Am J Cancer Res 2021;11(11):5726-5742
www.ajcr.us /ISSN:2156-6976/ajcr0136880

Original Article

Increased lactate in AML blasts upregulates TOX expression, leading to exhaustion of CD8$^+$ cytolytic T cells

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Received June 30, 2021; Accepted October 31, 2021; Epub November 15, 2021; Published November 30, 2021

Abstract: Recently, the role of lactate as merely an end product of cancer cell metabolism has been reassessed. Lactate has been implicated in more biological processes than previously understood and drives tumor progression. Here, we demonstrated that the bone marrow lactate concentrations in acute myeloid leukemia (AML) patients were substantially higher than those in their healthy control counterparts. Moreover, AML blasts from bone marrow expressed significantly higher lactate dehydrogenase-A (LDHA) levels. Further studies revealed that LDHA expression was regulated through the HIF1α pathway. Elevated lactate levels were indicative of alterations in CD8$^+$ T cell cytolytic phenotype and activity. An in vitro study showed that the lactate treatment group had significantly higher percentages of CD8$^+$ TEM and CD8$^+$ TEMRA cells as well as higher PD-1 expression in these cells than the control group. Lactate induced the loss of the effector function of CD8$^+$ T cells by altering lytic granule exocytosis. T cell dysfunction is characterized by an increase in terminally differentiated phenotypes, sustained expression of PD-1, and accelerated decline of cytolytic competence. Moreover, the TOX gene was found to be correlated with lactate production and implicated in CD8$^+$ T cell dysfunction. AML patients in complete remission after chemotherapy had markedly lower lactate concentrations, reduced CD8$^+$ TEM and CD8$^+$ TEMRA cells and PD-1 expression, and increased perforin and granzyme B. However, no difference was found in the relapsed patients. The study presented here has established lactate as a predictive biomarker for patient response to antitumor therapies and demonstrated that targeting this gene in AML patients could be a meaningful precision therapeutic strategy.

Keywords: CD8$^+$ T cell, lactate, programmed death-1, TOX, acute myeloid leukemia

Introduction

Acute myeloid leukemia (AML) is an aggressive haematological malignancy that is often associated with poor overall survival. Despite the significant efforts made in the treatment of AML, unmet clinical needs remain [1]. With the emergence of novel molecular technologies and improved genetic understanding, cancer immunotherapy has garnered significant research interest following initial successes in checkpoint blockade intervention and T cell engineering. Rapidly evolving immunotherapeutic modalities hold significant promise for transforming the treatment paradigm for AML [2-4]. However, curative treatment for AML remains a bottleneck. A deeper understanding of the interactions between tumor cells and T cells and the mechanisms underlying CD8$^+$ T cell functional attenuation is essential.

CD8$^+$ T cells are mainly comprised of four differentiation subsets, designated as follows: naïve T ($T_{naïve}$) cells, central memory T (TCM) cells, effector memory T (TEM) cells, and effector memory cells re-expressing CD45RA. The

linear differentiation model proposes that naïve CD8+ T cells first arise with coexpression of CD45RA and CCR7 and, following antigenic stimulation, differentiate into TCM and TEM subsets, characterized by lack of CD45RA and CCR7 expression, respectively. Upon further stimulation, T cells develop a terminally differentiated phenotype and re-express CD45RA [5, 6]. TEM and TEMRA cells exhibit distinct expression profiles and are associated with effector functions that mediate apoptosis of tumor cells [7]. Chronic antigen stimulation, however, can lead to gradual loss of effector function and T cell depletion [8, 9]. The exhausted phenotype of T cells is often associated with the coexpression of immune inhibitory signals (e.g., PD-1, CTLA4, Tim-3 and Lag-3) that suppress antitumor immunity [10]. Moreover, exhausted CD8+ T cells are often associated with reduced levels of cytotoxic proteins such as perforin and granzyme B, which are responsible for the maintenance of T cell proliferation and direct destruction of tumor cells [11]. The transcriptional regulator thymocyte selection-associated high-mobility group box protein (TOX) has been implicated in various stages of T cell differentiation. Several recent studies correlated TOX overexpression with T cell exhaustion in scenarios of persistent infection and tumors [12-16]. Overexpression of TOX has also been robustly correlated with high frequencies of PD-1 and Tim-3. In addition, TOX was found to be a critical regulator of the expression of several other inhibitory receptors (e.g., Pdcd1, Lag3, and Cd244) [17, 18].

Increasing research suggests that the release of tumor metabolites promotes immunosuppression through extracellular matrix remodeling and drives CD8+ T cells toward exhaustion [19]. For decades, lactate has been viewed merely as a by-product of cellular metabolism; however, its potential as a key regulator in cancer development has only been recognized in recent years. Abnormal lactate metabolism has been correlated with poor prognosis and disease-free survival in several cancer types [20-25]. Whereas lactate has been studied extensively as an immunosuppressive metabolite associated with both impaired innate and adaptive immunity [26-29], the mechanisms governing this process remain incompletely understood.

The present study demonstrates for the first time that lactate production in AML blasts promotes exhaustion of effector T cells and downregulation of perforin and granzyme B by overexpressing TOX in CD8+ T cells, thereby incapacitating the mechanisms required for the destruction of tumor cells.

Materials and methods

Patient and healthy donor material

The study protocol was approved by the ethics committee of the Affiliated Hospital of Guizhou Medical University. All patients recruited provided written informed consent to participate in the study in compliance with the Declaration of Helsinki. The baseline characteristics of these patients are presented in Table S1. Bone marrow mononuclear cells (BMMCs) were obtained from healthy donors and AML patients at diagnosis and after chemotherapy. Complete remission (CR) was achieved when the following criteria were met: blasts present in the bone marrow (BM) <5% as determined by morphological evaluation, neutrophil count $>1\times10^9$/L, and platelet count $>1\times10^{11}$/L.

Primary AML blast cells and cell lines

Enrichment of CD34+ cells from bone marrow was performed using human CD34+ microbeads purchased from Miltenyi biotec. OCI-AML3 and Kasumi-1 cells were kindly provided by Guizhou Provincial Laboratory of Haematopoietic Stem Cell Transplantation Center. Culturing was performed in RPMI 1640 medium comprising 10% foetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Each cell line was tested for the presence of mycoplasma, and STR analysis was implemented for identity confirmation.

In vivo model of acute myeloid leukemia

Female NOD/SCID mice (Model Organisms Centre, Shanghai, China) were used for the *in vivo* experiments. All protocol procedures on mice conformed to the institutional policy on the animal use of Guizhou Medical University, China. The *in vivo* model was established as previously reported [30]. The mice were irradiated at a dose of 2.5 Gy. On day 0, the mice received an intravenous administration of 2×10^5 OCI-AML3 cells. At day 3, the mice

received 10^7 in vitro preactivated T cells and were subsequently assigned to 3 groups by randomization, namely, 2 treatment groups and 1 control group, each containing 14 mice. After two weeks, six mice from each group were sacrificed, followed by femoral bone marrow aspiration. Survival analyses were performed using Kaplan-Meier curves.

CD8+ T cell isolation and in vitro stimulation

Mononuclear cells were isolated by density gradient centrifugation, followed by T cell purification using magnetically labelled beads from Miltenyi Biotech. Thereafter, autologous leukemic blasts with a stable knockout of LDHA that were exposed to 80 Gy of radiation were mixed with purified T cells (at a responder-to-stimulator ratio of 1:1), which were then incubated for 96 h in RPMI-1640 medium comprising 10 mM HEPES, 10% heat-inactivated FCS, 100 U/ml penicillin and 100 ng/mL streptomycin. The variance of individual data due to different donors was not statistically significant.

Flow cytometry

For staining, antibodies directed against CD3, CD8, CCR7 (CD197), CD45RA, PD-1 (CD279), and TOX were used. Detailed information on the antibodies and manufacturers is presented in Table S2. A fixation and permeabilization step were performed with the Intracellular Fixation and Permeabilization Buffer Set (eBioscience) following the manufacturer's instructions. Samples were analysed on an LSR II flow cytometer (Becton Dickinson), with at least 5,000 gated events per sample. For analysis of the data obtained, FlowJo Software was used. Sorting of CD8+ T cell subsets was performed based on the surface expression of CD45RA and CCR7 according to the recommendations in the guidelines for the use of flow cytometry and cell sorting in immunological studies.

