Bleximenib, the novel menin-KMT2A inhibitor JNJ-75276617, impairs long-term proliferation and immune evasion in acute myeloid leukemia

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Received: April 16, 2024.
Accepted: December 13, 2024.
Early view: December 19, 2024.

https://doi.org/10.3324/haematol.2024.285616

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Abstract

Acute myeloid leukemia (AML) remains challenging to treat, which in part relates to genetic heterogeneity of the disease, to the protective tumor microenvironment driving resistance to therapy, and also to immune evasion characteristics of leukemic cells. Targeting epigenetic programs in AML provides an attractive opportunity to impair long-term proliferation and induce differentiation. The novel inhibitor JNJ-75276617 (bleximenib) targets the menin-KMT2A interaction and has shown preclinical efficacy in AML.[1] Here, we provide mechanistic insights into how JNJ-75276617 impairs proliferation and drives differentiation of primary AML patient cells. A large-scale drug screen was set up in which genetic alterations and quantitative proteomics were compared with drug sensitivity in a preclinical setting, which revealed that granulocyte-macrophage progenitor (GMP)-like AML display the greatest sensitivity. Furthermore, we identified that *NPM1c/DNMT3A*^{mut} AML are sensitive, and some *NPM1*^{wt} AML subtypes without *KMT2A-MLLT3* rearrangements benefit from menin-KMT2A inhibition. Genome-wide chromatin immunoprecipitation-sequencing studies revealed patient-specific epigenetic alterations upon JNJ-75276617 treatment, uncovering a striking upregulation of MHC class I and class II expression as a consequence of epigenetic changes upon menin-KMT2A inhibition, independent of MEIS1 loss but involving CIITA activation. Functionally, this results in enhanced sensitivity of leukemic blasts to T-cell-mediated cytotoxicity in allogeneic and autologous settings. Our data indicate that JNJ-75276617 provides a potential therapeutic approach whereby not only proliferation is impaired and differentiation is induced, but whereby therapeutic benefit might also be achieved by reactivating the antigen presentation machinery.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease initiated by genetic mutations, whereby a block in differentiation leads to accumulation of hematopoietic stem and progenitor cells in the bone marrow.[2] Curative treatment has remained challenging, in part related to the complexity of the disease in which multiple, genetically distinct subclones frequently co-occur within individual patients, further complicating treatment efficacy.[3,4] While the usual backbone of treatment entails '7+3' chemotherapy, consisting of anthracyclines and cytarabine, new targeted therapies are emerging. For instance, specific inhibitors against *FLT3*, *BCL2*, *JAK2*, *IDH1* and *IDH2* mutations have now been added to treatment options for AML depending on mutational status and fitness of the patient. For patients with *KMT2A* (lysine methyltransferase 2A; previously mixed-lineage leukemia [MLL]) rearrangements and *NPM1* (nucleophosmin 1) mutations there are no Food and Drug Administration-approved targeted therapies available thus far.

The *KMT2A* gene is a member of the Trithorax group, which includes, among others, three pairs of family members *KMT2A/B*, *KMT2F/KMT2G* and *KMT2C/D*, and *KMT2E*, an enzymatically inactive protein.[5] Of these Trithorax proteins, only KMT2A and KMT2B can bind to menin, which serves as an adaptor between KMT2A/B and *PSIP1* (PC4 and SRSF1 interacting protein 1).[6] Menin-KMT2A inhibitors block the interaction between menin and KMT2A, resulting in displacement of the whole complex from chromatin. As a consequence, methyltransferase activity of the canonical

complex or fusion proteins is lost at these loci, and AML development is, therefore, impaired as shown in several *in vivo* model systems.[7-9] Known target genes of the KMT2A-complex, including the *HOX* genes and *MEIS1*, are all upregulated in patients with rearranged *KMT2A* and *NPM1* mutations.[10-15]

The importance of the tumor microenvironment in cancer disease development in general and also specifically in AML has become increasingly more evident.[16] By inducing alterations in the tumor microenvironment, leukemic blasts benefit in terms of drug resistance, driving relapse of disease.[17,18] One of the escape strategies of leukemic blasts is MHC class I and class II loss or decreased expression through genetic deletion or epigenetic downregulation, which renders the blasts invisible to T cells due to defective antigen presentation. Patients who relapse after allogeneic hematopoietic stem cell transplant (HSCT) show significantly lower expression of MHC class I and class II molecules, indicating a role for MHC class I and II antigen presentation in the effectivity of allogeneic HSCT and other immunotherapies.[18-20] It will be of great interest to develop new strategies to reverse the downregulation of MHC class I and II molecules to further improve the efficacy of immunotherapies.

Here, we show that the novel menin-KMT2A inhibitor JNJ-75276617 (bleximenib) impairs proliferation and drives differentiation of primary AML patient cells. We set up a drug screen in which genetic alterations and the quantitative proteome were compared with drug efficacy in a preclinical setting, which revealed that granulocyte-macrophage progenitor (GMP)-like AML subtypes displayed the greatest sensitivity. We found that not only *NPM1c/DNMT3A*^{mut} AML subtypes are sensitive, but that some *NPM1*^{wt} AML subtypes without *KMT2A-MLLT3* rearrangements also benefit from menin-KMT2A inhibition upon treatment with JNJ-75276617. Genome-wide chromatin immunoprecipitation (ChIP)-sequencing studies revealed patient-specific epigenetic alterations upon JNJ-75276617 treatment, revealing a striking upregulation of MHC class I and class II expression as a consequence of epigenetic changes upon menin-KMT2A inhibition, which functionally resulted in enhanced sensitivity of leukemic blasts to T-cell-mediated cytotoxicity.

Methods

Primary samples

AML blasts from peripheral blood or bone marrow from untreated patients were studied after informed consent. The protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen, The Netherlands, in accordance with the Declaration of Helsinki. Mononuclear cells were isolated via Lymphoprep™ separation and cryopreserved. Next-generation sequencing was performed to determine the mutation status of the primary AML cells using the TruSight Myeloid Sequencing Panel (Illumina) or exome sequencing. Neonatal cord blood samples were obtained from healthy full-term pregnancies at the obstetrics departments at the Martini Hospital and University Medical Center Groningen.

Menin-KMT2A inhibitor screen in primary acute myeloid leukemia samples

Cryopreserved mononuclear cells from patients with AML (*Online Supplementary Table S1)* were thawed, resuspended in newborn calf serum supplemented with DNase I (20 U/mL), 4 μM $MgSO_4$ and heparin (5 U/mL) and incubated for 15 min at 37°C. Mononuclear cells were either cultured in liquid medium supplemented with granulocyte colony-stimulating factor (Amgen), N-Plate (thrombopoietin) (Amgen) and interleukin-3 (Sandoz) (all 20 ng/mL) or co-cultured in Gartner medium supplemented with cytokines on MS5, which were confluent plated on 0.1% gelatin-coated 48-well plates and pre-treated with mitomycin C. Mononuclear cells were treated with dimethylsulfoxide (DMSO) or 0.03, 0.30 and 3.00 μM JNJ-75276617 inhibitor for 14 days. Fresh medium and inhibitor were added at day 7 after demi-population of the cells. On day 7 and 14 cells were stained with CD45-PECy7 (BioLegend; 304016), CD117-PE (ImmunoTools; 21271174X2), CD11b-FITC (ImmunoTools; 21279113X2), and DAPI (ThermoScientific) in a 96-well plate, and were incubated for 30 min at 4°C. Fluorescence measurements were taken using a MACSQuant X Flow Cytometer (Miltenyi Biotec).

Further details can be found in the *Online Supplementary Methods* section.

Results

Heterogeneity in sensitivity to menin-KMT2A inhibition across the diverse genetic landscape of acute myeloid leukemia

Recently, clinical trials with menin-KMT2A interaction inhibitors showed a positive response in *KMT2A*-rearranged and *NPM1*-mutated leukemias.[7,21,22] Although other AML subtypes, such as *NUP98*-rearranged leukemias[23] and *UBTF*-tandem duplication leukemia,[24] were sensitive to inhibitors of the menin-KMT2A interaaction, little has been revealed about the efficacy across the heterogeneous genetic landscape of AML and the mechanisms of action.

JNJ-75276617 is a novel, potent and selective inhibitor of the binding between menin and KMT2A.[1] To be able to link menin-KMT2A inhibitor sensitivity to protein expression profiles and genotypes we performed a drug screen in a panel of 37 primary AML blast samples for which we generated a full label-free quantitative proteome (N=22) and performed Illumina TruSight sequencing (N=30) to obtain mutation status. Samples were treated with 0.03-3.0 μM JNJ-75276617 in liquid culture or MS5 co-culture for 7 and 14 days, and

