

## Original Article

# STAT3 signaling pathway is involved in decitabine induced biological phenotype regulation of acute myeloid leukemia cells

Zhichao Zhu<sup>1\*</sup>, Xuzhang Lu<sup>2\*</sup>, Lijia Jiang<sup>1</sup>, Xiao Sun<sup>1</sup>, Haijun Zhou<sup>1</sup>, Zhuxia Jia<sup>2</sup>, Xiuwen Zhang<sup>2</sup>, Lingdi Ma<sup>3</sup>

<sup>1</sup>Laboratory Center, Changzhou No. 2 People's Hospital, Affiliated Hospital of Nanjing Medical University, No. 29 Xinglong Lane, Changzhou 213000, China; <sup>2</sup>Department of Hematology, Changzhou No. 2 People's Hospital, Affiliated Hospital of Nanjing Medical University, No. 188 Gehu Middle Road, Changzhou 213000, China; <sup>3</sup>Department of Laboratory Medicine, Huizhou No. 3 People's Hospital, Affiliated Hospital of Guangzhou Medical University, No. 1 Xuebei Street, Qiaodong Road, Huizhou 615000, China. \*Equal contributors.

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**Abstract:** Objective: This study aimed to investigate the role of signal transduction and transcriptional activator STAT3 and relevant signaling pathway in the DAC regulated biological phenotype of AML cells. Methods: The effect of DAC at different concentrations on the proliferation of HL-60 cells was determined. After DAC treatment for 48 h, the killing capability of NK cells against HL-60 cells and the protein expressions of STAT3, JAK1, JAK2, SOCS-1 and SOCS-3 were evaluated. Results: DAC markedly inhibited the proliferation of HL-60 cells. After the treatment of 48 hr with 0.2, 0.5 and 1.0 mol/L DAC, the HL-60 viability was reduced by 25±13%, 39±8% and 50±7% ( $P<0.01$ ), respectively, and the early apoptosis rate was increased to 24.77±7.5%, 27.1±4.48% and 30.53±3.93%, respectively (control: 3.11±0.12%,  $P<0.01$ ). DAC up-regulated the expression of MICA/B, ULBP-1 and ULBP-3 in HL-60 cells, and increased the killing activity of NK cells to HL-60 cells. DAC significantly induced the apoptosis of HL-60 cells and up-regulated the expression of NKG2D ligands in a dose dependent manner. Western blot assay showed the protein expression of STAT3, JAK, JAK2, phosphorylated STAT3, phosphorylated JAK1 and phosphorylated JAK2 decreased, while that of SOCS-1 and SOCS-3 increased in HL-60 cells after DAC treatment. Conclusion: In HL-60 cells, DAC can markedly inhibit their proliferation and up-regulate the expression of NKG2D ligands, and DAC also increase the cytotoxicity of NK cells to HL-60 cells, which may be related to the STAT3 related signaling pathway.

**Keywords:** Decitabine, STAT3, SOCSs, acute myeloid leukemia, natural killer cells, NKG2D ligand

## Introduction

Acute myeloid leukemia (AML) is a malignant hyperplasia disease of the hematopoietic system and has been the most common type of acute leukemia in adults. The incidence of AML is increasing over year. There are 12,330 new cases in the United States, and 18000 cases in Europe. The 5-year survival rate of adult AML patients is only about 50%. For patients older than 70 years, the 5-year survival rate is below 10%, although intensive chemotherapy has been used [1, 2]. Along with the in depth epigenetics studies, it is found that aberrant epigenetics is also involved in the pathogenesis of AML in addition to the change in cytogenetics. It can cause genomic instability, and thus lead

to cytogenetic abnormalities with the involvement of environment factors [3]. Murai et al found that there was hypermethylation of apoptosis related gene BNIP3 in patients with acute lymphoblastic leukemia, AML and multiple myeloma patients, which silenced BNIP3 expression [4]. Hasegawa et al also reported hypermethylation of p15 gene in acute leukemia, plasma cell disorder, non-Hodgkin's lymphoma and myelodysplastic syndromes [5]. However, Jiang et al found that the abnormal methylation of DNA was a major mechanism, which led to the silencing of tumor suppressor gene TSG and the development of myelodysplastic syndrome of AML [6], and thus to regulate the hypermethylation might be a target for the treatment of AML.