RNA isolation and TCRβ sequencing

Following enrichment of CD8+ T cells using the Dynabeads™ CD8 Positive Isolation Kit (Thermo Fisher Scientific), RNA extraction was performed with the Ultrapure RNA Kit (Cwbiotech, Beijing, China) according to the manufacturer's protocol. The RNA samples were quantified using a Qubit RNA HS Assay Kit (Thermo Fisher Scientific). RNA integrity was checked with the Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer. Reverse transcription (20 ng of total RNA) was performed (SuperScript IV VILO Master Mix; Thermo Fisher Scientific), followed by target amplification (25 ng cDNA) using the Oncomine™ TCR Beta-LR Assay Kit (Thermo Fisher Scientific). Purified library samples were diluted 1:100, and after an elution step, they were quantified using the Ion Library Quantitation Kit (Thermo Fisher Scientific). The enriched samples were loaded onto an Ion 530 chip for sequencing, and the sequencing data were analysed by Ion Reporter Software.

Repertoire analysis, including the indexes of diversity and clonality, was performed using the Oncomine™ TCR Beta-LR Assay in Ion Reporter™ Software. The frequency and sequence profiles were established by the Shannon index. Shannon entropy was calculated as $-\sum_{i=1}^{R} p_i \log_2(p_i)$, where p_i indicates the frequency of the i^{th} clone, and R indicates the total number of clones. Samples with many clones of similar frequencies have high Shannon diversity.

Luciferase reporter assay

Luciferase activities were measured using the luciferase assay system from Promega. Briefly, the wild-type and mutant LDHA HRE constructs were inserted into luciferase reporter plasmids. The cells were harvested at 24 hours after transfection and resuspended in lysis buffer. Cell lysates were cleared by centrifugation, and 10 ml of supernatants was mixed with an equal volume of Luciferase Assay Reagent and subsequently analysed for luciferase activity with a luminometer.

Western blotting

The pellets were resuspended in RIPA lysis buffer solution (P0013E, Beyotime). The total protein content was quantitatively measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The extracted proteins were separated on SDS-PAGE gels and then transferred to PVDF membranes (Millipore) before incubation in the presence of primary antibodies at 4°C overnight. Thereafter, the membranes were washed in phosphate-buffered saline three times for 10 min prior to incubation

with secondary antibodies at RT for 2 h. Tubulin and histone H3 were used as protein loading controls. Detailed information on the antibodies and manufacturers is presented in Table S2.

Immunofluorescence

AML or $CD8^+$ T cells were harvested after incubation and then fixed in 4% paraformaldehyde. Afterward, the cells were co-cultured with primary antibodies overnight at 4°C. After rinsing in PBS, fluorophore-labelled secondary antibodies were added. The cells were washed with PBS again prior to staining with DAPI for 20 min. Finally, 15 μl of mounting media and sterile coverslips were applied to the slides.

Gene interference construct

Stable knockdown of HIF-1α, LDHA and TOX was performed using lentiviral transduction particles (Sigma-Aldrich). For HIF-1α, the following lentiviral transduction particles were used: TRCN0000003808, TRCN0000003809, TRCN0000003810, TRCN0000003811, and TRCN0000010819; for LDHA, the lentiviral transduction particles used were: TRCN0000164922, TRCN0000165175, TRCN0000158441, TRCN0000026538 and TRCN0000026554; and for TOX, the lentiviral transduction particles used were: TRCN0000019475, TRCN0000019476, TRCN0000019478, and TRCN0000019477. Lentiviruses that stably overexpress HIF1α and LDHA were purchased from Gene Pharma (Shanghai, China). The cells were transduced on RetroNectin (Clontech Laboratories)-coated 12-well plates at a density of 100 μg/ml following the supplier's protocol. Next, the wells were blocked with 2% (w/v) BSA in PBS for 30 min at RT before BSA aspiration, and lentiviral particles were added to each well at a volume of 0.5 ml. The plates were then spun at 3,700 r.p.m. for 2 h at RT to increase the interaction between the lentiviral particles and RetroNectin. After harvesting the supernatant by centrifugation, 1-4 million cells were added per well in 1 ml of medium. This was followed by an additional centrifugation step at RT, 1,300 r.p.m. for 10 min prior to incubation at 37°C to improve integration with the lentiviral particles [31].

Measurement of lactate

The lactate concentration was measured in the bone marrow fluid or cell culture supernatant using a lactate assay kit (Abcam, ab65331) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) and then quantified via a Nanodrop spectrophotometer (Thermo Fisher Scientific). LDHA gene expression levels were determined using SYBR Green Supermix (Thermo Fisher Scientific) by qPCR conducted in an ABI 7500 instrument. The expression levels of the LDHA gene (F: GGAGATCCATCATCTCTCC; R: GGAGATCCATCATCTCTCC) and TOX gene (F: CCCCATGAACCATAATGGCCT; R: CCCAGCATATTGGAGACTGTGA) were determined by qRT-PCR, and Rplp0 was used for normalization of gene expression data. The relative gene expression level in a target sample was calculated by the $2^{-\Delta\Delta Ct}$ formula described in an earlier study [32].

Chromatin immunoprecipitation (ChIP) assay

Cell fixation was proceeded with incubation in PBS containing 1% formaldehyde for 10 min, followed by the addition of lysis buffer on ice. Chromatin was then fragmented by sonication to 200-500 bp in length. The resultant DNA was immunoprecipitated using anti-lysine-lactylation antibody (PTM-1401-RM, PTM Biolabs) and Dynabeads. Once complete, chromatin fragments containing the target protein were enriched and TOX promoter sequence was analysed (F: GTGGAACGGATGAAGAACT; R: TGTCCTTGGTGAGAGGTC).

ELISAs

ELISA kits (Thermo Fisher Scientific and RayBiotech) were used to investigate the concentration levels of perforin and granzyme B. After harvesting 10^5 cells in each group, a standard sandwich ELISA was utilized to quantitatively determine the LDHA levels with a commercially available kit (Aviva Systems Biology).

Statistical analysis

To examine the significance of differences between the two independent groups, the Mann-Whitney U-test was applied. A multiple group comparison procedure was performed using analysis of variance (ANOVA). Correction for multiple tests was performed using the false discovery rate method. Matched paired comparisons were performed using the Wilcoxon signed-rank test.