effects on proliferation, viability and differentiation were monitored by flow cytometry (*Online Supplementary Figure S1A, B*). Area under the curve (AUC) values were determined based on viable DAPI^{-}/CD45^{dim} blast counts, which revealed strong efficacy but also heterogeneity in responses divided into high, late, early and low sensitivity to JNJ-75276617 (Figure 1A, *Online Supplementary Figures 1C* and *S2*). We also screened a panel of AML cell lines and our primary cord blood-derived *KMT2A-AF9* AML model[15] which again revealed heterogeneity in responses, with the *KMT2A*-rearranged and *NPM1c* AML being the most sensitive (Figure 1B, C). Although slight differences in sensitivity were observed between liquid cultures and MS5 co-cultures for some AML samples, these did not reach significance and in general we observed that when AML samples were sensitive, this was true under both liquid culture as well as MS5 bone marrow stromal co-culture growth conditions (*Online Supplementary Figure S3C, D*). Normal healthy CD34^{+} cells were considerably less sensitive compared to AML cells, both under liquid culture and MS5 stromal co-culture conditions, with AUC values well above 2.5 (Figure 1D). We considered samples with an AUC below 2.5 to be sensitive. To gain more insight into the genetic component of the differential sensitivity to menin-KMT2A inhibition, we compared AUC values with mutational status. *NPM1c/DNMT3A*^{mut} was the strongest predictor of sensitivity, particularly at day 14 (Figure 1E, *Online Supplementary Figure S3A*). Interestingly, however, we also identified strong efficacy in several *NPM1*^{wt} GMP-like AML samples without *KMT2A-MLLT3* rearrangements (Figure 1A, AML22, AML11, AML15, AML4 and AML45). AML22 did have a *KMT2A-EP300* rearrangement, in line with the notion that *KMT2A*-rearranged AML is sensitive to menin-KMT2A inhibition.[7,25,26] AML4 had a *CEBPA* mutation and AML15 had a *NUP98*-rearrangement and a *CEBPA* mutation, which both have also been shown in cell lines to be sensitive to menin-KMT2A inhibition by VTP50469 (*NUP98*) or MI-463 and MI-503 (*CEBPA*).[23,27] Four other AML samples with a *CEBPA* mutation (AML61, AML17, AML62 and AML3) were slower responders and showed a decrease in proliferation at day 14 in liquid culture. AML11 and AML45 both had a *RUNX1* mutation, which has also been shown to be sensitive to menin-KMT2A inhibition in one case in the clinical trial of ziftomenib (KO-539).[28] Less sensitive *RUNX1*- and/or *CEBPA*-mutated AML samples in our cohort often coincided with *TET2* mutations and in AML60 with a *RAS* mutation. To further investigate the efficacy of JNJ-75276617 in AML patient samples with fusion-protein aberrations, we additionally treated primary AML samples from four cases with *KMT2A-MLLT3*, one with a *NUP98*-rearrangement and two with *NPM1-MLF* (*Online Supplementary Figure S3B*, also added to Figure 1A). After 7 days of treatment of these seven samples, two of the liquid-cultured samples and four of the MS5 co-cultured samples were sensitive to the inhibitor. Although no effect was observed on proliferation after 7 days of treatment of menin-KMT2A in AML35 and AML33, an effect on differentiation measured by CD11b expression was found. AML37 and AML38 not only showed reduced proliferation after menin-KMT2A treatment, but also displayed a strong induction of CD11b expression. This would suggest that a combination read-out of proliferation (AUC values) and differentiation induction would be advisable. Therefore, the effects of menin-KMT2A inhibition on differentiation were investigated in further detail. CD11b was significantly increased upon menin-KMT2A inhibition, which was observed for AML samples where proliferation was also strongly inhibited (Figure 2A, *Online Supplementary Figure S4A*, black curves), but also in cases in which proliferation was less affected by menin-KMT2A inhibition (Figure 2A, *Online Supplementary Figure S4A*, red curves). To address this further, AUC (proliferation) values and change in CD11b expression were compared, revealing AML samples that responded in terms of inhibition of proliferation, induction of differentiation, or both (Figure 2B, *Online Supplementary Figure S4B*). To gain more insight into these phenomena, the CD45^{dim} blast population was distinguished from the more myeloid committed/monocytic CD45^{high}/SSC^{high} population by flow cytometry (Figure 2D), and effects on proliferation *versus* differentiation were analyzed which again revealed heterogeneous responses. For example, in the more immature AML17, JNJ-75276617 treatment resulted in a clear block in proliferation of the CD45^{dim} blast population while a strong increase in more myeloid committed/monocytic CD45^{high}/SSC^{high} differentiated cells was observed (Figure 2C), coinciding with a strong increase in CD11b (Figure 2E). AML13 was a more committed AML subtype with a larger proportion of CD45^{high}/SSC^{high} cells at diagnosis. Here, total cell counts of both the blast-like cells as well as the more committed monocyte-like cells were reduced (Figure 2C), again coinciding with an increase in CD11b (Figure 2C-E). A strong induction in CD11b expression was also noted in our cord blood *KMT2A-AF9* model[15] (*Online Supplementary Figure S4C*), coinciding with clear morphological myeloid differentiation (*Online Supplementary Figure S4D*). The induction of differentiation was most pronounced in *NPM1c* and *DNMT3A*^{mut} cases (*Online Supplementary Figure S4E*).

Quantitative proteome analyses link menin-KMT2A inhibitor sensitivity to more committed granulocyte-macrophage progenitor-type acute myeloid leukemias

To obtain a better understanding of the protein expression programs that underlie menin-KMT2A inhibitor sensitivity we analyzed the full proteome of a set of 22 primary AML samples. We calculated Pearson correlations between the quantitative proteome dataset and AUC values of liquid culture day 7 (*Online Supplementary Figure S5A*) or MS5 day 7 (*Online Supplementary Figure S5D*). Ranked Pearson correlation lists were then used for gene set enrichment analysis (GSEA), which revealed that menin-KMT2A inhibitor-sensitive AML cells were enriched for signatures related to L-GMP, neutrophil degranulation, *KMT2A*-rearranged

leukemias and *NPM1c*-mutated leukemias; this was seen in both liquid cultures as well as MS5 co-cultures (Figure 3A, *Online Supplementary Figure S5B, C, E*). These data suggest that somewhat more committed, L-GMP-type AML are most sensitive to menin-KMT2A inhibition. To further analyze these findings, we performed single-sample GSEA on the full proteome to identify the maturation status of the AML samples (Figure 3B) and subsequently compared

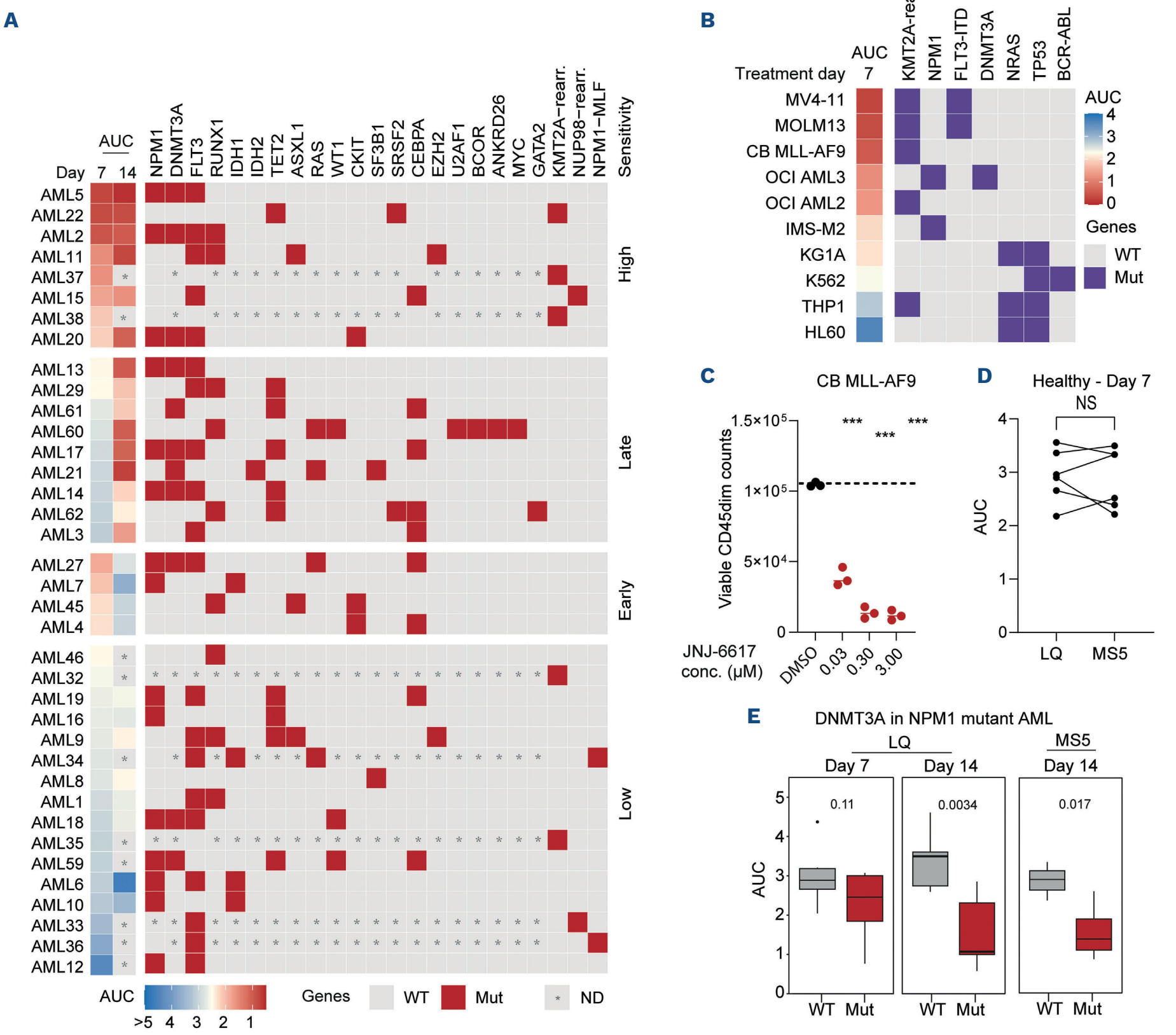


Figure 1. Heterogeneity in sensitivity to menin-KMT2A inhibition across the diverse genetic landscape of acute myeloid leukemia. (A) Area under the curve (AUC) values at day 7 and day 14 of the JNJ-75276617 inhibitor drug screen in primary acute myeloid leukemia (AML) patients' samples with various mutations in liquid culture. AUC values were calculated to show the effect of the inhibitor on proliferation. AML samples are ordered based on high, late, early and low sensitivity. Gray tiles with a star (*) annotate not determined (ND) samples. Wild-type genes are annotated with a gray tile (WT) and mutated genes are annotated with a red tile (Mut). (B) AUC values of the JNJ-75276617 inhibitor drug screen in ten AML cell lines. Wild-type genes are annotated with a gray tile (WT) and mutated genes with a purple tile (Mut). (C) Dose-dependent effects on cell viability upon treatment of the cord blood *KMT2A-AF9* model with JNJ-75276617 under liquid culture (LQ) conditions. (D) Paired analysis of AUC values calculated for the effect of JNJ-75276617 inhibitor treatment on LQ and MS5 co-cultured healthy CD34$^+$ cells on day 7 (N=6, biological replicates). (E) Boxplots showing AUC values of *DNMT3A*^{mut} *versus* *DNMT3A*^{WT} in *NPM1c* primary AML patients' samples on LQ day 7, 14 and MS5 day 14. Statistical analysis by the unpaired Student *t* test or paired for panel (D). DMSO: dimethylsufoxide; NS: not statistically significant. *P<0.05, **P<0.01, ***P<0.001.

