NAM on SIRT6 activity and generate more NAD + to enhance the deacetylation of SIRT6, which in turn promotes the binding of CDKs to cyclins to help tumor cells maintain a high level of cell cycle progression. On the other hand, this metabolic alteration can also produce more ADO as an immunosuppressant. In this study, our data suggests that the NK cells were the most affected immune cell population.

ADO acts as a negative immune regulator in a variety of tumors, and its accumulation during immune checkpoint therapies, such as those involving PD1/PDL1 inhibitors, can impair the therapeutic effects [35]. However, little attention has been paid to the enzymes that play roles in adenosine production and function. We found that the action of adenosine on tumor cells can significantly increase the protein levels of NAM-ADO-metabolizing enzymes as well as promote the expression of immune checkpoint-related molecules (Fig. 6J, K). This effect can lead to a positive cycle of adenosine's effects on cells and further result in a suppressed immune microenvironment.

These data provide preliminary proof for the vital roles of NAMPT and other enzymes in AML immunomodulation.

Discussion

Most previous studies have focused on the abnormal expression of NAMPT and other NAD + synthesis rate-limiting enzymes, such as NAPRT, in a large class of tumors based on the higher demand for ATP by cancer cells and the shorter half-life of NAD+. In addition to epigenetic silencing of the frequently mutated PPM1D-driven NAPRT, which confers sensitivity to NAMPT inhibition in pediatric gliomas, and IDH1-mutated glioma types that are extremely sensitive to NAMPT loss [36, 37], no oncology research has delved into the relationship between specific mutations, NAMPT expression, and tumor susceptibility to NAMPT inhibition. Because of the huge differences in AML lineage sources, different subtypes of AML depend on NAD + and its synthetic pathways at varying degrees. Therefore, NAMPT-targeted therapy for AML has not been further developed to date.

First, this study explored the effects of DNMT3A-R882H mutation on cellular metabolism at the genomic, transcriptomic, and metabolomic levels. We found that DNMT3A mutation can cause metabolic reprogramming in leukemia cells through the upregulation of the NAM-metabolizing enzyme NAMPT. The use of the NAMPT inhibitor FK866 can depolymerize the cyclin-CDK complexes by inhibiting the deacetylation of the NAD+-dependent deacetylase SIRT6 and finally inducing cell cycle arrest. Next, this study provides a screening method for the targeted therapy of FK866 in AML; that is, high expression of NAMPT mostly occurs in AML-M4/5 leukemia. This kind of AML may be the preferred type for FK866 therapy. Finally, our data showed that DNMT3A mutation can form an immunosuppressive microenvironment through NAM-NAD+-ADO metabolic reprogramming, thus providing an explanation for the poor prognosis of DNMT3A-mutated leukemias that are more prone to immune escape.

In addition to intracellular NAMPT (iNAMPT), extracellular NAMPT (eNAMPT) secreted through non-canonical pathways is also considered as a novel soluble factor that exhibits cytokine/adipokine/DAMP-like effects and may act in a non-enzymatic manner. Studies have shown that eNAMPT is carried by extracellular vesicles and participates in systemic circulation in mice and humans. Extracellular vesicles - carried eNAMPT can be internalized into cells to enhance NAD + synthesis. Moreover, the deacetylation of sirtuins can affect the secretion of eNAMPT [38, 39]. Levels of eNAMPT (particularly serum NAMPT) have also been shown to increase in cancer and possibly lead to poor prognosis [40]. eNAMPT secreted by cancer cells may contribute to angiogenesis and induction of an inflammatory cancer microenvironment [41]. Researchers have found that eNAMPT has an immunosuppressive and pro-tumor microenvironment in chronic lymphocytic leukemia and is important for the differentiation of monocytes into tumor-supporting M2 macrophages [42]. NAMPT and NAD + levels are significantly increased during pro-inflammatory M1 macrophage activation [43].

This study focused on the differential expression of iNAMPT caused by DNMT3A mutation and its effect on the malignant proliferation of tumor cells and the formation of an immunosuppressive microenvironment through metabolic reprogramming. However, the possible impact of DNMT3A mutation on the secretion and delivery of eNAMPT by tumor cells need to be further explored. Revealing the relationship between DNMT3A mutation and eNAMPT may help clarify the interaction between iNAMPT and eNAMPT in tumors that carry this mutation and thus determine the potential of a combination of iNAMPT inhibitors and eNAMPT neutralizing antibodies as a therapeutic strategy.