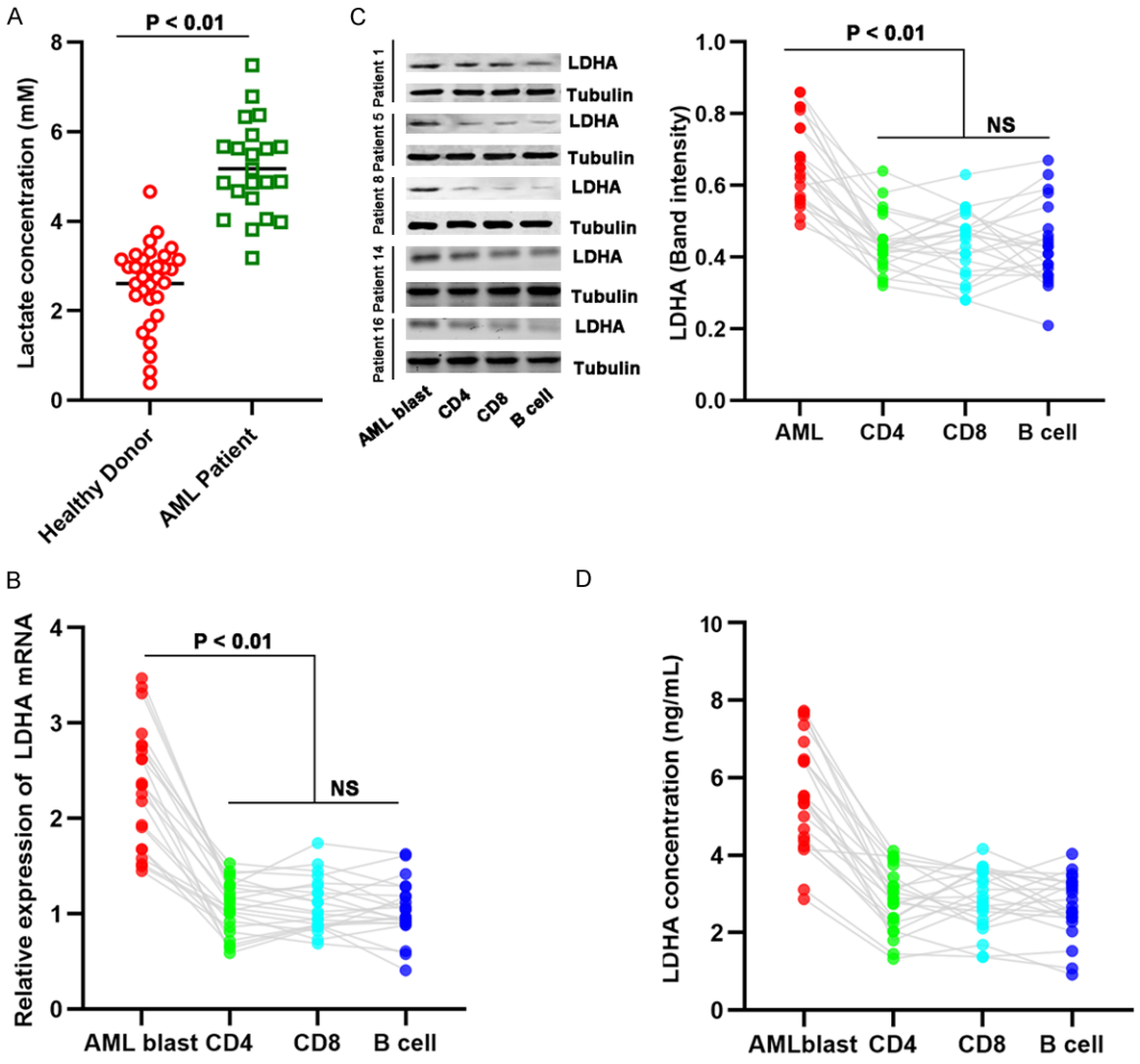

Figure 1. Notably higher lactate levels in newly diagnosed AML patients. A. Lactate concentrations in the bone marrow of 22 AML patients and 32 healthy donors. B. Comparison of relative mRNA expression levels of LDHA across several cell types (AML blast, $CD8^+$/$CD4^+$ T and B cells) derived from AML BM samples. C. Comparison of the protein expression levels of LDHA across several cell types (AML blast, $CD8^+$/$CD4^+$ T and B cells) derived from AML BM samples. Left panel: Protein expression levels of LDHA detected by Western blotting in 5 AML samples. Right panel: LDHA quantification values in all samples. D. Comparison of LDHA protein concentrations across several cell types (AML blast, $CD8^+$/$CD4^+$ T and B cells) derived from AML BM samples by ELISA. ns, not significant.

Results

Notably higher lactate levels in newly diagnosed AML patients

For the purpose of lactate measurement, bone marrow sampling was performed in 22 newly diagnosed AML patients and 32 healthy control participants. The mean BM lactate concentration of the AML patients was higher than the mean BM lactate concentration of the healthy donors (**Figure 1A**). Among the various cell populations in bone marrow, AML blast, $CD4^+$ T, $CD8^+$ T and B cells were assessed for relative LDHA mRNA and protein expression levels. The relative mRNA and LDHA protein expression levels in AML blast cells were found to be much higher than the relative mRNA and LDHA protein expression levels in other cell types (**Figure 1B-D**).

HIF1α activation upregulates LDHA expression and induces lactate production in AML blast cells

Motivated by the above results, we propose that AML blast cells are responsible for sustained glycolysis and pronounced lactate pro-

duction. Lactate dehydrogenase A (LDHA) is a critical cytosolic enzyme, an essential hallmark of tumor progression, and a significant regulator of the glycolytic process, primarily responsible for pyruvate-lactate reactions [33, 34]. The interconnection between LDHA and HIF1α was analysed by constructing a protein-protein interaction (PPI) network using the STRING v10 database (**Figure 2A**). Previous studies have identified HIF1α binding sites that are enriched for an ACGTG motif [35]. To determine the potential regulatory role of HIF1α in LDHA expression at the transcriptional level, we performed promoter analysis, with a focus on the search for the presumed HIF1α binding sites. HIF1α overexpression was found to be associated with enhanced LDHA reporter activity. Two putative regions bound to HIF1α were confirmed, and either mutated region was found to reverse the enhancement of promoter activity. The mutation of one binding site moderately decreased the promoter activity, but the mutation of both binding sites completely ablated the promoter activity (**Figure 2B**).

We next performed lentiviral transduction for the stable knockdown or overexpression of the target cells. **Figure 2C** and **2D** show the expression levels of HIF1α and LDHA, respectively, determined by immunofluorescence assays after HIF1α knockdown or overexpression. Western blot analysis and sandwich enzyme immunoassay showed decreased LDHA protein levels in the Kasumi-1 cell line, OCI-AML3 cell line and primary AML blast cells after HIF1α knockdown. In contrast, the expression levels of LDHA protein were upregulated after overexpression of HIF1α (**Figure 2E**, **2F**). After *in vitro* incubation for 24 hours, the lactate content in the cultures of the overexpression group was significantly higher than the that in the cultures in the control group; in contrast, the opposite was observed in the knockdown group, where the lactate content in the cultures was significantly lower than the lactate content in the cultures in the control group. However, no significant differences were observed for the lactate levels between the culture results (48 h vs. 72 h) in either the overexpression or control group (**Figure 2G**).

Elevated levels of lactate are linked to the loss of CD8+ cytotoxic T cells

The addition of lactate at all concentrations tested (0, 2.5, 5.0, 7.5 mM) during incubation did not increase the frequency of apoptotic CD8+ T cells (**Figure 3A**). Local acidosis is an important hallmark of the tumor micromilieu and is a result of enhanced glycolytic metabolism and sustained accumulation of lactate. To examine the potential association between lactate and T-cell proliferation, graded concentrations of lactate were added to cultures of CD8+ T cells, and no pro-inhibitory effect was observed (**Figure 3B**). Cytotoxic T cells induce apoptosis mainly through the perforin/granzyme B pathway [11]. Thus, the potential relationship between lactate levels and the expression index of perforin and granzyme B in AML patients was examined. Remarkably, a strong correlation with perforin (r=0.748, P<0.01) and granzyme B (r=0.752, P<0.01) was noted (**Figure 3C**). Tumor-derived lactate indeed exerted an exposure-dependent inhibitory effect on perforin and granzyme B secretion in activated CD8+ T cells (**Figure 3D**, **3E**). In earlier studies, CD8+ and CD4+ T cells were shown to express Slc16a1 and Slc5a12 [36], which are major lactate transporters involved in the suppression of T cell function. This finding was validated in the present study (**Figure 3F**). Slc16a1 expression was not changed after exposure to lactate (**Figure 3G**). Pharmacological blockade of Slc16a1 using specific inhibitors resulted in elevated percentages of perforin and granzyme B in CD8+ T cells (**Figure 3H**). These findings were consistent with earlier studies indicating that import of lactate into CD8+ T cells is dependent on the interaction with Slc16a1, leading to the downregulation of perforin and granzyme B.

Elevated lactate is associated with TOX upregulation and dysfunctional CD8+ T cells

The decrease in naïve T cells and TCM subsets together with the increase in TEM and TEMRA subsets in CD8+ T cells have been attributed to dysfunctional and exhausted T cells after prolonged exposure to the tumor microenvironment, limiting their ability to proliferate and produce cytokines [37]. The frequencies of TEM, TEMRA and TEM plus TEMRA cells were congruous with the levels of lactate production (**Figure 4B**). In addition, the expression levels of PD-1 in these cell subsets were also found to be significantly associated with lactate concentrations (**Figure 4C**). These findings were further confirmed in an *in vitro* model in which lactate mediated upregulated PD-1 expression (**Figure 4D**, **4E**).