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Decitabine (DAC) is a new epigenetic drug and can enter DNA after phosphorylation *in vivo* to induce the demethylation of DNA by inhibiting the activity of DNA methyltransferase (DNMT). DAC can induce genomic DNA methylation and local hypermethylation of CpG islands, which inhibits the expression of some highly activated abnormal intracellular genes, and activates that of tumor suppressor genes silenced due to the methylation of promoter region. In addition, DAC can increase the expression of cell differentiation-related genes, and finally inhibit the growth of tumor cells [7, 8]. Clinical studies have confirmed that about 47% of patients achieved complete remission after 3 courses of intravenous infusion of low dose DAC (20 mg/m<sup>2</sup>/d) for 10 days, and the overall response rate increased to 64%. In addition, complete remission occurred in 52% of patients with normal karyotypes and 50% of patients with abnormal karyotypes. The median overall survival time and disease-free survival time significantly increased [9, 10]. DAC is especially suitable for the elderly who are not a candidate for high-dose therapy and the transplanted patients, and has become a first-line treatment for old AML patients [11, 12].

Although the therapeutic efficacy of DAC has been confirmed in some clinical studies, the role of DAC in the treatment of AML is still inconclusive. Long-term studies also indicate that DAC has no significant advantages on the removal of residual leukemic cells and the reduction of leukemia relapse over traditional chemotherapeutics. Hence, more studies are needed to confirm the anti-leukemia mechanisms of DAC to provide the theoretical basis for the clinical application of DAC.

In this study, human AML HL-60 cells were employed, and the effects of DAC on the *in vitro* growth and immunophenotype of HL-60 cells were investigated. Our results showed DAC not only significantly induced the apoptosis and proliferation of HL-60 cells, but increased the expression of NKG2D ligands of natural killer (NK) receptor on the HL-60 cells, leading to the elevated killing activity of NK cells to HL-60 cells *in vitro*. Moreover, the effects of DAC on the HL-60 cells were related to the inhibition of JAK/STAT3 signaling pathway.

### Materials and methods

#### Materials

DAC (Sigma, USA) was dissolved in DMSO (10 mmol/L), sterilized by filtration through a 0.22- $\mu$ m filter, and stored at -20°C for use. Fetal calf serum (FCS), horse serum, PRMI1640 and  $\alpha$ -MEM were obtained from Gibco. Antibodies against NKG2D ligands of human NK cell receptor (anti PE-MICA/B, PE-ULBP1, APC-ULBP2, and PE-ULBP3 marked with fluorescent), and the isotype control IgG1 conjugated with PE and APC were obtained from R&D System Company. Reagents for flow cytometry were obtained from the BD Company (BD Biosciences FACSCalibur). The Quantitative Determination Kit for BCA protein (BCA Protein Assay Kit, 71285-3) was obtained from Merck Chemical Technology Company (Shanghai), America. Antibodies against STAT3, phospho-STAT3, JAK1, phospho-JAK1, JAK2, phospho-JAK2, SOCS, SOCS3 and actin were obtained from Cell Signaling Technology Company. Chemiluminescence Amersham ECL Plus<sup>TM</sup> Kit was purchased from GE Healthcare Company, America.

#### Cell culture

HL-60 cells (human acute granulocytic leukemia cell line) were obtained from Cell Resource Center, the Institute of Life Sciences, Chinese Academy of Sciences, and maintained in PRMI1640 including 10% FCS, 100 IU/mL penicillin and doxorubicin. NK92 cells (human NK cell line) were kindly provided by Prof. Ji KH from Xinguangwuhuoshi Hospital, Taipei, and grown in  $\alpha$ -MEM including 12% FCS, 100 U/mL IL-2, 100 IU/mL penicillin and doxorubicin. The density of HL-60 cells was adjusted to  $2 \times 10^5$ /mL with RPMI1640 including 10% FBS. Then, DAC was added at a final concentration of 0.2, 0.5 or 1  $\mu$ mol/L. Cells were maintained for 24 or 48 hr in 5% CO<sub>2</sub> at 37°C before measurements.

#### Measurement of cell proliferation by CCK-8 assay

After treatment with different concentrations of DAC (0.2, 0.5 and 1  $\mu$ mol/L) for 24 and 48 hr, HL-60 cell were incubated with CCK-8 (10  $\mu$ L per well) for 1-4 hr at 37°C. Then, the absor-

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bance was determined at 450 nm (reference wavelength at 600 nm). Meanwhile, the blank hole (zero hole) without cells was added with the same volume of cell culture medium and CCK-8, and the control well with cells was added with the same volume of PBS. The experiments were repeated thrice and cell viability was determined: cell viability (%) =  $(OD450_{\text{sample}} - OD450_{\text{blank control}}) / (OD450_{\text{control}} - OD450_{\text{blank control}})$ .