Declarations

Competing interests: All authors declared no competing interests.

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Author Contributions

All authors contributed to the manuscript. X.J.Y and Y.Y.W conceived the project and designed the experiments. X.J.Y and X.W performed the experiments and analyzed the data. Y.Y, Z.Y.L, Y.S.C and S.Q.S assisted with some experiments and data analysis; X.J.Y and X.W wrote the paper. Y.Y.W supervised the execution of the experiments and revised the paper. All authors discussed the results and approved the final manuscript.

Competing Interests

The authors report there are no competing interests to declare.

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Tables

Table 1 is available in the Supplementary Files section.

Figures

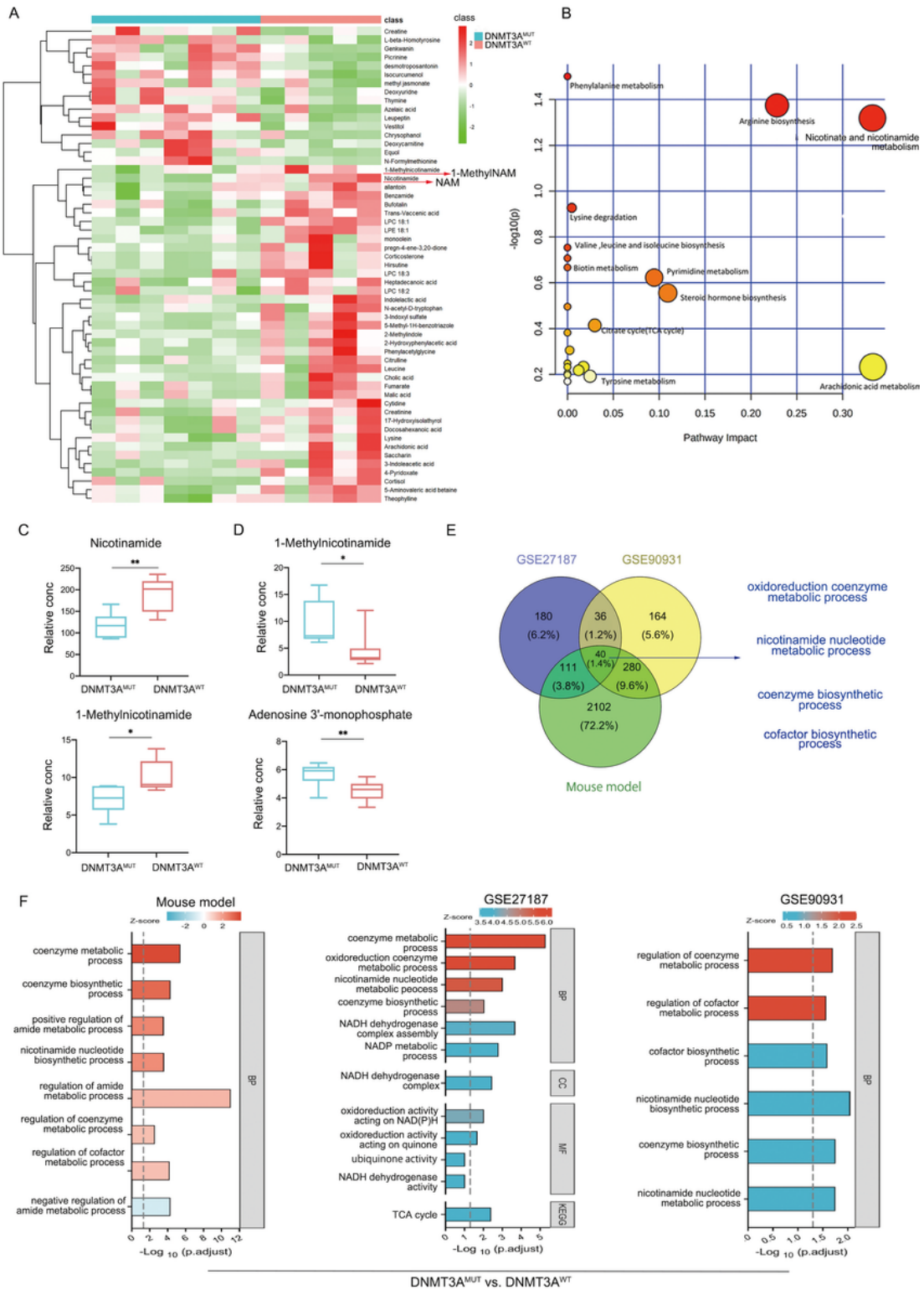

Figure 1

DNMT3A mutation leads to abnormal metabolism of nicotinamide (NAM).

A UHPLC-HRMS/MS detection showed 55 differential metabolites in the serum of mice in the DNMT3A^{MUT} group compared with the DNMT3A^{WT} group. **B** GOKEGG enrichment of the 55 differential metabolites. **C** Levels of NAM and 1-methylnicotinamide in mouse serum. **D** Levels of 1-methylnicotinamide and Adenosine 3'-monophosphate in mouse bone marrow cells. **E** GOKEGG enrichment of the differential genes of DNMT3A^{MUT} vs DNMT3A^{WT} in the mouse model, GSE27187 and GSE90931, and 40 common differential pathways are in all of them, of which the highlighted ones are the NAM-NAD+-related metabolic pathways. **F** Differential pathway analysis of NMN synthesis and coenzyme metabolism in the mouse leukemic cells, GSE90931 and GSE27187.