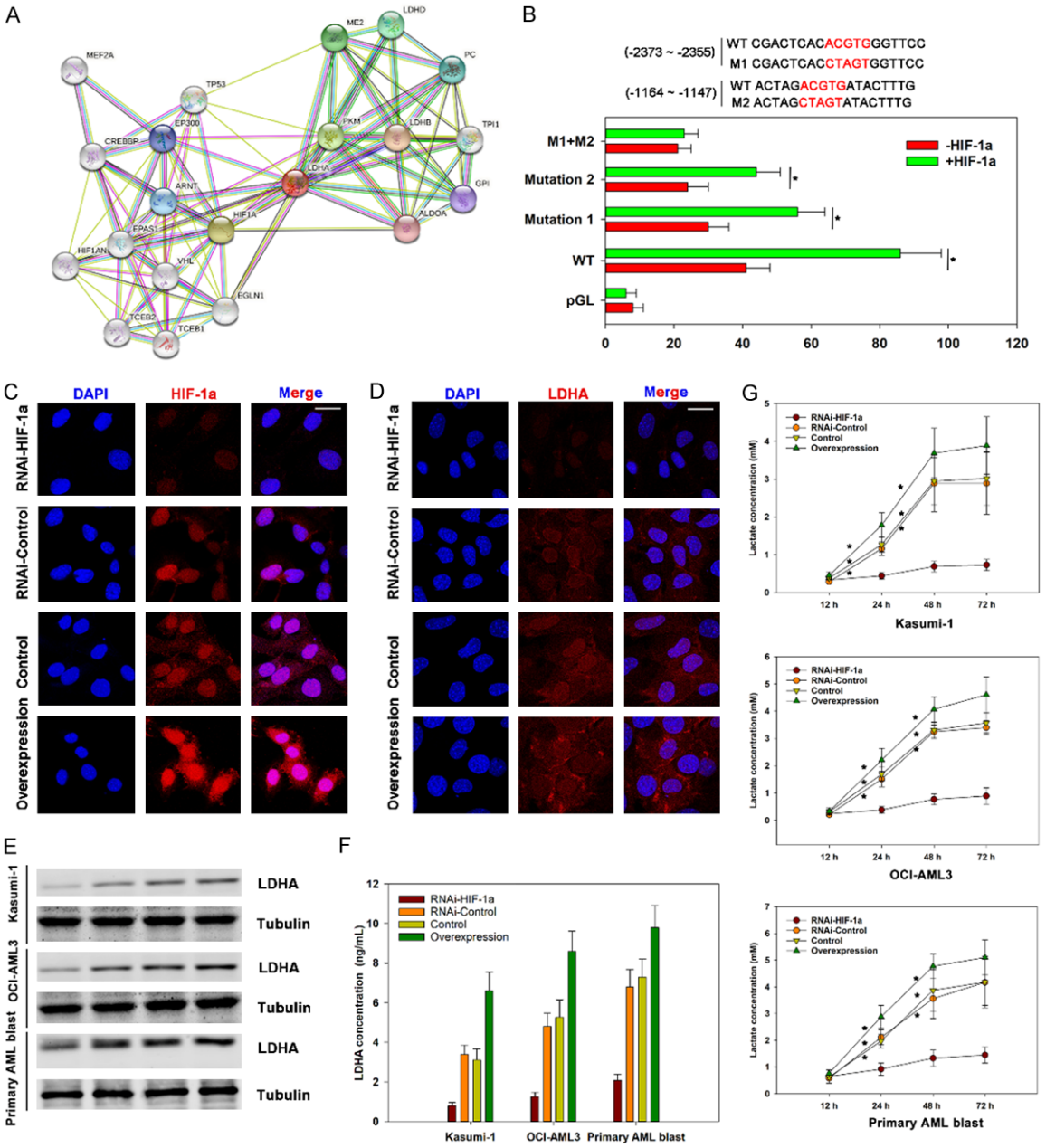

Figure 2. HIF1α activation upregulates LDHA expression and induces lactate production in AML blast cells. A. Prediction of functional associations between LDHA and HIF1α through protein-protein interaction (PPI) network construction using the STRING database. B. Induction of luciferase activity in cells transfected with the HIF1α promoter-reporter construct and an empty vector control. The "X" represents the mutant-type consensus sequences. The red letters in the binding domain indicate the presumed or mutated consensus sequences. M, mutant; WT, wild-type. C, D. Analysis of HIF1α and LDHA expression in the Kasumi-1 cell line by quantitative immunofluorescence. Kasumi-1 cells were transduced with a lentivirus containing HIF1α-shRNA or control-shRNA constructs. Cells were labelled with HIF1α or LDHA antibody. DNA in nuclei was counterstained with DAPI dye. Scale bar: 10 μm. E, F. Protein expression levels of LDHA in Kasumi-1, OCI-AML3 and primary AML blast cells after knocking down or overexpressing HIF1α. G. The three sets of cells were cultured for 12, 24, 48 and 72 hours after knocking down or overexpressing HIF1α. The culture supernatant was then assayed for lactate concentration. *P<0.01.

CD8+ T cells with cytotoxic effector functionality trigger apoptosis of tumor cells through T cell receptor (TCR) activation. The diversity of the TCR repertoire may reflect the general propensity of tumor antigen recognition [38-40]. Restricted TCR diversity and persistent expand-

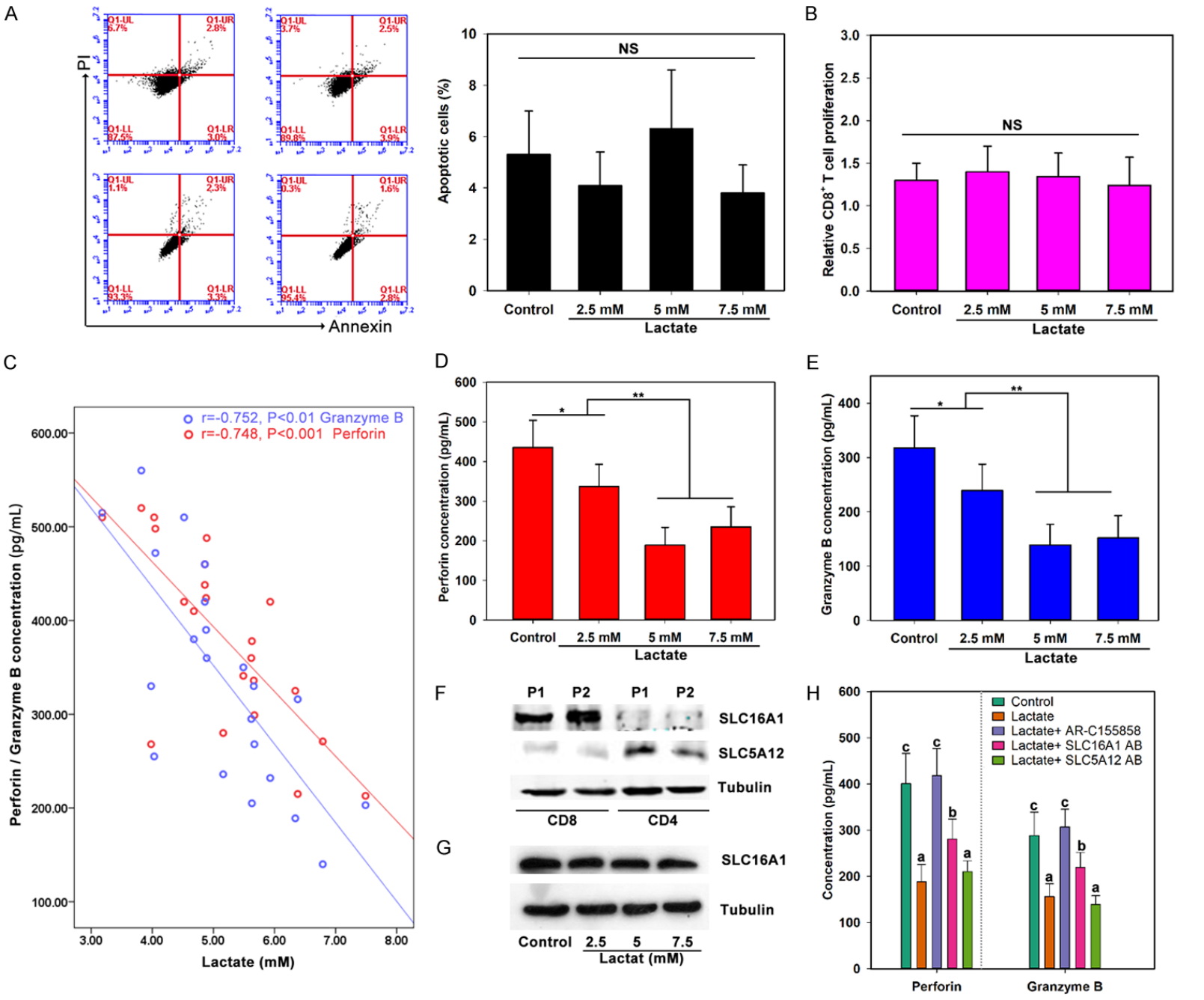
A
PI
Annexin
Q1-UL 6.7%
Q1-UR 2.8%
Q1-LL 87.5%
Q1-LR 3.0%
Q1-UL 3.7%
Q1-UR 2.5%
Q1-LL 89.8%
Q1-LR 3.9%
Q1-UL 1.1%
Q1-UR 2.3%
Q1-LL 93.3%
Q1-LR 3.3%
Q1-UL 0.3%
Q1-UR 1.6%
Q1-LL 95.4%
Q1-LR 2.8%
B
Apoptotic cells (%)
NS
Control
2.5 mM
5 mM
7.5 mM
Lactate
Relative CD8+ T cell proliferation
NS
C
r=-0.752, P<0.01 Granzyme B
r=-0.748, P<0.001 Perforin
Perforin / Granzyme B concentration (pg/mL)
Lactate (mM)
D
Perforin concentration (pg/mL)
E
Granzyme B concentration (pg/mL)
F
P1
P2
SLC16A1
SLC5A12
Tubulin
CD8
CD4
G
Control
2.5
5
7.5
Lactat (mM)
H
Control
Lactate
Lactate+ AR-C155858
Lactate+ SLC16A1 AB
Lactate+ SLC5A12 AB
Concentration (pg/mL)
Perforin
Granzyme B