### *Detection of apoptotic cells by Annexin-V/PI double staining*

HL-60 cells were treated with different concentrations of DAC (0.2, 0.5 and 1  $\mu\text{mol/L}$ ) for 24 and 48 hr, and HL-60 cells without treatment served as a control group. Cells were collected, suspended in 100  $\mu\text{L}$  of 1 $\times$ Annexin binding buffer at  $1 \times 10^6/\text{mL}$ , and then mixed with 5  $\mu\text{L}$  of Annexin-V and 1  $\mu\text{L}$  of PI. In control group, 5  $\mu\text{L}$  of Annexin-V and 1  $\mu\text{L}$  of PI were also added. Incubation was done in dark for 15 min, and then 400  $\mu\text{L}$  of 1 $\times$ Annexin-binding buffer was added and mixed on ice in dark, followed by flow cytometry. The experiment was repeated at least thrice.

### *Detection of NKG2D ligands expression on NK cells by flow cytometry*

Cells in experiment group were collected, washed once with PBS, and suspended in 100  $\mu\text{L}$  of PBS. Then, anti-human NKG2D ligand antibodies (MICA/B, ULBP1, ULBP2 and ULBP3) conjugated with 4  $\mu\text{L}$  of fluorescein were added, followed by incubation in dark for 30 min at 4°C. Then, the cells were washed twice with precold PBS, and suspended in 100  $\mu\text{L}$  of PBS. The expression of antibodies conjugated with fluorescein on cells was determined by flow cytometry, and the values were expressed the medial fluorescence intensity (MFI). The isotype IgG1 (BD/Pharmingen) was used as a negative control. The experiment was performed at least thrice.

### *Determination of cytotoxicity of NK cells by CFSE*

Human NK92 cells were used as effector cells, and HL-60 cells as target cells. CFSE staining was performed as follows: well-conditioned HL-60 cells were centrifuged at room temperature for 5 min and washed twice with PBS, and then the cell density was adjusted to  $2 \times 10^5/$

mL. CFSE was added to the system at a final concentration of 5  $\mu\text{mol/L}$ . Then, the system was pipetted gently and incubated for 10 min at 37°C. Then, 5 times volume of cold RPMI 1640 was added, followed by incubation at 4°C for 5 min. The labeled cells were washed thrice and suspended in RPMI 1640, and the cell density was adjusted to  $2 \times 10^5/\text{mL}$ . Then, the effector cells and target cells were mixed at a ratio of 5:1 or 10:1. After incubation at 37°C for 4 hr, cells were collected, followed by washing twice with PBS. After addition of 5  $\mu\text{L}$  of PI (50  $\mu\text{g}/\text{mL}$ ), incubation was done at room temperature in dark for 15 min. Eventually, the cytotoxicity of by NK cells to HL-60 cells (CFSE+PI+HL-60) was determined by flow cytometry. The experiment was repeated thrice.

### *Detection of expressions of JAKs, STAT3 and SOCSs by Westetn blot assay*

Total protein was extracted from HL-60 cells and protein concentration was detected with BCA method. Then, 20  $\mu\text{g}$  of proteins was then loaded, separated by 10% SDS-PAGE and then transferred to a nitrocellulose membrane for 120 min at 400 mA. The membrane was blocked with 5% non-fat milk and incubated with antibodies against SOCS1, SOCS3, phosphor-JAK, phosphor-STAT3 and Actin independently. All the bands were visualized with horseradish peroxidase-conjugated secondary antibodies by using the enhanced chemiluminescence system. Then, quantification of protein bands was done with Image J software.

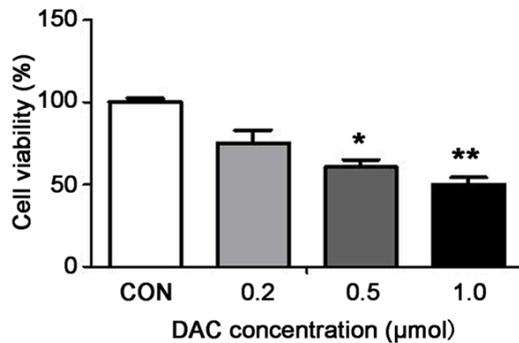
### *Statistical analysis*

Statistical analysis was performed with SPSS 16.0 statistical software. Data are presented as mean  $\pm$  standard deviation from at least three experiments. One-Way analysis of variance (ANOVA) was employed for the comparisons between groups. A value of  $P < 0.05$  was considered statistically significant.