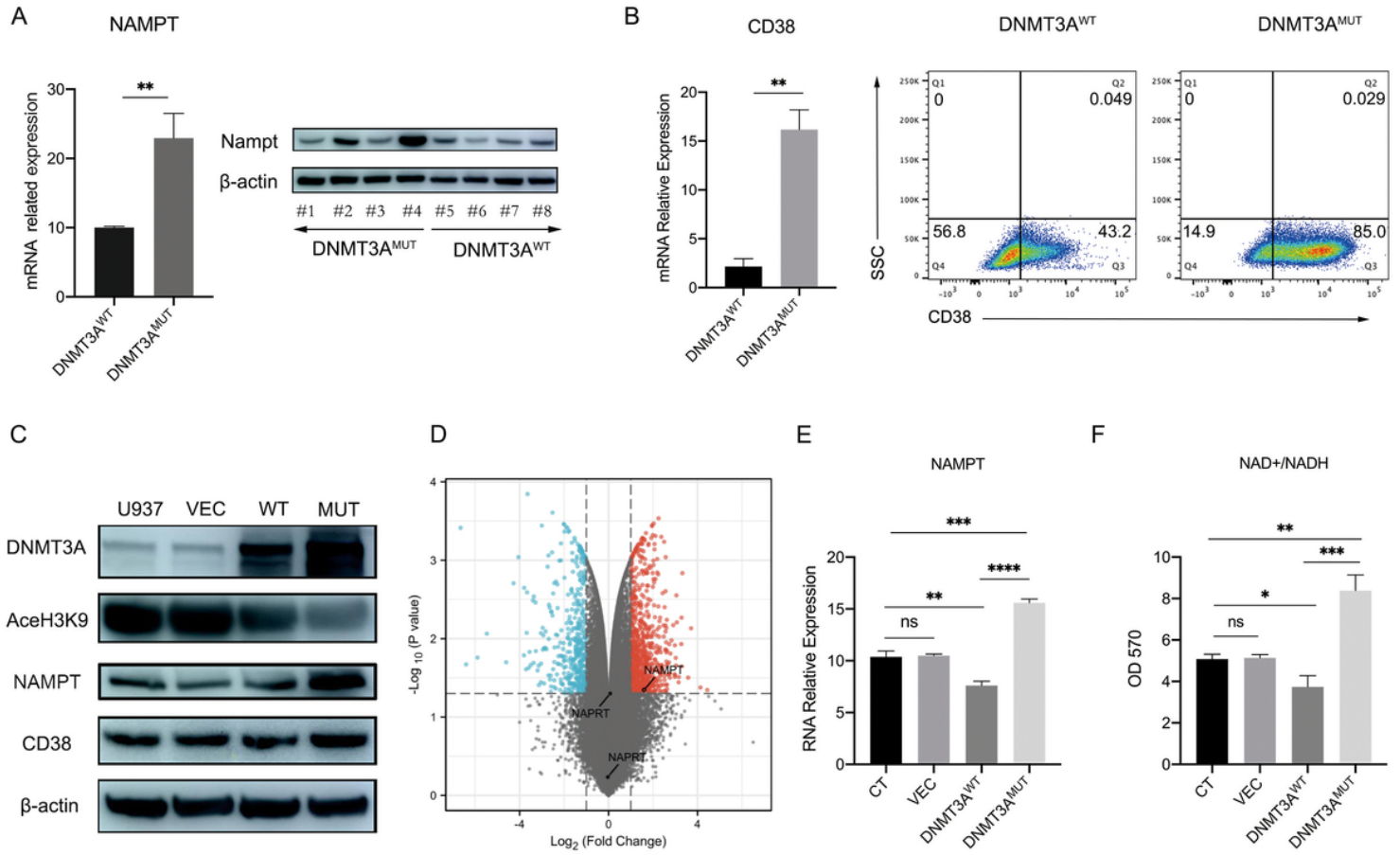

Figure 2

DNMT3A mutation results in high expression of NAMPT and increased NAD+/NADH in cell lines.

A–B NAMPT and CD38 are highly expressed in mice harboring the DNMT3A-R878H mutation. **C–E** In U937 cells, the DNMT3A-R882H mutation leads to a marked increase in NAMPT at the transcriptional and translational levels. **F** In U937 cells, the DNMT3A-R882H mutation results in the increased intracellular NAD+/NADH content. The mean ± SD of minimum n = 3 independent experiments is displayed, representative images shown *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 (Student's T-test).