Figure 3. Elevated levels of lactate are linked to the loss of CD8+ cytotoxic T cells. A. Lactate levels and CD8+ T cell apoptosis. For apoptotic cell evaluation, CD8+ T cells were exposed to increasing doses (0, 2.5, 5.0, 7.5 mM) of lactate for 72 hours, and flow cytofluorometric analysis was performed per the Annexin V/PI protocol. B. Lactate levels and T cell proliferative effect. CD8+ T cells were exposed to increasing doses (0, 2.5, 5.0, 7.5 mM) of lactate for 72 hours, and carboxyfluorescein succinimidyl ester (CFSE) was used to assess the division frequency of CD8+ T cells. C. Significant correlation between lactate concentrations and perforin and granzyme B production. D, E. Association between lactate levels and perforin/granzyme B production. Upon stimulation, CD8+ T cells were exposed to increasing doses (0, 2.5, 5.0, 7.5 mM) of lactate for 72 hours, and the culture supernatant was harvested and assayed for perforin and granzyme B production. F. Western blotting determination of Slc16a1 and Slc5a12 protein expression levels in CD4+ and CD8+ T cells from AML specimens. G. Association between lactate levels and Slc16a1 protein expression. CD8+ T cells were exposed to increasing doses (0, 2.5, 5.0, 7.5 mM) of lactate for 72 hours, and the expression levels of Slc16a1 were assessed by Western blotting. H. Upon stimulation, CD8+ T cells were treated with lactate (5 mM) alone or in combination with AR-C155858 (20 nM), anti-Slc16a1 antibody (2.5 μg/ml) or Slc5a12 antibody (2.5 μg/ml) for 72 hours. The culture supernatant was harvested and assayed for perforin and granzyme B production. ns, not significant. **P*<0.05, ***P*<0.01.

ed clones were observed in BM samples from AML patients [41-43]. Importantly, no congruence was noted between the Shannon indexes and lactate production or between the expression of highly expanded clones (HECs, defined as clonotypes with abundance greater than 0.1% in each sample [44]) and lactate concentrations (**Figure 4F**, **4G**).

To substantiate the regulatory role of lactate, we next investigated whether lactate mediated its effect on TOX expression. Elevated lactate levels were shown to upregulate TOX expression, and this effect was subdued after treatment with the Slc16a1 inhibitor AR-C155858 (Figures S1A and **5A-C**). Recent studies have established lactate as a crucial regulator of gene transcription via histone lactylation. To confirm this finding, ChIP experiments were carried out to determine histone lactylation on the promoter region of *TOX*. Lactate treatment was observed to have increased the histone lactylation levels in the *TOX* promoter (Figure S1B, S1C), corroborating the regulatory role of lactate and the close involvement of histone lactylation [45-47]. The knockdown of TOX reversed the upregulatory effect of lactate on TEM and TEMRA ratios and PD-1 expression in TEM plus TEMRA cells (**Figure 5D**, **5E**).

In a murine model, following the injection of OCI-AML3 and CD8+ T cells, a remarkable increase in the expression levels of TOX and PD-1 in TEM and TEMRA subsets was noted in the LDHA overexpression group at week 2 relative to the control group. Meanwhile, the expression levels of TOX and PD-1 in the LDHA knockdown group were lower than the expression levels of TOX and PD-1 in the control group (**Figure 5G**, **5H**). The Kaplan-Meier plot showing the survival of the mice after receiving a tail vein infusion of OCI-AML3 cells revealed that overexpression of LDHA largely reduced mouse survival, while stable knockdown of LDHA significantly increased mouse survival (37 and 57.5 days, respectively, versus 50 days for the control) (*P*=0.021).

Changes in lactate levels and CD8+ T cell distribution in AML patients before and after chemotherapy

Detailed information on the paired samples is listed in **Figure 6A**. Four out of a total of 22 newly diagnosed patients were lost to follow-up. Half of the remaining patients achieved remission, and the other half relapsed after chemotherapy. Lactate concentrations and LDHA protein concentrations of AML blast cells were compared in the 18 patients against the following metrics: TOX and PD-1 expression, perforin and granzyme concentrations and differentiation of CD8+ T cells. The results indicated that for patients who achieved complete remission after chemotherapy, taking the data at diagnosis as baseline, the lactate concentrations, TOX expression in CD8+ T cells, fraction of TCM plus TEMRA cells in the total CD8+ T cells, and PD-1 expression all decreased after chemotherapy (*P*=0.018, 0.024, 0.025, and 0.012, respectively), and perforin and granzyme B concentrations increased (*P*=0.044 and 0.042, respectively). For patients who relapsed after chemotherapy, there was no difference in the above parameters (*P*>0.05). For the LDHA protein concentration of AML blast cells in relapsed patients before and after chemotherapy, no significant difference was found. Moreover, in both patient groups, there was no difference in the LDHA protein concentration of CD8+ T cells at diagnosis or after chemotherapy.