AUC values between GMP-like, mixed and hematopoietic stem cell (HSC)-like AML, which showed a significantly higher sensitivity to JNJ-75276617 in GMP-like AML cultured on MS5 (Figure 3C) and a trend towards sensitivity in liquid-cultured samples (*Online Supplementary Figure S6D*). Since we identified that AML patient samples with *NPM1c* and *DNMT3A* mutations showed an increased sensitivity to menin-KMT2A inhibition, we wondered whether *NPM1c* and *DNMT3A*^{wt} samples were more HSC-like and double-mutant AML samples more GMP-like. While *NPM1c* mutations were present in all three populations, *NPM1c/DNMT3A*^{wt} AML samples were exclusively found in HSC-like and mixed populations (Figure 3B). In addition, GMP-like *NPM1c* AML samples were always *DNMT3A*^{mut} as well. To further substantiate these findings in larger cohorts of patients we performed GSEA in The Cancer Genome Atlas and our own quantitate proteome dataset; comparing *NPM1c/DNMT3A*^{mut} AML subtypes with *NPM1c/DNMT3A*^{wt} AML subtypes revealed that *NPM1c/DNMT3A*^{mut} AML subtypes were indeed significantly more GMP-like (*Online Supplementary Figure S6E, F*). Reversely, we observed that lower sensitivity was associated with more immature phenotypes linked to GSEA terms such as "HSC UP", "poor prognosis AML genes", "RRNA processing" and "chromatin modifying enzymes" (Figure 3B, *Online Supplementary Figure S5B-D*). These analyses were repeated focusing only on *NPM1*^{wt} AML samples, and also within this genetic subgroup we observed that more committed L-GMP-type AML were the most sensitive while immature leukemic stem cell (LSC)-type AML were less sensitive to menin-KMT2A inhibition (*Online Supplementary Figure S6C*). MEIS1, HOXA proteins and IGF2BP2 are frequently upregulated in *NPM1c* AML (*Online Supplementary Figure S5F*), and *MEIS1* and *IGF2BP2* are also downregulated upon menin-KMT2A inhibition (Figure 4B, *Online Supplementary Figure S7)*. However, we did not observe correlations between JNJ-75276617 inhibitor sen-

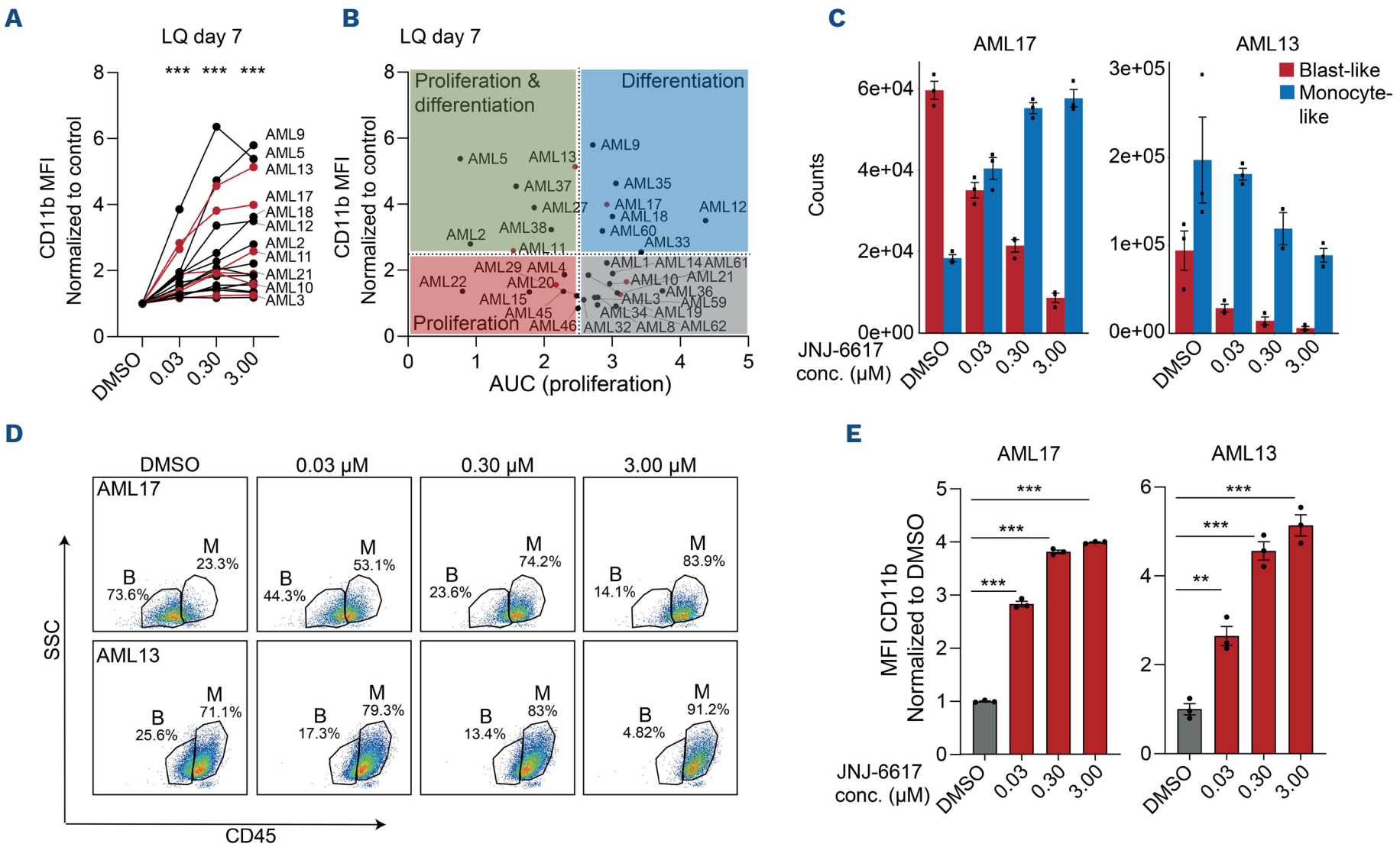
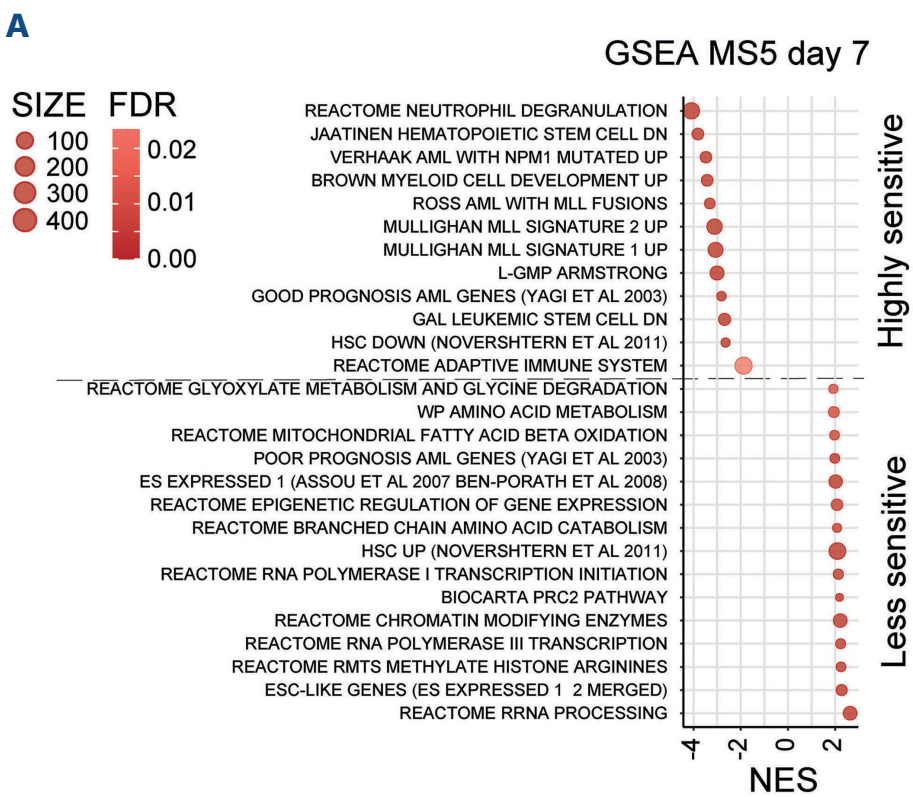