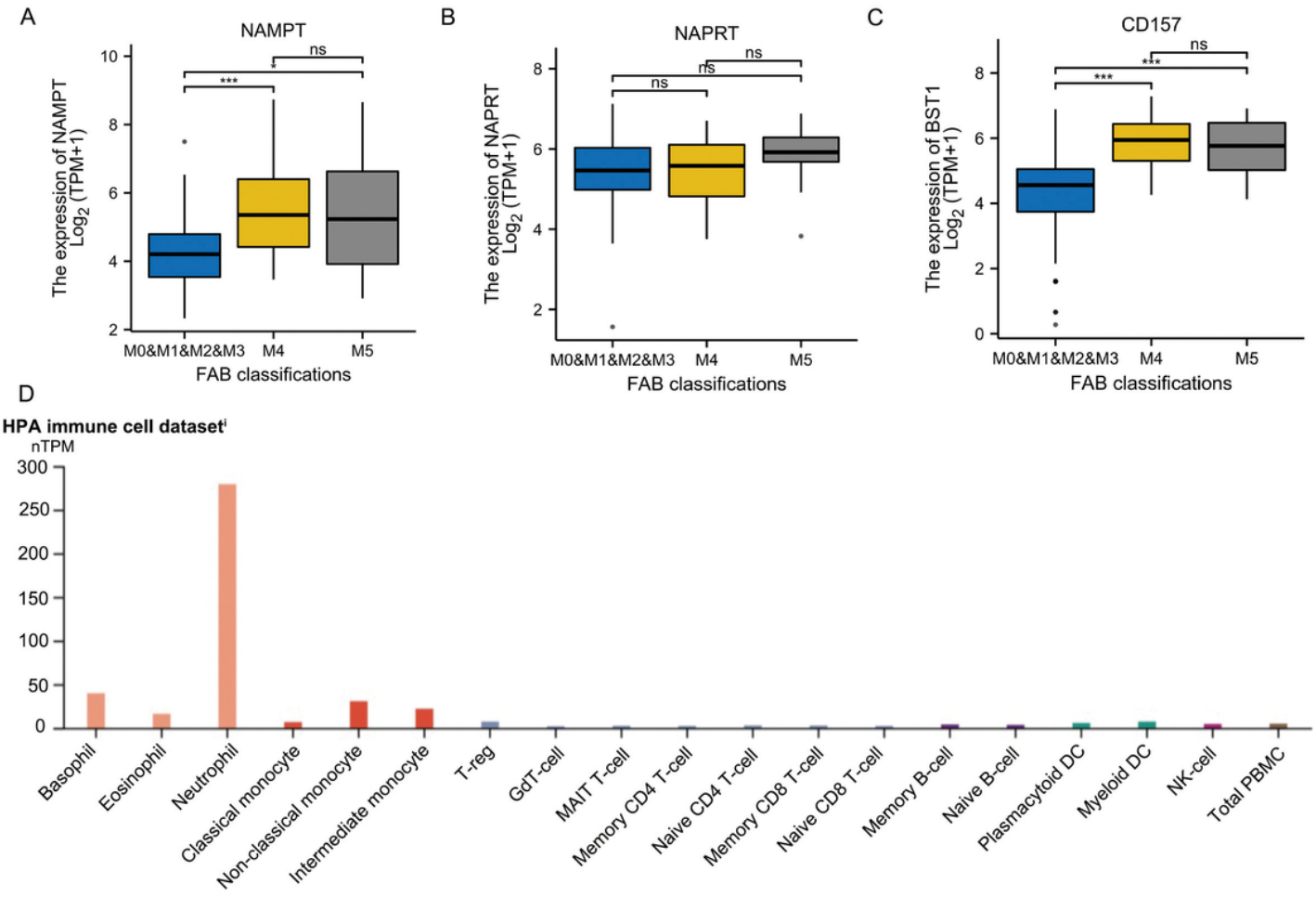

Figure 3

NAMPT is highly expressed in AML-M4/5 cases in the TCGA database.

A The expression of NAMPT in various types of AML in the TCGA-AML database. **B** The expression of NAPRT in various types of AML in the TCGA-AML database. **C** The expression of CD157 in various types of AML in the TCGA-AML database. **D** Expression levels of NAMPT in various immune cells from HPA database.