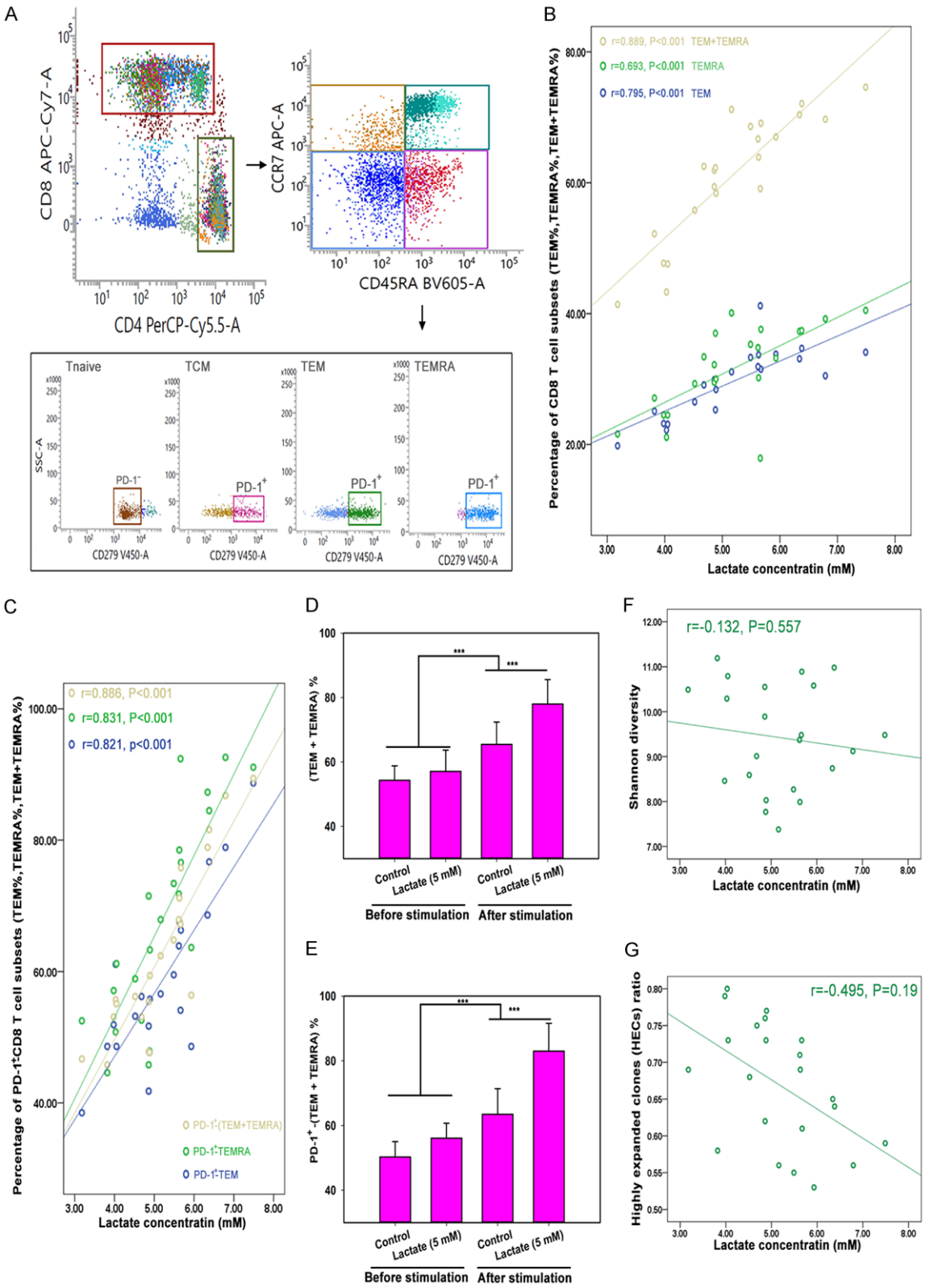

Figure 4. Lactate concentrations and CD8+ T cell differentiation/PD-1 expression. A. Top: Gating strategy of flow cytometry to discriminate TN, TCM, TEM, and TEMRA cells. Bottom: PD-1 expression in TN, TCM, TEM, and TEMRA cells. B. Correlation between lactate production and TEM, TEMRA and TEM plus TEMRA subset frequencies in AML patients. C. Correlation between lactate concentrations and PD-1 expression ratios in TEM, TEMRA and TEM plus TEMRA cells. D. TEM plus TEMRA subset frequencies in response to lactate treatment. Upon stimulation, CD8+ T

cells were incubated in the presence of 5 mM lactate for 72 hours and then collected for flow cytometry analysis. E. Association between lactate levels and PD-1 expression in TEM and TEMRA cells. Upon stimulation, CD8+ T cells were incubated in the presence of 5 mM lactate for 72 hours and then collected for flow cytometry analysis. F. Lactate concentrations were not significantly correlated with the Shannon indexes, a measure of TCR diversity, in TEM and TEMRA cells. G. Lactate concentrations were not significantly correlated with the ratios of highly expanded clones (HECs, clonotypes with a frequency of more than 0.1% in each sample) in TEM and TEMRA cells.

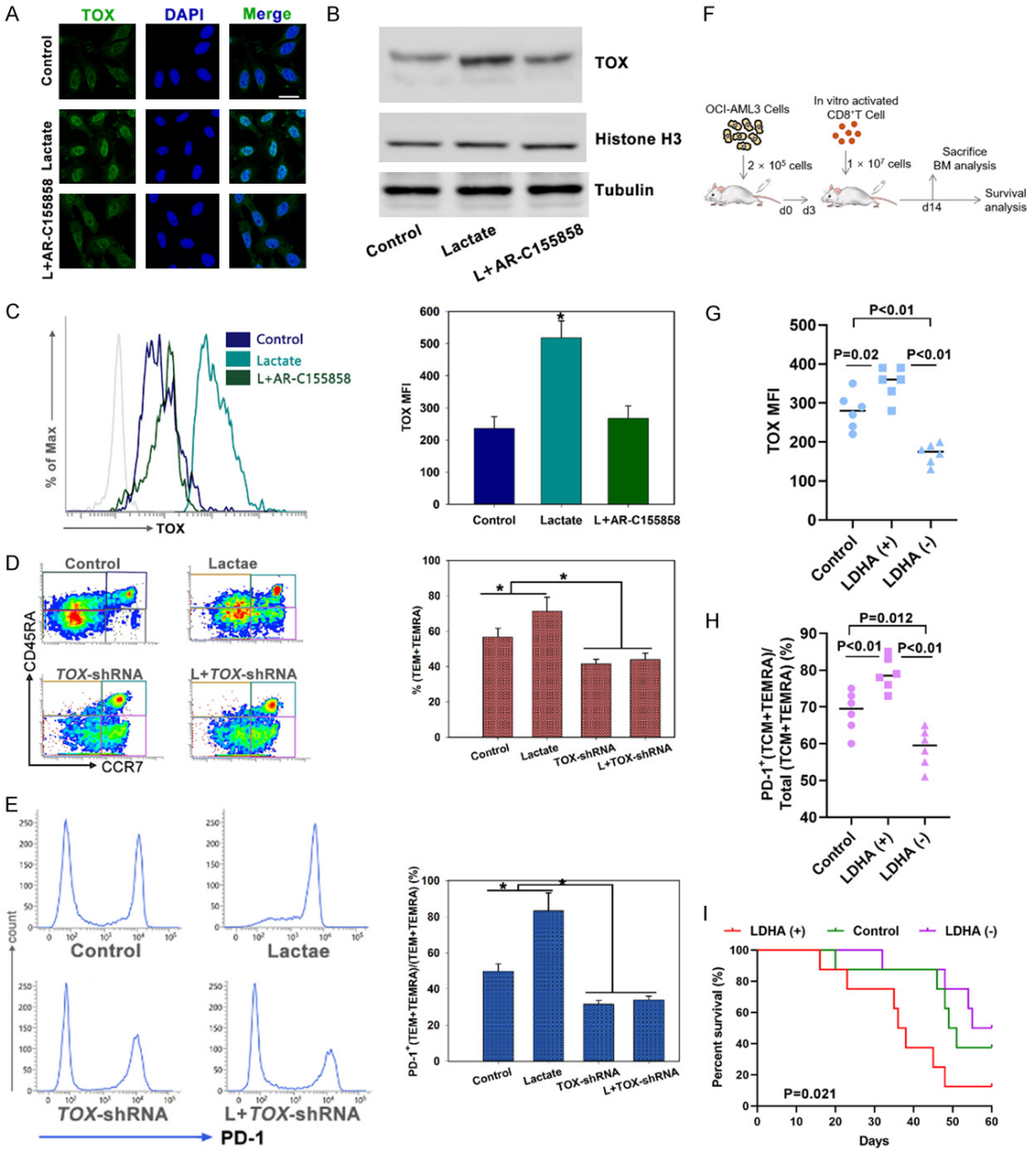

Figure 5. Lactate upregulates TOX expression. A. Quantitative immunofluorescence of TOX expression in CD8+ T cells. Upon stimulation, CD8+ T cells were incubated with 5 mM lactate or subjected to treatment with 20 nM Slc16a1 inhibitor (AR-C155858) for 72 hours. Cells were stained with TOX antibody. DNA in nuclei was counterstained with DAPI dye. Scale bar: 10 μm. B. TOX protein expression analysis by Western blotting. CD8+ T cells were treated in the same manner as stated above. C. Representative flow cytometry histograms and quantification of TOX expression in CD8+ T cells are shown in the left and right panels, respectively. D. Potential pro-differentiation role of lactate in CD8+ T cells through TOX expression. Left: Flow cytometry plots showing coexpression of CD45RA and CCR7 on CD8+ T cells. Right: Fraction of TEM plus TEMRA cells among total CD8+ T cells. E. Lactate-mediated PD-1 expression