Figure 2. Menin-KMT2A inhibition induces differentiation in leukemic blast cells. (A) Line plots showing the mean fluorescent intensity (MFI) of CD11b normalized to the dimethyloxidesulfoxide (DMSO) control for liquid culture (LQ) day 7 (N=19). Black curves represent samples in which JNJ-75276617 also blocked proliferation, red curves represent samples in which proliferation was less affected by JNJ-75276617 treatment. (B) Comparison between AUC and CD11b expression (normalized to DMSO control) identifying four groups: proliferation, differentiation and proliferation, differentiation only, and weak responders. Red dots represent samples in which proliferation was less affected by menin-KMT2A inhibition. (C) Absolute cell counts for gates drawn for blast-like and monocyte-like cell populations. showing the dose-dependent effect on proliferation of the JNJ-75276617 inhibitor on LQ day 7. (D) Flow cytometry plots of AML17 and AML13 on LQ day 7. CD45 expression and side scatter (SSC) are depicted for DMSO, 0.03, 0.3 and 3.0 μM JNJ-75276617 inhibitor. Gates are drawn for blast-like (B) and monocyte-like (M) cell populations. (E) Bar plots depicting the MFI of CD11b normalized to the DMSO control for AML17 and AML13 on LQ day 7. Statistical analysis by unpaired Student *t* test. *P<0.05, **P<0.01, ***P<0.001.

sitivity and MEIS1, HOXA10 or IGF2BP2 baseline expression (*Online Supplementary Figure S6A, B*).

Chromatin immunoprecipitation sequencing reveals common and acute myeloid leukemia-specific targets upon menin-KMT2A inhibition

To further evaluate the targets of menin-KMT2A we performed chromatin immunoprecipitation (ChIP) sequencing in three primary AML patient samples (AML2, *NPM1c*/*DNMTA*^{mut}/*FLT3*-ITD/*RUNX1*^{mut}; AML5, *NPM1c/DNMTA*^{mut}/*FLT3*-ITD and AML22, *TET2*^{mut}/*SRSF2*^{mut}/*KMT2A-EP300*) and the OCI-AML3 cell line (*NPM1c*) after 4 days of treatment with DMSO or JNJ-75276617 inhibitor. We first analyzed genome-wide changes after menin-KMT2A inhibition and noticed that H3K4me3



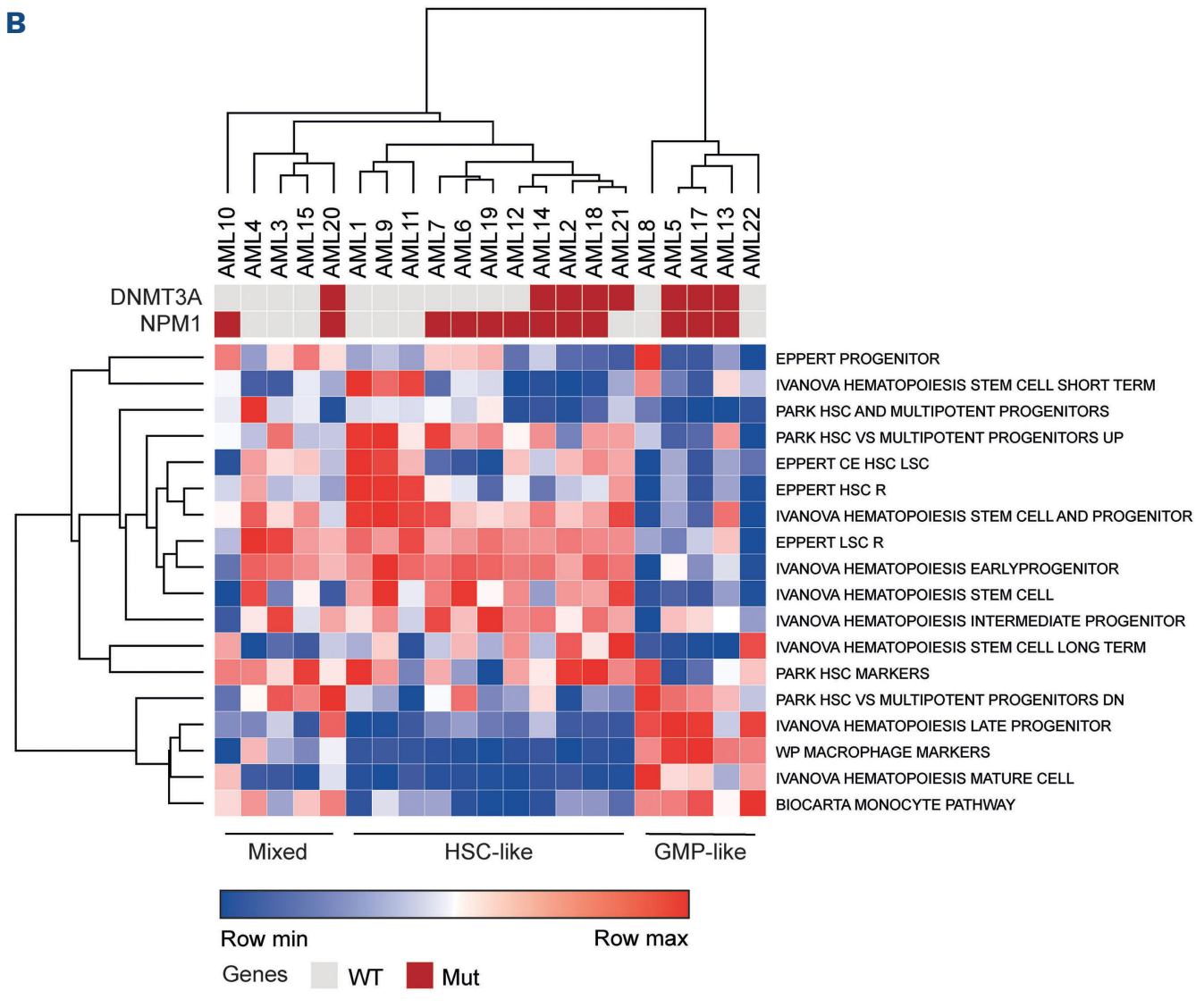


Figure 3. Quantitative proteome analyses link menin-KMT2A inhibitor sensitivity to more committed granulocyte-macrophage progenitor-type acute myeloid leukemias. (A) Dot plot of gene set enrichment analysis (GSEA) signatures enriched in sensitive and less sensitive primary acute myeloid leukemia (AML) samples at MS5 day 7. (B) Selection of single-sample GSEA terms from proteome dataset AML samples to identify maturation state. Hematopoietic stem cell (HSC)-like, granulocyte-macrophage progenitor (GMP)-like and mixed samples were identified. *DNMT3A* and *NPM1c* were annotated, with gray representing wildtype (WT) and red representing mutated (Mut). (C) Comparison between AUC values in GMP-like, mixed and HSC-like groups of MS5 day 7. Statistical analysis by the unpaired Student *t* test. NES: normalized enrichment score.

marks were slightly reduced in AML2, while they were slightly increased in the other samples (Figure 4A). H3K27ac marks were overall increased in three out of the four cases, but reduced in AML5. We then analyzed specific loci, and observed a consistent loss of H3K27ac marks at the *MEIS1* locus in all samples, coinciding with a loss of H3K4me3 marks (Figure 4B). At the *HOXA* locus we observed that epigenetic changes were less prominent. We noted that the active H3K4me3/H3K27ac state of the *MEIS1* locus was reverted to a Polycomb-repressed state characterized by an increase in H3K27me3 upon menin-KMT2A inhibition (Figure 4B).

When comparing common and AML-specific targets it was clear that *MEIS1* plays a very central role upon menin-KMT2A inhibition, and other previously described targets, such as *FLT3*, *PBX3*, *TCF4* and *SATB1*, also displayed reduced H3K4me3 levels in the majority of cases (Figure 4C). Furthermore, the known menin-KMT2A target *IGF2BP2*, which was recently identified as a therapeutic target in AML, was found in our dataset as well (Figure 4B, C).[24,29-31] Loss of H3K27ac and H3K4me3 at *MEIS1* and *IGF2BP2* loci correlated with significant reductions in mRNA expression, albeit with some heterogeneity, as determined by quantitative real-time polymerase chain reaction analysis in proliferation-sensitive (e.g., AML22), differentiation-sensitive (e.g., AML35) or proliferation and differentiation-sensitive AML samples (e.g., AML5) (*Online Supplementary Figure S7A*). *HOXA9*