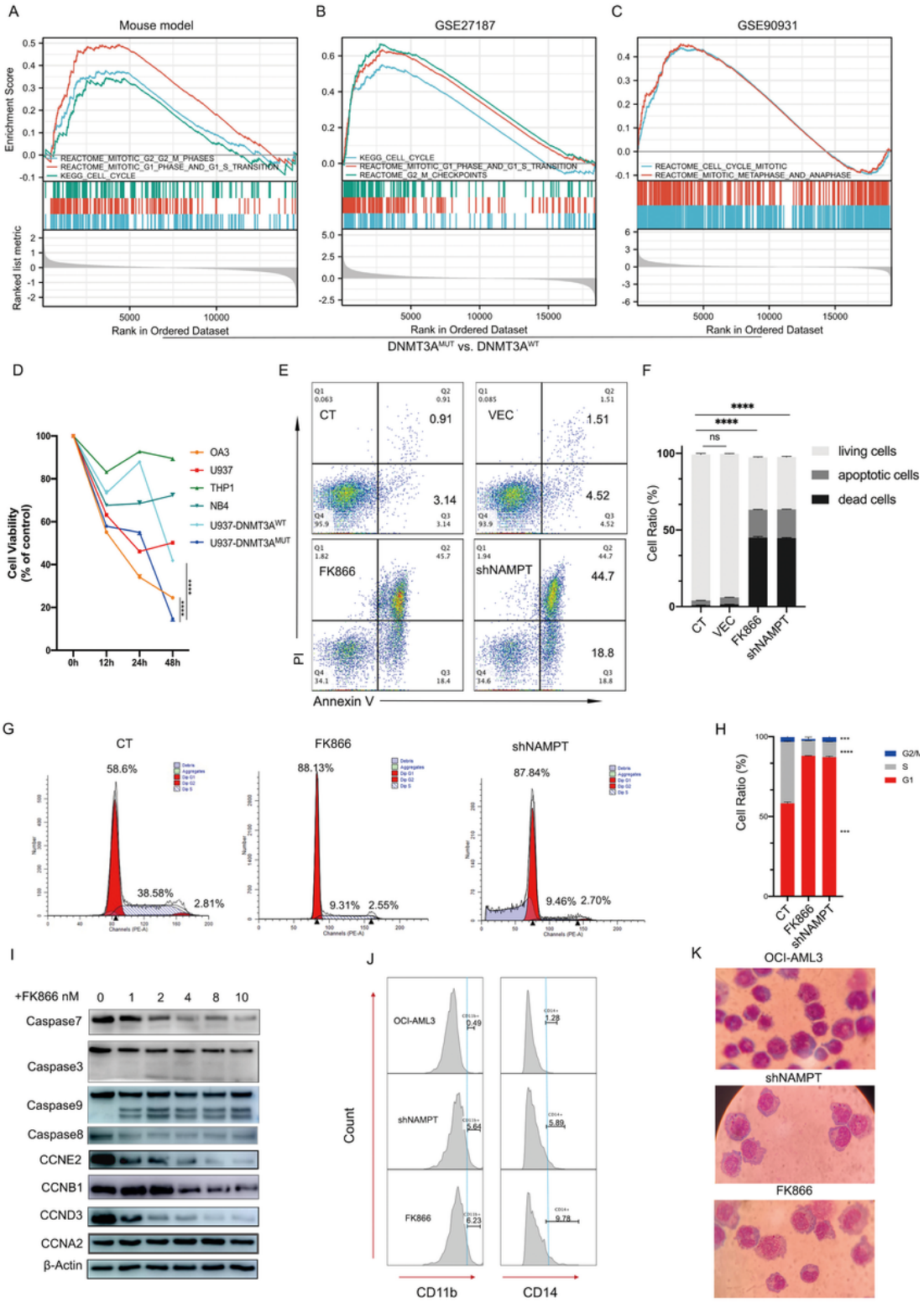

Figure 4

Inhibition of NAMPT induced apoptosis, cell cycle arrest, and cell differentiation in AML cells.

A-C GSEA enrichment of cell cycle-related signaling pathways between samples that carried DNMT3A^{MUT} and DNMT3A^{WT}. **D** Cell viability after NAMPT inhibition using FK866. **E-F** Cell apoptosis after shNAMPT or NAMPT inhibition using FK866. **G-H** Cell cycle arrest after shNAMPT or NAMPT inhibition using FK866.

I Apoptosis and cell cycle-related protein changes after NAMPT inhibition using FK866. **J-K** Cell differentiation-related markers and cell morphological changes after shNAMPT or NAMPT inhibition using FK866. The mean ± SD of minimum n = 3 independent experiments is displayed, representative images shown *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 (Student's T-test).