## **Results**

### *DAC inhibited HL-60 cells proliferation*

Results showed that DAC significantly inhibited the proliferation of HL-60 cells *in vitro*. After treatment with 0.2  $\mu\text{mol/L}$  DAC for 48 h, the viability was  $25 \pm 13\%$ , which slightly lower than that in control group. For cells treated with 0.5



**Figure 1.** Effect of DAC at the different concentrations on the proliferation of HL-60 cells. DAC (0.2, 0.5 and 1.0 µM) significantly inhibited proliferation of HL-60 cells after 48-h treatment. When compared with control group, the cell viability declined by 40.9±8% and 50±7% ( $P<0.01$ ) in 0.5 µM DAC group and 1.0 µM DAC group, respectively ( $P<0.05$ ). Data are reported as mean ± SD. \* $P<0.05$  vs control group; \*\* $P<0.01$  vs control group.

and 1.0 µmol/L DAC, the viability significantly declined by 40.9±8% and 50±7% ( $P<0.01$ ), respectively, as compared to control cells (**Figure 1**). These suggest that DAC inhibits the proliferation of HL-60 cells in a dose-dependent manner.

#### *DAC induced HL-60 cells apoptosis*

After treatment of DAC for 24 hr, the number of apoptotic cells remained unchanged regardless the concentrations of DAC. After DAC treatment for 48 hr, cells of early apoptosis were observable and the early apoptosis rate of HL-60 cells was 24.77±7.5% and 27.1±4.48% in 0.2 µmol/L group and 0.5 µmol/L group, respectively, which were significantly higher than in control group (3.11±0.12)%, ( $P<0.01$ ). However, the early apoptosis rate in 1.0 µmol/L group (30.53±3.93%) was comparable to 0.2 µmol/L group and 0.5 µmol/L group ( $P>0.05$ ) (**Figure 2**). It indicates that DAC induced HL-60 cells apoptosis was more closely related to the time of DAC treatment than the DAC concentration. Although the apoptosis rate enhanced with the increase in DAC concentration, DAC at a concentration of higher than 0.5 µmol/L may not further increase apoptosis rate.

#### *DAC increased NKG2DL expression on HL-60 cells*

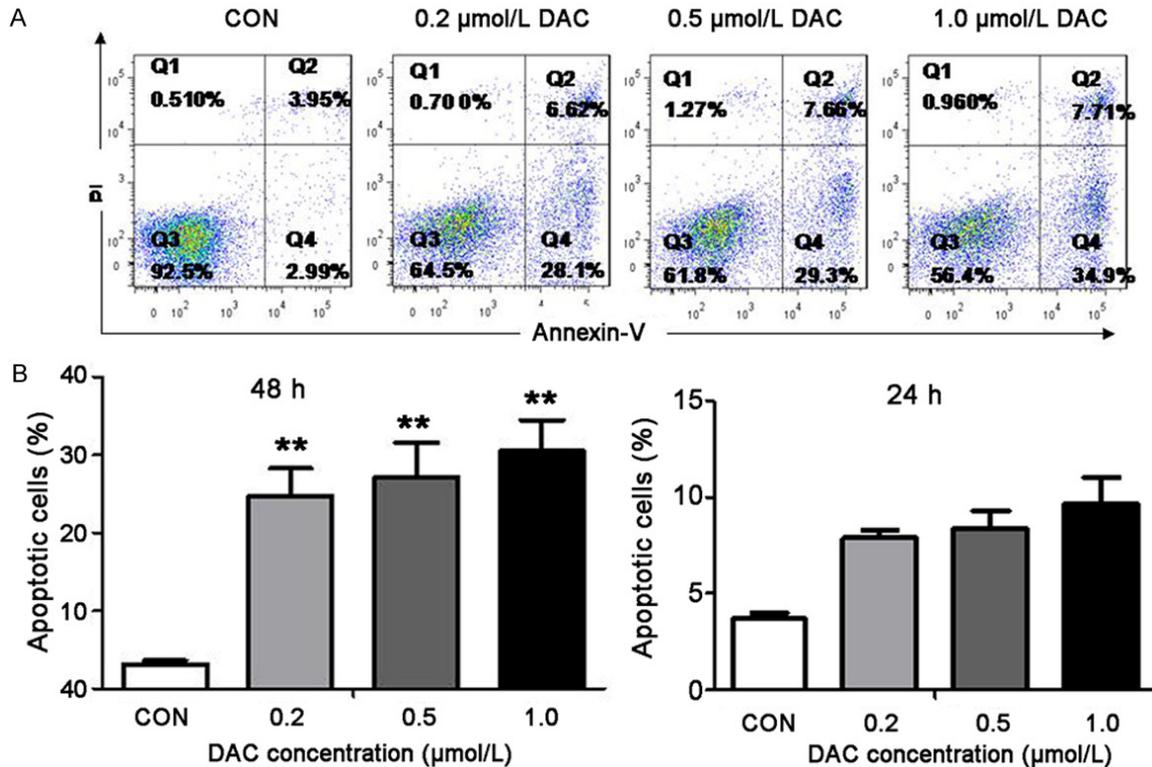
NKG2D is a main activated receptor on the NK cells. It can bind to relevant ligands on the tar-