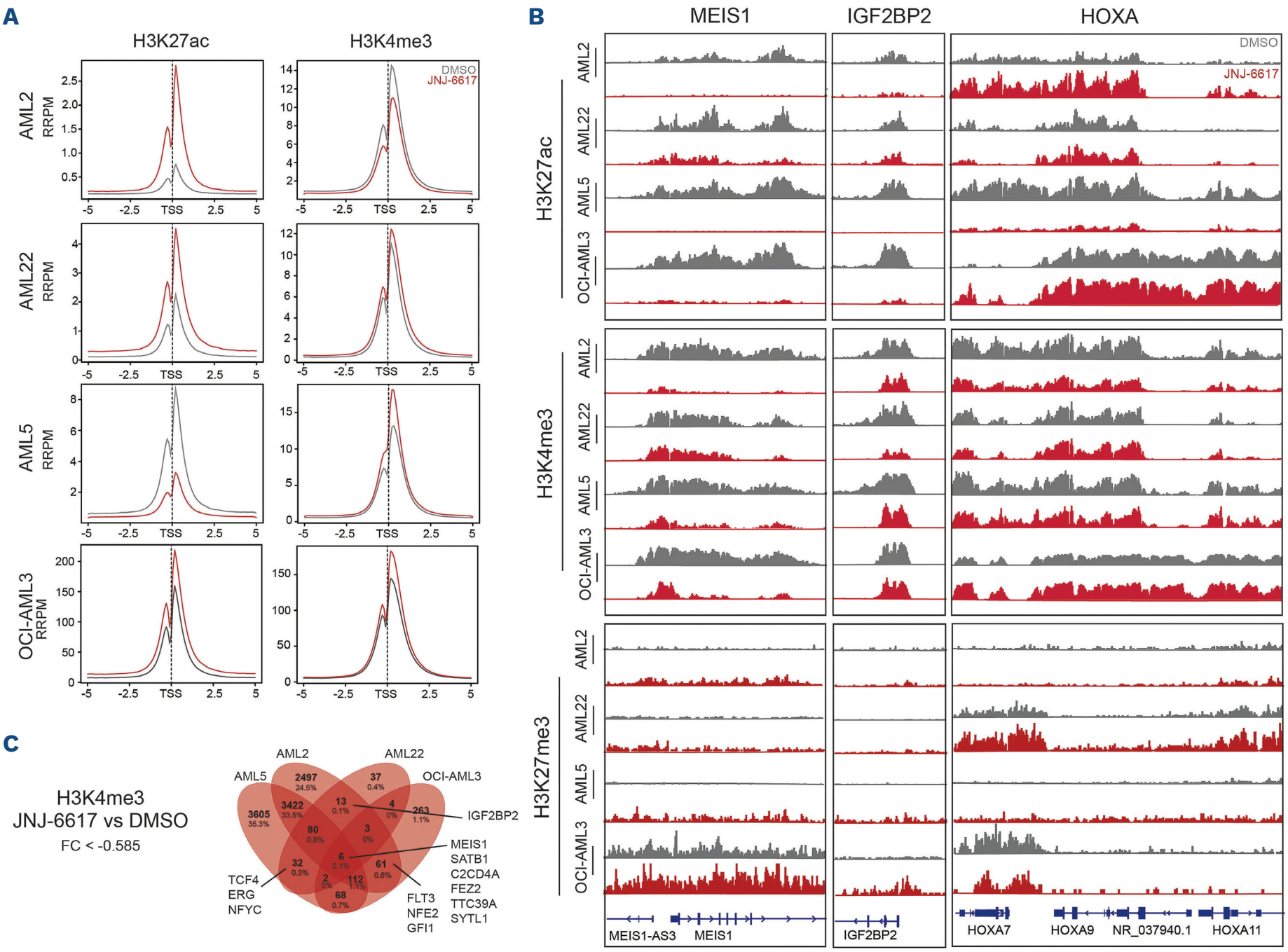


Figure 4. Chromatin immunoprecipitation sequencing reveals common and acute myeloid leukemia-specific targets upon menin-KMT2A inhibition. (A) Average plots showing genome-wide H3K27ac and H3K4me3 changes after menin-KMT2A inhibition for 4 days in AML2, AML22, AML5 and OCI-AML3 cells. (B) H3K27ac, H3K4me3 and H3K27me3 chromatin immunoprecipitation sequencing tracks of AML2, AML22, AML5 and OCI-AML3 cells treated with dimethylsulfoxide (DMSO) or 0.03 (AML22)/0.3 μM JNJ-75276617 inhibitor for 4 days showing *MEIS1*, *IGF2BP2* and the *HOXA* cluster. (C) Venn diagrams showing overlap in genes with a fold change (FC) < -0.585 for H3K4me3 expression in menin-KMT2A inhibitor JNJ-75276617-treated *versus* DMSO-treated cells. RRPM: reference normalized reads per million mapped reads.

mRNA expression was less consistently affected across all AML samples upon menin-KMT2A inhibition, with significant downregulation in five samples but significant upregulation in two samples, which appeared to be independent of the effect of the inhibitor on proliferation or differentiation (*Online Supplementary Figure S7A*). Given the fact that *MEIS1* is transcriptionally repressed after menin-KMT2A inhibition, we wondered whether re-expression of MEIS1 would rescue the observed impaired proliferation, as described previously. Indeed, both OCI-AML3 and MOLM13 cells displayed significantly increased cell counts in the presence of JNJ-75276617 upon MEIS1 overexpression compared to DMSO-treated cells (*Online Supplementary Figure S7B, C*). We also investigated whether re-expression of IGF2BP2 would rescue sensitivity to menin-KMT2A inhibition, and while a slight recue in proliferation was observed, this was not as strong as compared to that seen in the MEIS1 rescue experiments, indicating that IGF2BP2-independent pathways downstream of MEIS1 must exist as well (*Online Supplementary Figure S7D-E*). Inhibition of *MEIS1* expression upon treatment with JNJ-75276617 is not prevented by IGF2BP2 overexpression, while *BMI1*, which is a target gene of IGF2BP2, is restored upon IGF2BP2 overexpression (*Online Supplementary Figure S7F*).

Besides these more common targets, it was interesting to note that a lot of the epigenetic changes induced upon menin-KMT2A inhibition were relatively patient-specific. Already at baseline, prior to menin-KMT2A inhibition, the epigenetic landscapes differed considerably between patients, in line with what we and others observed previously,[32,33] potentially also as a consequence of differences in maturation stage,[41] and we therefore hypothesize that the epigenetic changes induced upon menin-KMT2A inhibition are also different per patient (Figure 4C, *Online Supplementary Figure S7A*). Despite these patient-specific features, gene ontology analyses on loci at which H3K4me3 or H3K27ac were gained showed significant enrichment for processes associated with differentiation, lineage specification and myeloid commitment (*Online Supplementary Figures S8C, D* and *S9A*). Loci at which H3K4me3 marks were lost were significantly enriched for processes associated with cell cycle, apoptosis and response to growth factors (*Online Supplementary Figure S8C*). GSEA revealed that HSC/LSC and *NPM1c* signatures were generally lost (*Online Supplementary Figure S8A*, *Online Supplementary Table S2*), while differentiation signatures were gained, which also included terms associated with MHC binding, antigen processing and presentation, phagocytosis and interferon-γ responses (*Online Supplementary Figure S8B*, *Online Supplementary Table S2*). Similar changes were observed in OCI-AML3 cells (*Online Supplementary Figure S9B*).

Menin-KMT2A inhibition drives HLA expression in a MEIS1-independent but CIITA-dependent manner in the case of MHC class II

Since we observed an increase in H3K4me3 marks on loci related to immunity and MHC proteins (*Online Supplementary Figures S8B, D,* and *S9B*), we wished to explore this further. We focused on MHC class I (*HLA-A*) and MHC class II (*HLA-DPA1/B1* and *HLA-DRA*) loci, and observed an increase in H3K4me3 and H3K27ac levels upon menin-KMT2A inhibition (Figure 5A). This resulted in increased mRNA expression of *HLA-A* and *HLA-DR* in a larger panel of AML undergoing 4 days of treatment with a menin-KMT2A inhibitor (Figure 5B), as well as at the protein level determined by flow cytometry (Figure 5C), although there was clear variation between samples from individual AML patients as well. HLA-DR was upregulated in the majority of cases, HLA-A was upregulated in 9/25 cases, and in 5/25 cases a consistent significant upregulation of both MHC class I and MHC class II was noted *(Online Supplementary Figure S10A, B*). In addition, the mRNA expression of *CIITA*, the known transcription factor of MHC class II genes, was significantly upregulated after menin-KMT2A inhibition. When correlating the fold change of *CIITA* and *HLA-DR* between cells treated with JNJ-75276617 inhibitor or DMSO, we identified a strong positive correlation (R^2=0.748) (*Online Supplementary Figure S10C*). Since *MEIS1* was significantly reduced in JNJ-75276617 inhibitor-treated cells (*Online Supplementary Figure S7*), we considered whether *MEIS1* could be a negative regulator of HLA-DR and other MHC class II genes, as described by Eagle *et al.*[34] However, when we compared the fold change in *MEIS1* expression *versus HLA-DR* expression no correlation was observed (*Online Supplementary Figure S10D*) and HLA-DR was still upregulated upon JNJ-75276617 inhibitor treatment in MEIS1 overexpression models, as determined by flow cytometry (*Online Supplementary Figure S10E*). These data argue that menin-KMT2A controls MHC class II expression in a MEIS1-independent manner. Therefore, we performed ChIP quantitative polymerase chain reaction and confirmed binding of menin at the *CIITA* and *HLA-DR* loci (Figure 5D). Subsequently, we generated heterozygous and homozygous *CIITA* knockout MV4-11 cells (*CIITA*$^{+/-}$ and *CIITA*$^{-/-}$, respectively), which we treated with 0.03, 0.30 or 3.00 μM JNJ-75276617 inhibitor for 4 days. At baseline, HLA-DR expression was significantly lower in *CIITA*$^{+/-}$ and *CIITA*$^{-/-}$ cells than in MV4-11 scrambled (SCR) cells (Figure 5E-G). Upon JNJ-75276617 treatment, HLA-DR expression was dose-dependently upregulated in MV4-11 SCR, but not in *CIITA*$^{-/-}$ cells. These data indicate that the menin-KMT2A complex directly regulates *HLA-DR* loci in a CIITA-dependent manner.