upregulation in TEM and TEMRA cells. Representative flow cytometry histograms and quantification of PD-1 expression in CD8+ T cells are shown in the left and right panels, respectively. Data are representative of at least three independent experiments. F. According to the experimental protocol, the mouse experimental design was as follows. On day 0, 2×10^5 OCI-AML3 cells were injected into the lateral tail vein of NOD/SCID mice, which were pre-exposed to γ irradiation at a dose of 2.5 Gy. On day 14, mice (n=6) in each group were euthanized under general anaesthesia. Immediately thereafter, BM aspirates were collected from the femora of the hind legs. G. Quantification of TOX expression in CD8+ T cells. TOX expression in CD8+ T cells was quantified by flow cytometry. H. Determination of PD-1 expression in TEM and TEMRA subsets. I. Kaplan-Meier plot showing the survival probability of the mice. L, Lactate.

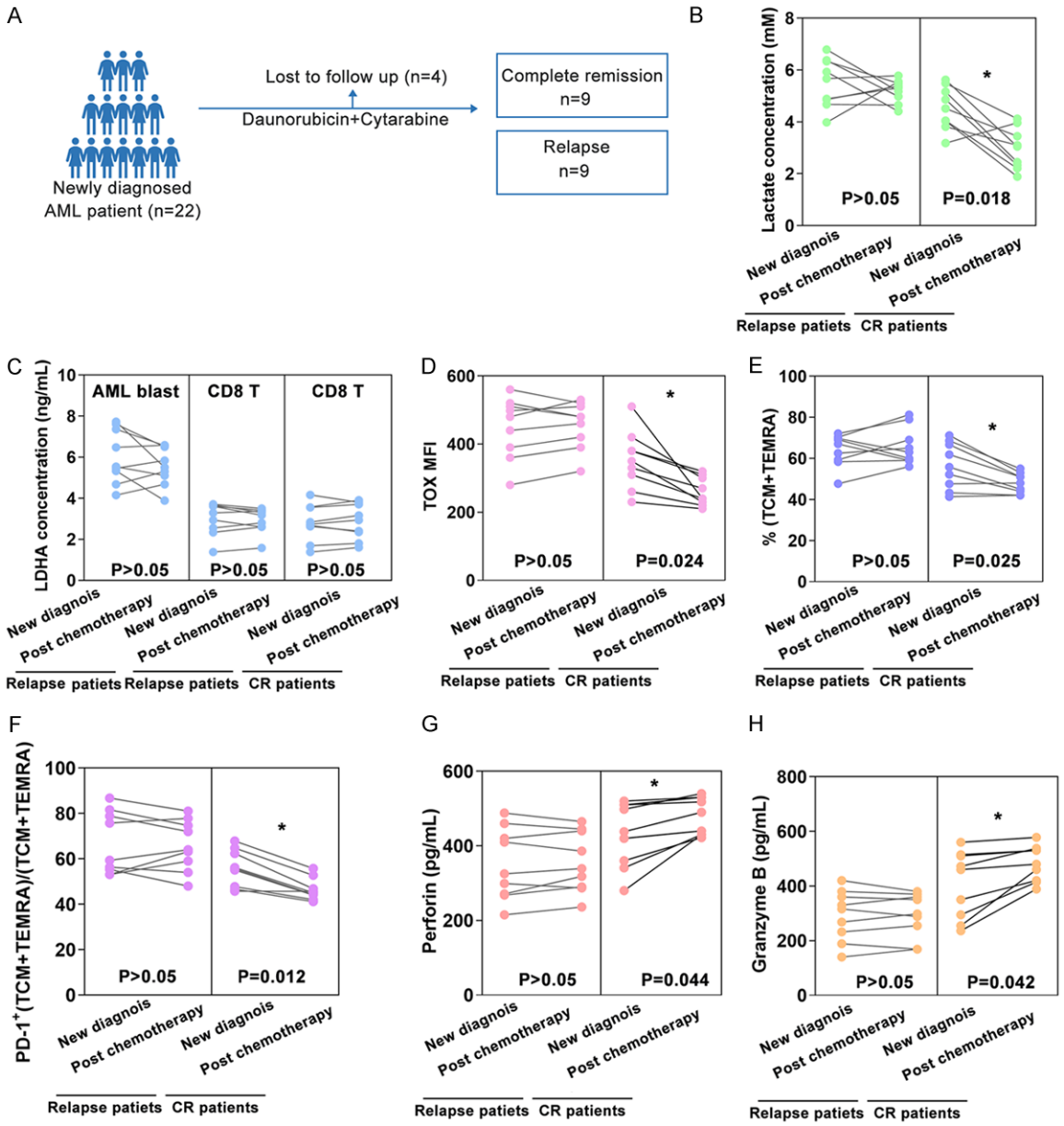

Figure 6. Changes in lactate levels and CD8+ T cell distribution in AML patients before and after chemotherapy. A. Schematic representation of matched samples collected at diagnosis and post-chemotherapy. Samples from 9 patients who relapsed and 9 patients who achieved CR after chemotherapy were collected and paired for analysis. B, C. Quantitative measurement of lactate levels and LDHA protein concentrations using ELISA. D-F. Flow cytometry quantification of TOX expression levels, proportion of TEM plus TEMRA cells, and PD-1 expression in TEM and TEMRA cells. G, H. Quantitative measurement of perforin and granzyme B production using ELISA.

Discussion

Proteins that promote effector T cell suppression are broadly categorized into three groups: inhibitory receptors (iRs) during T cell activation, e.g., programmed cell death protein 1 (PD1); immunological tumor-promoting cells within the microenvironment, including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs); and a diverse range of soluble factors, e.g., indoleamine-2,3-dioxygenase (IDO), nucleotides, arginase, and tumor-derived metabolites [19, 48, 49]. AML blasts arise from myeloid progenitors and accumulate in the bone marrow, an environment favorable for their maturation and differentiation [50]. The high recurrence rates after chemotherapy, limited donor availability in hematopoietic stem cell transplantation (HSCT), and lack of breakthroughs in immunotherapy for AML have prompted extensive investigation into the role of AML blasts in the bone marrow microenvironment in mediating $CD8^+$ T cell dysfunction.

Excessive lactate production has been hitherto reported in several solid tumors [22-25]. J. Feng et al. demonstrated the role of tumor-derived lactate in the regulation of PD-L1 promoter activity through the GPR81 signaling pathway. The production of lactate in lung cancer cells was shown to attenuate the cAMP signaling system by GPR81 receptor stimulation, thereby contributing to $CD8^+$ T cell exhaustion [51]. A recent study by Karin Fischer et al. measured lactate concentrations in several malignancies and revealed that the maximum serum lactate levels were notably higher in patients than in healthy individuals (8.7 mM vs. 2.2 mM) [23]. The present study measured lactate concentrations in bone marrow samples from AML patients and healthy donors. Consistently, the lactate concentrations in the bone marrow samples from AML patients ranged from 3.18-7.49 mM (mean 5.18 mM), whereas the lactate concentrations in the bone marrow samples from healthy donors ranged from 0.64-4.66 mM (mean 2.60 mM). In an *in vitro* model, Karin Fischer et al. demonstrated that increasing the concentration of lactate from 5 to 15 mM could effectively inhibit the proliferation of human cytotoxic T lymphocytes (CTLs) and limit the cytotoxic activity to a great extent. In addition, Mendler et al. showed that 10 mM lactate triggered the activation of the JNK/c-Jun and p38 pathways and interfered with IFN-γ production [52]. Consistent with their results, $CD8^+$ T cells treated with 10 mM lactate significantly inhibited cytokine production, and IFN-γ production was disrupted when the concentration was increased to 20 mM (data not shown). A major consideration in the design of the present study is the suitability of the lactate concentrations used for *in vitro* imitation of the intra-tumoral micromilieu. In this context, concentration gradients (2.5, 5.0, and 7.5 mM) were used for subsequent *in vitro* experiments. Following exposure to 2.5, 5.0, and 7.5 mM lactate, no changes were noted regarding T cell proliferation and apoptosis. However, 5 mM lactate was sufficient to induce a significant inhibition of perforin and granzyme B production.