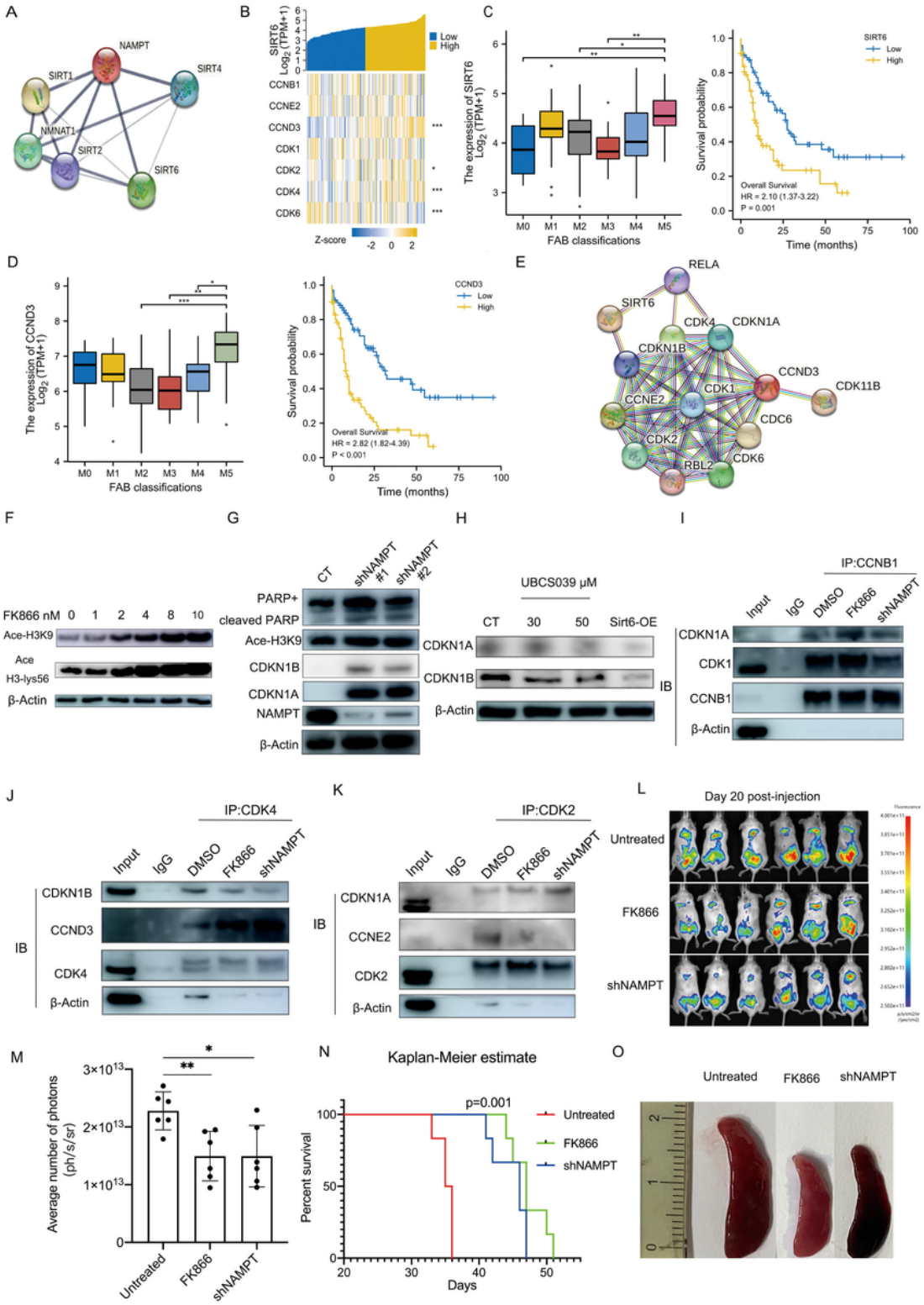

Figure 5

NAMPT promotes cell cycle by promoting cyclin-CDKs complexes formation via Sirt6.

A String prediction shows links between NAMPT and the Sir2 family. **B** Correlation between SIRT6, CCND3, and its CDKs in TCGA AML database. **C, D** SIRT6 and CCND3 are highly expressed in M4/5 leukemia and associated with poor prognosis. **E** String prediction shows that SIRT6 may have an effect on CCND3 and its CDK complexes formation through CDKN1A/B. **F** Inhibition of NAMPT effectively inhibits the deacetylation ability of SIRT6. **G** shNAMPT reduces the deacetylation ability of SIRT6 and increases the expression of CDKN1A/B. **H** Agonism or SIRT6 overexpression promotes CDKN1A/B degradation. **I-K** shNAMPT or FK866 inhibits the formation of the CDK1-CCNB1, CDK4-CCND3, and CDK2-CCNE2 complexes. **L-M** Tumor fluorescence imaging and mean photon counts of untreated, FK866-treated tumor-bearing mice or mice injected with shNAMPT tumor cells. **N**Kaplan–Meier survival curve of untreated, FK866-treated tumor-bearing mice or mice injected with shNAMPT tumor cells. **O** Splenic infiltration of tumors of untreated, FK866-treated tumor-bearing mice or mice injected with shNAMPT tumor cells. The mean ± SD of minimum n = 3 independent experiments is displayed, representative images shown *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 (Student's T-test).