Menin-KMT2A inhibition enhances T-cell cytotoxicity

Next, we functionally evaluated whether JNJ-75276617 inhibitor treatment would increase sensitivity to immune cells. First, the AML cell lines MOLM13 and MV4-11 were pre-treated for 4 days with DMSO or 0.3 μM and 0.1 μM JNJ-75276617 inhibitor, after which peripheral blood mononuclear cells were added at different effector:target ratios for an additional 72 hours. As observed before, menin-KMT2A inhibition resulted in an increase in HLA-DR expression

(Figure 6A). Furthermore, menin-KMT2A inhibition impaired cell proliferation as noted before (Figure 6B, 0:1 ratio), but upon the addition of peripheral blood mononuclear cells a significant further decline in viable cell counts was observed in both cell lines (Figure 6B). Next, we functionally studied T-cell cytotoxicity in an allogeneic setting. Primary

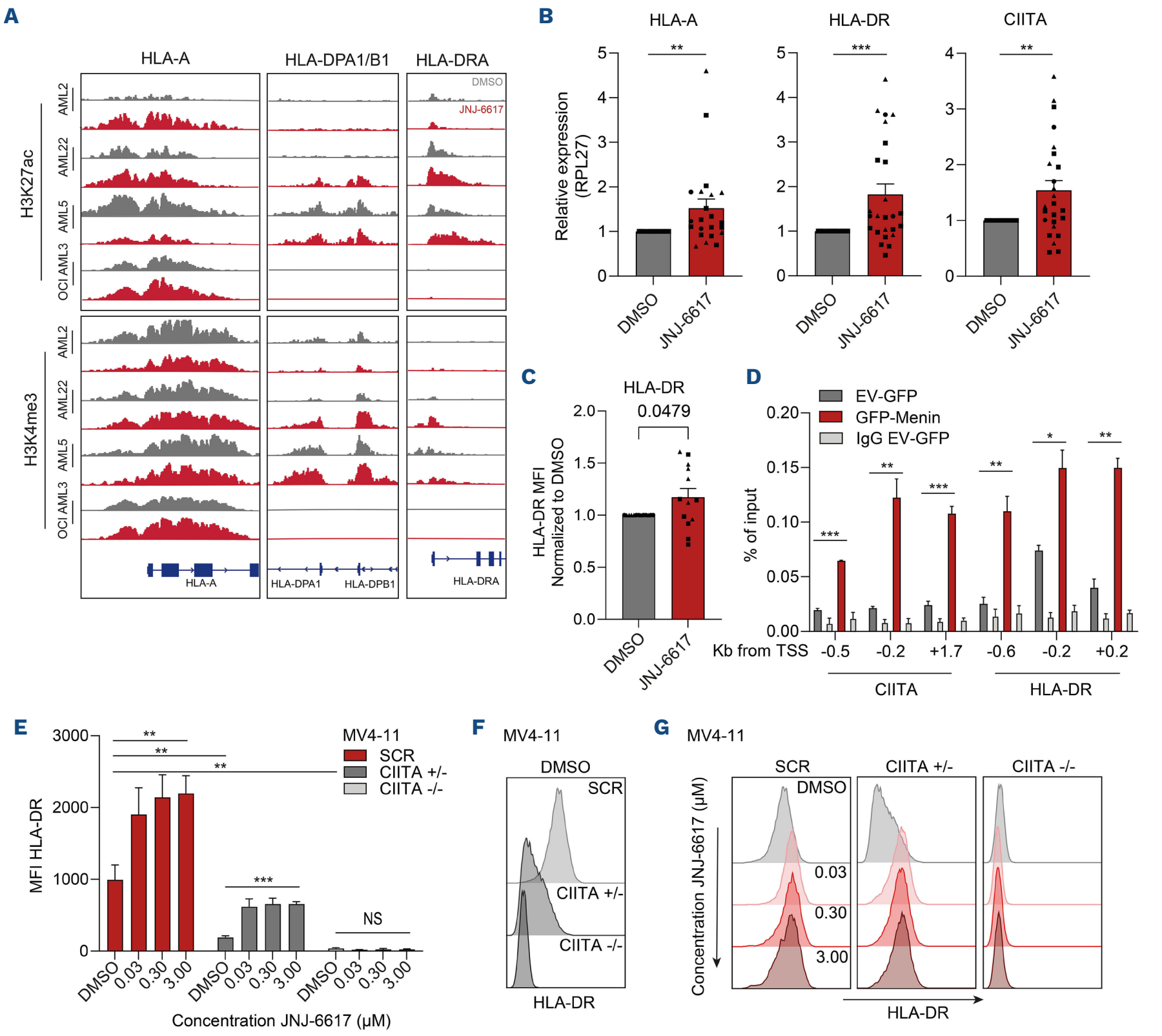


Figure 5. Menin-KMT2A inhibition drives HLA expression in a MEIS1-independent but CIITA-dependent manner in the case of MHC class II. (A) H3K27ac and H3K4me3 chromatin immunoprecipitation (ChIP) sequencing tracks of AML2, AML22, AML5 and OCI-AML3 cells treated with dimethylsulfoxide (DMSO) or 0.03 (AML22)/0.3 μM JNJ-75276617 inhibitor for 4 days showing *HLA-A*, *HLA-DPA1/B1* and *HLA-DRA* loci. (B) Bar plots showing relative mRNA expression of *HLA-A*, *HLA-DR* and *CIITA* in 4-day JNJ-75276617 inhibitor-treated primary acute myeloid leukemia (AML) patients' samples normalized to DMSO. Triangles annotate *NPM1c* samples. (C) Bar plot depicting mean fluorescent intensity (MFI) of HLA-DR in 4-day DMSO- or JNJ-75276617 inhibitor-treated primary AML patients' samples normalized to DMSO. Triangles annotate *NPM1c* samples. (D) Bar plot showing ChIP quantitative polymerase chain reaction data of MOLM13 cells with empty vector (EV)-green fluorescent protein (GFP) and GFP-menin using antibodies against GFP. (E) Bar plot showing MFI of HLA-DR in MV4-11 scrambled (SCR), *CIITA* heterozygous knockout (*CIITA*$^{+/-}$) and CIITA homozygous knockout (*CIITA*$^{-/-}$) cells treated with DMSO, 0.03, 0.30 or 3.00 μM JNJ-75276617 inhibitor for 4 days (N=3). (F) MFI of HLA-DR in MV4-11 scrambled, *CIITA*$^{+/-}$ and *CIITA*$^{-/-}$ in control cells. (G) MFI of HLA-DR in MV4-11 scrambled, *CIITA*$^{+/-}$ and *CIITA*$^{-/-}$ cells treated with DMSO, 0.03, 0.30 or 3.00 μM JNJ-75276617 inhibitor for 4 days. Error bars represent mean ± standard error of the mean. Statistical analysis by the unpaired Student *t* test or simple linear regression. NS: not statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001.

AML samples (N=8) with different mutational backgrounds were pretreated with 0.3 μM JNJ-75276617 inhibitor for 4 days, after which cells were washed and antiCD3/CD28 activated T cells from healthy donors were added at different effector:target ratios for an additional 3 days. Four out of eight AML patient samples were found to have an increased sensitivity to allogeneic T cells after menin-KMT2A inhibition (Figure 6C, *Online Supplementary Figure S11A*). Interestingly, unlike the other four less sensitive primary AML samples, all four sensitive AML samples carried *NPM1c* and *DNMT3A* mutations (Figure 6D). T-cell counts were also diminished after co-culture with menin-KMT2A-inhibitor pretreated AML that showed increased T-cell cytotoxicity, suggestive of activation-induced cell death after target cell recognition (*Online Supplementary Figure S11B*).[35] Finally, we evaluated T-cell cytotoxicity in an autologous setting. CD45$^+$ blast populations and CD3$^+$ T-cell populations were sorted from primary *NPM1c* AML patients' samples (N=9) (*Online Supplementary Table S1*). The AML blasts were then cultured in either the absence (depleted) or presence (enriched) of their own T cells at a fixed ratio, in the absence or presence of 0.3 μM JNJ-75276617 inhibitor. As we observed before, menin-KMT2A inhibition resulted in cell intrinsic cytotoxicity in the absence of T cells, but upon menin-KMT2A inhibition a further significant enhanced T-cell cytotoxicity was observed (Figure 7A-C), coinciding with increased MHC class I and MHC class II expression (Figure 7D, E). The strength of the T-cell cytotoxic response was not directly correlated to the level of MHC upregulation, indicating that other mechanisms, such as potential tumor cell intrinsic immune evasion mechanisms, might also play a role that will need to be investigated further. In AML54 JNJ-75276617 treatment did not result in increased HLA-A upregulation but did induce strong upregulation of