AML blasts are highly dependent on the bone marrow niche to facilitate their maturation and proliferation. The bone marrow is highly heterogeneous and home to a wide array of cell types [50]. As a result, it is crucial to assess the LDHA protein expression of the main cell types to determine the origin of lactate. LDHA has been shown in earlier studies to be expressed on cancer cells and enhance glycolysis by converting pyruvate into lactate. Earlier studies have established elevated LDHA levels as a predictor of tumor progression and poor survival outcomes [33, 34, 53, 54]. We observed notably higher LDHA protein expression in AML blasts than in other cell types analysed, i.e., $CD4^+$ T, $CD8^+$ T and B cells. However, one major limitation of this study is that although AML blasts were identified as the subset with the highest LDHA expression, due to different total cell counts and varying ratios of cell types, the specific fraction of lactate derived from AML blasts was not determined. Thus, our findings need to be validated in additional studies with refined approaches.

In contrast to TEM and TEMRA cells, T naïve and TCM cells, despite their proliferative nature and ability to induce IL-2 production, have limited effector function [6]. Immunophenotypic analyses revealed a marked increase in the abundance of TEM and TEMRA cells and a decrease in the abundance of naïve T and TCM cells in AML patients compared with healthy controls. Moreover, elevated expression of PD-1 and Tim-3 was also noted in AML patients, which was associated with T cell exhaustion

[37, 55, 56]. Our study identified strong correlations between lactate concentrations and percentages of CD8+ TEM and CD8+ TEMRA cells as well as PD-1 expression levels. *In vitro* experiments indicated that lactate could substantially increase the overall percentages of CD8+ TEM and CD8+ TEMRA cells. Meanwhile, lactate was also shown to promote PD-1 expression in TEM and TEMRA cells. Hence, lactate appears to work in both ways; it may increase the ratio of effector T cells, and it may also upregulate the expression of PD-1 in such effector T cells, driving them toward exhaustion. TOX has been previously reported as an essential biomarker for T cell differentiation and tumor progression [13, 18]. Recent research has revealed that TOX is implicated in T cell survival of exhausted T cells in chronic infections [12]. Knockdown of the TOX gene led to reduced PD-1 mRNA and protein expression and reinforced the phenotype of effector T cells [17]. Sekine et al. showed that TOX expression was overwhelmingly found in TEM and TEMRA cells [15, 57]. These findings indicated that both the exhausted T cell phenotype and the effector T cell phenotype can express TOX. After knocking down the TOX gene, the previously observed effects of lactate on the TEM/TEMRA ratio and PD-1 expression subsided, suggesting that lactate exerted its proapoptotic effect on CD8+ T cells through the TOX gene.

Immune checkpoint blockade has shown clinical efficacy in solid tumors. CAR-T cell therapy has also achieved initial success by harnessing the potential of the immune system. Despite advances in solid tumors, these forms of immunotherapy have limited efficacy against AML. The results of the present study showed that increased lactate levels in AML blasts upregulate TOX expression in CD8+ T cells and trigger T cell exhaustion, potentially hampering cytolytic function. Lactate secreted by AML blasts mediates the inhibition of cytotoxic T cells, which may partially be responsible for the low success rate of immunotherapy. Our data add to a growing body of literature in support of lactate as a major determinant of antitumor responses in immunotherapy.

Acknowledgements

This work received grants from the National Natural Science Foundation of China. (Grant No. 81660616 and 81960032), the Social Development Research Project of Guiczhou Province (Grant No. [2015]3036) and the National Centre for Clinical Medicine of Hematological Diseases (2020ZKPB03).

Disclosure of conflict of interest

None.

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Table S1. AML patients and donors characteristics

Domain	Patients Total (n=22)	Donors Total (n=32)
Age (year)		
Median	51	45
Range	33-66	30-56
Gender		
Male	12	15
Female	10	17
WBC ($\times 10^9$/L)		
Mean	25.58	6.05
Range	6.15-53.33	5.12-7.70
PB blasts (%)		
Mean	31.47	
Range	18.36-53.35	
PB Absolute blasts count ($\times 10^9$/L)		
Mean	12.57	
Range	1.98-24.54	
BM blasts (%)		
Mean	60.39	
Range	23.30-86.91	
BM Absolute blasts count ($\times 10^9$/L)		
Mean	19.53	
Range	7.25-46.38	

WBC, white blood cell; PB, peripheral blood; BM, bone marrow. AML type accords to WHO (World Health Organization) 2016.

Table S2. Primary antibodies used in this study

Antibody	Source	Cat. Number	Dilution			
			IF	WB/Chip-PCR	FCM	Culture
HIF-1α	CST	79233	1:200			
LDHA	CST	3582	1:100	1:500		
SLC16A1	CST	85680		1:1000		2.5 μg/mL
SLC5A12	Abcam	ab262934		1:1000		2.5 μg/mL
TOX	Thermo	PA553781		1:500		
Lysine-Lactylation	PTM Biolabs	PTM-1401-RM		WB: 1:1000 CHIP: 1:500		
Histone H3	Abcam	ab1791		1:5000		
Tubulin	Abcam	ab6046		1:5000		
CD3	Biolegend	317306			5 μl/10^7 cells	
CD4		317428				
CD8		301016				
CD279 (PD-1)		329920				
CD197 (CCR7)		353214				
CD45RA		304134				

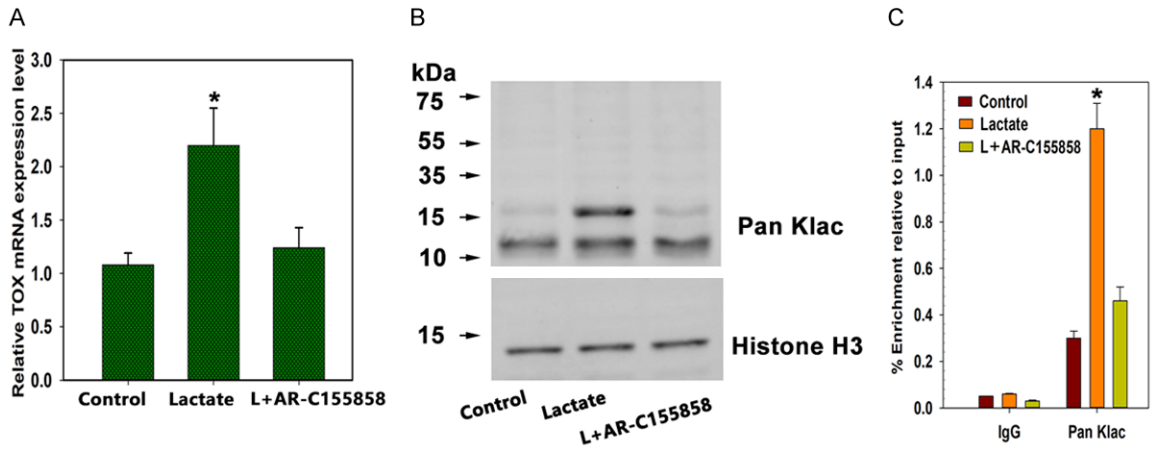

Figure S1. Lactate drives TOX mRNA expression and enhances histone lactylation in the *TOX* promoter. A. Quantitative measurement of TOX mRNA expression in CD8+ T cells. Stimulated CD8+ T cells were exposed to 5 mM of lactate and treated with 20 nM of Slc16a1 inhibitor (AR-C155858) for 72 h. B. Western blotting assays using pan anti-Kla antibody in CD8+ T cells. Cells were subjected to the same treatment as described above. C. Anti-lactylated histone levels in the *TOX* promoter. L, Lactate. *P<0.05.