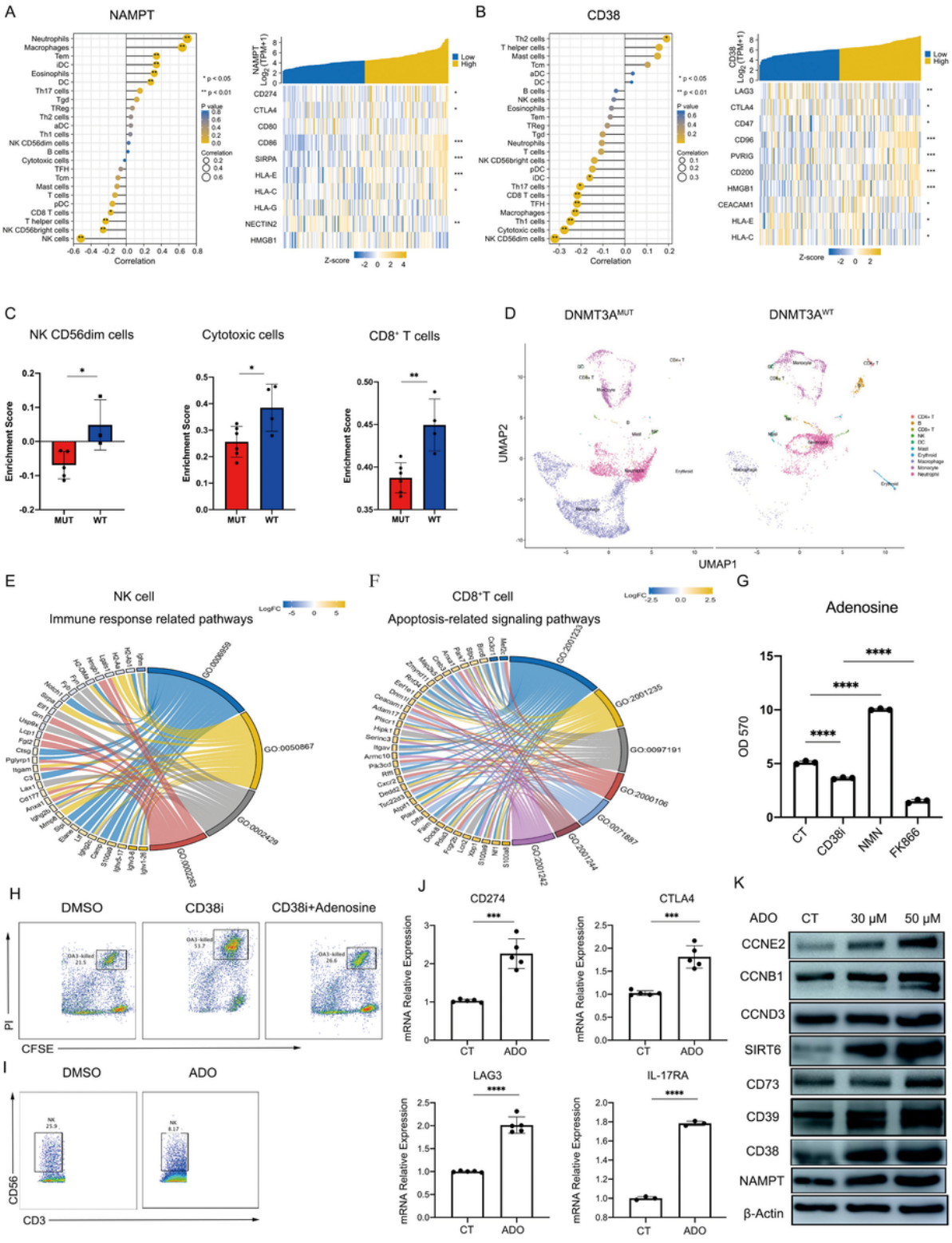

Figure 6

AML with high NAMPT expression mediates immunosuppression.

A–B Tumors with high NAMPT and CD38 expression mediate the abnormal immune infiltration of NK, NK CD56 bright/dim cells and abnormal expressions of immune checkpoint molecules. **C** M5 patients with DNMT3A^{MUT} have abnormal NK cell, cytotoxic cell, and CD8^{+} T cell infiltration. **D** Mice bearing

DNMT3A^{MUT} have abnormal NK and CD8^{+} T cell infiltration. **E-F** Mice carrying DNMT3A^{MUT} have abnormal NK cell immune responses and abnormal CD8^{+} T cell apoptosis. **G–H** Inhibition of CD38 or NAMPT reduces the level of ADO produced by cells, which can significantly weaken the killing effect of PBMC on tumor cells. **I–J** ADO reduces the proportion of NK cells in PBMC culture and increases the expression level of immune checkpoints on tumor cells. **K** ADO increases the expression level of NAM-ADO metabolic enzymes in tumor cells. The mean ± SD of minimum n = 3 independent experiments is displayed, representative images shown *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 (Student's T-test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterials.pdf
- Table1oncogene.xls