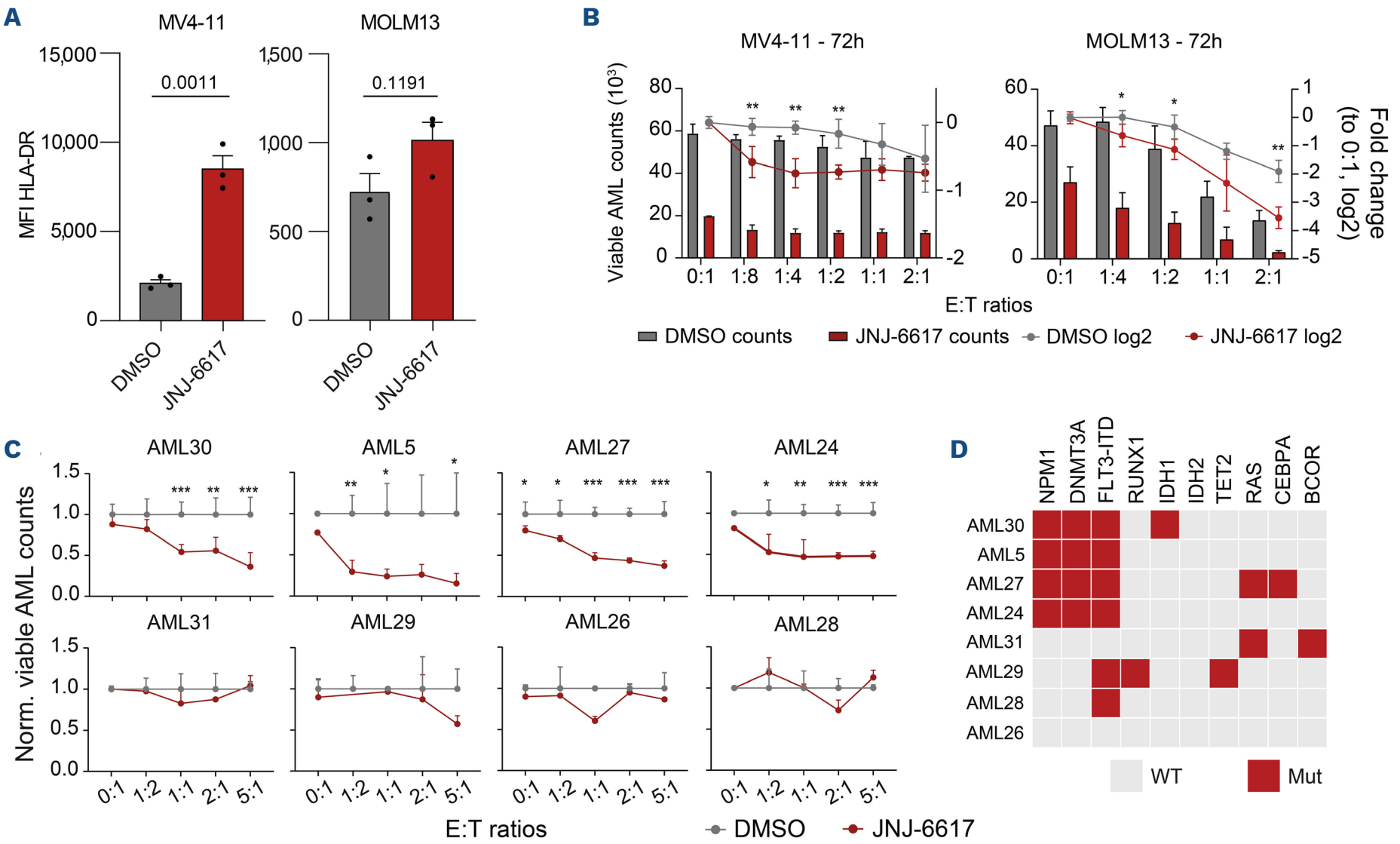


Figure 6. Menin-KMT2A inhibition enhances T-cell cytotoxicity. (A) Bar plots depicting mean fluorescent intensity of HLA-DR in dimethylsulfoxide (DMSO) or 0.3 μM JNJ-75276617 inhibitor-treated MV4-11 and MOLM13 cells normalized to DMSO. (B) Bar and line plots showing viable acute myeloid leukemia (AML) counts (bar plot) determined by annexin-V and Zombie NIR™ of different effector:target (E:T) ratios in DMSO or JNJ-75276617 pre-treated MV4-11 and MOLM13 cells co-cultured with allogeneic T cells for 72 hours. MV4-11 cells were pre-treated with 0.1 μM JNJ-75276617 and MOLM13 cells with 0.3 μM. E:T ratios were normalized to 0:1 (line plot). (C) Line plots showing normalized viable cell counts, determined by annexin-V and Zombie NIR™, of different E:T ratios in eight primary AML samples, which were pre-treated with DMSO or 0.3 μM JNJ-75276617 and co-cultured with allogeneic T cells for 72 hours. E:T ratios were normalized to their DMSO control. (D) Tile plot depicting the mutational status of primary AML samples used in panel (C). Error bars represent mean ± standard error of the mean. Statistical analysis by the unpaired Student *t* test. *P<0.05, **P<0.01, ***P<0.001.

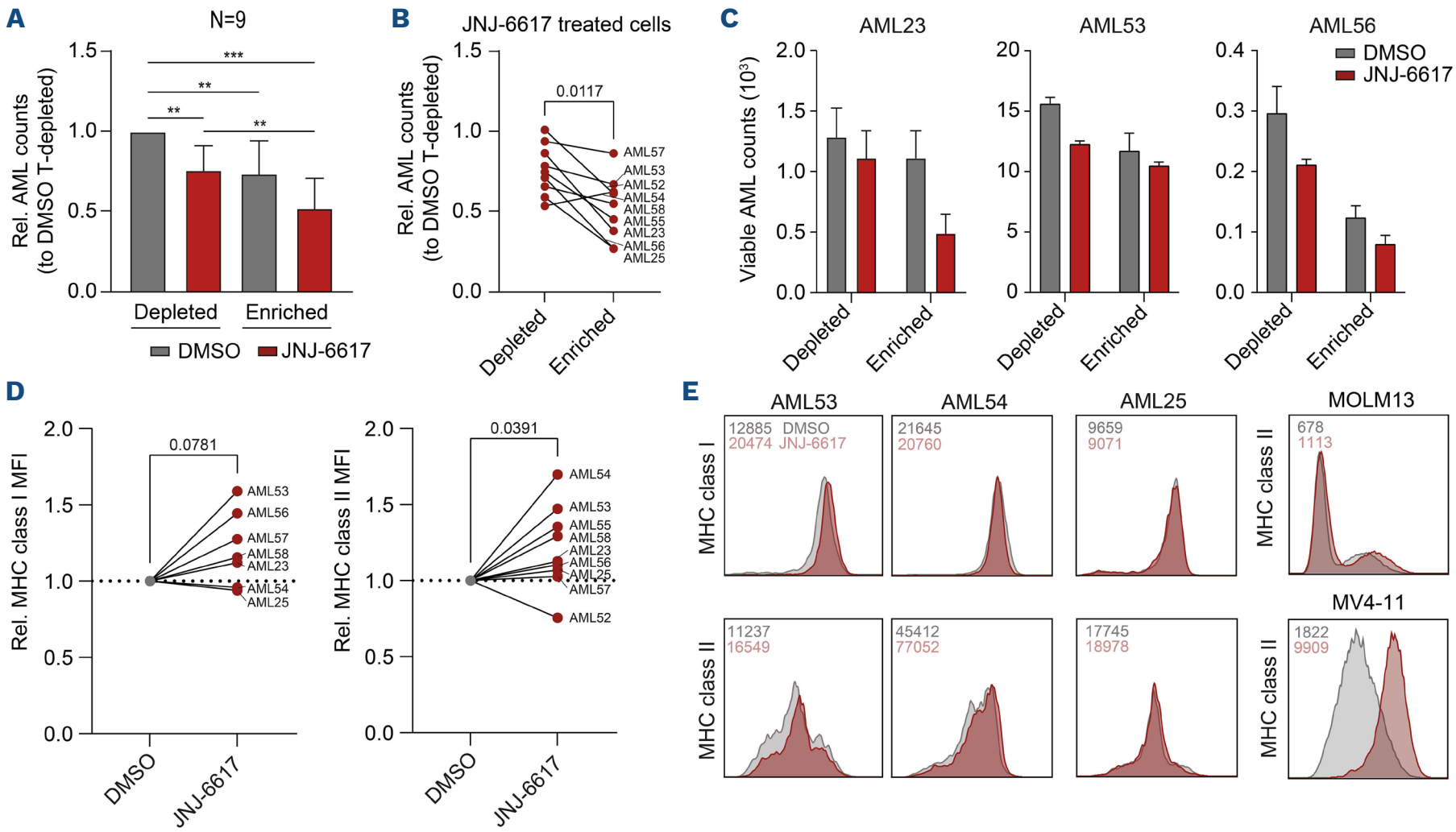


Figure 7. Menin-KMT2A inhibition enhances T-cell cytotoxicity. (A) Bar plot showing relative acute myeloid leukemia (AML) counts in primary AML samples (N=9) treated with dimethylsulfoxide (DMSO) or 0.3 μM JNJ-75276617 inhibitor cultured without T cells (depleted) or with T cells (enriched). (B) Paired comparison of JNJ-75276617-treated primary AML samples cultured without or with T cells. (C) Three examples of panel (B). (D) Paired comparison of MHC class I and MHC class II mean fluorescent intensities (MFI) of primary AML samples shown in (B) upon treatment with 0.3 μM JNJ-75276617 inhibitor. Expression is relative to the DMSO samples. (E) MHC class I and MHC class II expression of three primary AML samples shown in (D) treated with DMSO or 0.3 μM JNJ-75276617 inhibitor and MHC class II expression of MOLM13 and MV4-11 cells treated with DMSO or 0.3 μM JNJ-75276617 inhibitor. Error bars represent mean ± standard error of the mean. Statistical analysis by the unpaired Student *t* test. **P*<0.05, ***P*<0.01, ****P*<0.001.

HLA-DR. Nevertheless, T-cell cytotoxicity was not further enhanced and AML cells even expanded slightly in the presence of allogeneic T cells (Figure 7B, C), which might be related to the observation that intrinsic cell viability was already strongly affected by menin-KMT2A inhibition or that AML cells might respond to the secretion of cytokines, such as interferon-γ upon T-cell activation. However, additional studies are required to obtain further insight into the exact underlying mechanisms. No change in CD4/CD8 ratio or CD69 expression was noted upon JNJ-75276617 treatment (*Online Supplementary Figure S11C, D*). Together, these data indicate that menin-KMT2A inhibition not only impairs long-term self-renewal and drives differentiation of leukemic blasts via cell intrinsic mechanisms, but may also decrease their immune evasion capacity.

Discussion

Inhibitors that target the menin-KMT2A interaction have appeared as a potential new therapeutic approach for patients harboring *KMT2A*-rearrangements or *NPM1c* mutations. Various in-human trials have now been initiated, but much is still unknown about the exact cell biological processes and downstream molecular mechanisms that mediate inhibitor-induced phenotypes across the heterogeneous AML landscape. Here, we describe that the novel menin-KMT2A inhibitor JNJ-75276617 impairs proliferation and drives differentiation of primary AML patient samples, with GMP-like AML subtypes displaying the greatest sensitivity. Inhibitor sensitivity is not restricted to *NPM1c/DNMT3A*^{mut} and *KMT2A*-rearranged AML subtypes, but also *NPM1*^{wt} AML subtypes that carry *CEBPA* mutations or *NUP98*-rearrangements were sensitive to JNJ-75276617. Furthermore, inhibitor sensitivity should not only be determined by reduction in proliferation; a combination read-out with induction of differentiation and proliferation would be advisable. Mechanistically, we reveal that T-cell-mediated cytotoxicity is enhanced in leukemic blasts as a consequence of MHC class I and MHC class II upregulation.
Several menin-KMT2A inhibitors are currently being tested in clinical trials to evaluate their safety and efficacy

in relapsed or refractory AML with *NPM1c* or *KMT2A* rearrangements, including KO-539, SNDX-5613, DS-1594b and JNJ-75276617.[36-38] In the phase I trial of SNDX-5613 (revumenib) 68 patients were enrolled and treated: 46 patients had *KMT2A*-rearrangements, 14 had *NPM1c* and eight patients lacked both mutations.[22] Within these 68 patients, no responses were found in the eight patients without *KMT2A*-rearrangements or *NPM1* mutations. Two patients had a *NPM1* and *DNMT3A* mutation of whom one responded to revumenib. In the KO-539 trial one patient harboring *NPM1*, *DNMT3A* and *KMT2D* mutations treated with 200 mg/day achieved a minimal residual disease-negative complete remission for >100 weeks.[28] Interestingly, in this trial responses were also reported in patients who did not carry *NPM1* mutations or *KMT2A*-rearrangements. Our data would argue that a particular interest should be given to patients who carry both *NPM1c* and *DNMT3A* mutations or *KMT2A*-rearrangements, but that a subset of *NPM1*^{wt}/*KMT2A*^{wt} patients might also benefit, particularly in the context of *CEBPA* and *RUNX1* mutations or *NUP98*-rearrangements.

Our data indicate that a common signature that is affected in all tested cases upon menin-KMT2A inhibition entails the known targets *MEIS1* and *IGF2BP2*. Expression of these genes is strongly downregulated upon JNJ-75276617 treatment, resulting in loss of long-term proliferation of leukemic blasts, coinciding with an induction of differentiation. MEIS1 has been identified as an important transcription factor that controls self-renewal of HSC and LSC.[14,39-41] One of its targets is IGF2BP2, which was recently shown to control the transcriptional state and maintenance of HSC[42] and was identified as a potential therapeutic target in AML.[43] Mechanistically, IGF2BP2 acts as an m^{6}A reader, thereby regulating mRNA abundance in HSC, and deficiency of IGF2BP2 resulted in loss of HSC function.[42] We observed that *MEIS1* expression is directly correlated with *IGF2BP2* expression levels, and while re-expression of exogenous MEIS1 was able to partially rescue cell proliferation of JNJ-75276617-treated cells, in line with previous data,[21,23] we only observed a minor rescue in proliferation and target gene expression upon IGF2BP2 overexpression. These data clearly indicate that multiple pathways downstream of menin are relevant in conveying phenotypes, which cannot exclusively be explained by *IGF2BP2* changes.

Besides loss of H3K4me3 marks on targets such as *MEIS1* and *IGF2BP2* upon inhibition of the interaction between menin and KMT2A, we also found that various loci become activated, as shown by increases in H3K27ac and H3K4me3. There was considerable heterogeneity in upregulated targets between individual AML patients upon menin-KMT2A inhibition. At baseline, prior to menin-KMT2A inhibition, the epigenetic landscapes already differed considerably between patients, in line with what we and others observed previously,[32,33] potentially also as a consequence of differences in maturation stage[41] and we therefore hypothesize that the epigenetic changes induced upon menin-KMT2A inhibition are also rather AML patient-specific. But what was common to all cases is that genes associated with myeloid differentiation programs and immune functions become activated, in line with what we observed phenotypically. While further studies are needed to obtain mechanistic insights driving gene activation, a possibility could be that loci with increased H3K4me3 expression undergo a switch from occupancy by menin-KMT2A to SETD1A and SETD1B with KMT2A, as was shown by Sparbier *et al*. for MHC class I expression.[44] Extensive further analyses, including the development of CRISPR knockout models, would be required to causally link SETD1A and/or SETD1B to *CIITA* expression control. In addition, it was recently elegantly shown, using CRISPR screens in murine *KMT2A-AF9* leukemia models, that durable menin-KMT2A inhibition-induced phenotypes depend on KMT2C/D-UTX.[45] Upon inhibition, the menin-KMT2A complex was replaced by the KMT2C/D-UTX complex which was necessary to maintain expression specifically of differentiation genes. However, KMT2A-AF9-driven MEIS1 expression did not depend on UTX. It will be interesting to determine whether the menin-KMT2A to KMT2C/D-UTX switch also drives differentiation programs in human AML patient samples, and also whether potential resistance to menin-KMT2A inhibitors would be linked to a failure to install this switch. In the same study, it was noted that all members of the non-canonical PRC1.1 complex were required to maintain long-term sensitivity to menin-KMT2A inhibition, since loss of PCGF1, BCOR or KDM2B rendered murine *KMT2A-AF9* cells resistant.[45] It is tempting to speculate that loci that become downregulated upon menin-KMT2A inhibition, such as the *MEIS1* locus, depend on Polycomb group proteins to maintain long-term repression, and we indeed observed an increase in H3K27me3 marks at the *MEIS1* locus, which we aim to investigate further in future studies.

Finally, we found that treatment with the JNJ-75276617 inhibitor resulted in upregulation of HLA-A and HLA-DR in our primary AML cohort and cell lines. While a previous report suggested that MEIS1 would be a negative regulator of HLA-DR expression,[34] we were unable to validate this regulatory mechanism in our setting as there appeared to be no correlation with loss of MEIS1 expression and upregulation of HLA-DR. Rather, we observe strong correlations between HLA-DR and expression of its transcriptional regulator CIITA, and we therefore propose that the induction of MHC class II molecules is MEIS1-independent. In line, re-expression of MEIS1 failed to impair JNJ-75276617-induced upregulation of HLA-DR, and we also observed direct binding of menin to both the *CIITA* and *HLA-DR* loci. Functionally, we observed that the upregulation of MHC class I and MHC class II molecules resulted in enhanced cytotoxicity, both in allogeneic and autologous T-cell cytotoxicity assays. Sparbier *et al*. also reported that treatment with a menin-KMT2A inhibitor resulted in upregulation of MHC molecules.[44] In K562 cells in which MHC class I genes are in a bivalent state, characterized

by both H3K4me3 and H3K27me3 marks, it was shown that both Polycomb group proteins as well as expression of the menin-KMT2A complex are required to maintain repression of the locus. Functionally, it was shown that pre-treatment of cells with the menin-KMT2A inhibitor VTP-50469 before co-culture with OT-I T cells enhanced MHC class I-mediated tumor cell killing significantly, thereby providing a direct link between MHC class I upregulation, antigen presentation and increased immunogenicity upon inhibition of menin-KMT2A.[44] After initial chemotherapy treatment the majority of patients receive an allogeneic HSCT but unfortunately ~20% of the patients still relapse within 5 years.[46] It was found that these relapsed patients have a significantly lower expression of MHC class I and II molecules after allogeneic HSCT.[18] Patients harboring a *NPM1c* mutation have a relatively favorable prognosis in the absence of *FLT3*-ITD mutations and in *NPM1c*-mutated patients without *FLT3*-ITD mutations allogeneic HSCT in first-line treatment seems to be beneficial for survival.[47,48] This might be because mutated NPM1 is localized to the cytoplasm, where it can be processed by the MHC class I or II degradation pathway leading to the presentation of NPM1-mutant peptides by HLA molecules[49] initiating a leukemia-specific autologous T-cell cytotoxicity against these NPM1c peptides.[36,50] Obviously, the efficacy of cytotoxicity depends heavily on appropriate expression of MHC class I and MHC class II molecules, and our data indicate that menin-KMT2A inhibition can potentially add therapeutic benefit by reactivating antigen presentation machinery.

Disclosures

MCK, AK, and UP are currently employees of Janssen Research & Development and may own stock/stock options in Johnson & Johnson. The other authors have no conflicts of interest to disclose.

Contributions

SMH, DML, NLR, ATJW, FAJvdH, VvdB and JJS performed research. SMH, DML, NLR, MCK, ATJW, FAJvdH, VvdB, AK, UP, GH and JJS discussed and analyzed data. MCK, AK and UP provided compounds. SMH and JJS wrote the manuscript, which was edited and approved by all authors.

Funding

This work was supported by a grant from the Dutch Cancer Foundation to JJS (11013) and a PPP grant from Health Holland/TKI (LSHM200204)

Data-sharing statement

All data are available in the main text or the Online Supplementary Materials. ChIP-sequencing data can be found at GSE237834 and proteome data at PXD030487.

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