get cells, and activate NK cells, by transducing activated signals to NK cells and inhibiting the inhibitory signals mediated by NK inhibitory receptor. Tumor cells often escape from immune surveillance of NK cells by reducing or disordering the expression of NKG2D ligand, and avoiding the recognition and killing by NK cells [13]. DNA methylation in the promoter region CpG plays an important role in the regulation of NKG2D and its ligand expression. Demethylation drugs may induce the expression of NKG2D and its ligand. To further investigate whether DAC is able to regulate the expression of NKG2D ligand in NK cells, the expression of four NKG2D ligands on HL-60 cells was detected by flow cytometry. Results showed DAC (0.2, 0.5, and 1.0 µmol/L) treatment for 24 hr failed to significantly change the expression of four NKG2D ligands (MICA/B, ULBP-1, ULBP2 and ULBP-3). However, after treatment for 48 hr, even in a low concentration (0.2 µmol/L), DAC significantly increased the expression of four NKG2D ligands. When compared with control group, the expression of MICA/B, ULBP-1 and ULBP-3 increased 189%, 162% and 180%, respectively ( $P<0.05$ ). Although the NKG2D ligand expression increased significantly, DAC at a higher concentration (1.0 µmol/L) failed to further increase the NKG2D ligand expression, but a slight decline (ULBP1 & ULBP3) was observed, which was consistent with previous findings that the apoptosis-inducing effect of DAC on HL-60 cells was dose-independent. The dose-dependent effect of DAC implies that DAC at different concentrations might work by different molecular mechanisms (**Figure 3**).

#### *DAC enhanced the cytotoxic sensitivity of HL-60 cells to NK cells*

To further investigate whether the up-regulated expression of NKG2D ligand is related to the cytotoxic sensitivity of HL-60 cells to NK cells, the killing activity of NK cells to HL-60 cells was determined by flow cytometry after 48-hr treatment with DAC at different concentrations. When the E:T was 5:1, the percentage of killed HL-60 cells was 7.93±4.41%, 21.37±6.94% and 24.7±5.65%, after treatment with 0.2, 0.5, 1.0 µmol/L DAC, respectively, which was significantly higher than in control group (6.59±2.66)% ( $P<0.01$ ). When the E:T was 10:1, the killing activity of NK cells remained unchanged. It indicates that DAC enhances the killing activity of

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**Figure 2.** Early cell apoptosis of HL-60 cells following DAC treatment. A. HL-60 cells were treated with different concentrations of DAC (0.2, 0.5 and 1.0 μM) and apoptotic cells were determined by flow cytometry. After DAC treatment for 48 hr, the early apoptosis rate of HL-60 cells was  $24.77 \pm 7.5\%$  and  $27.1 \pm 4.48\%$  in 0.2 μmol/L group and 0.5 μmol/L group, respectively, which were significantly higher than in control group ( $3.11 \pm 0.12\%$ ) ( $P < 0.01$ ). Q1: Advanced apoptosis cells. Q2: Dead cells. Q3: Living cells. Q4: Early apoptotic cells. B. Cell apoptosis of HL-60 cells following DAC treatment. After 24-hr treatment, the number of apoptotic cells remained unchanged. After 48-hr treatment, cells of early apoptosis were observable, but the early apoptosis rate was comparable among DAC groups ( $P > 0.05$ ). Data are reported as mean  $\pm$  SD. \* $P < 0.05$  vs control group; \*\* $P < 0.01$  vs control group.

NK cells by increasing the NKG2D ligand expression on HL-60 cells (Figure 4).

### DAC inhibited the expression of molecules in JAK/STAT3 signaling pathway

STAT3 is an important participant in the JAK/STAT3 signaling pathway which has been found to be involved in the occurrence and development of leukemia and activated in leukemia cells [14]. STAT3 is mainly activated by cytokines such as interleukin 6 (IL-6), which plays an important role in the cell survival, proliferation, differentiation, as well as the development and differentiation of hematopoietic cells [15, 16]. Furthermore, STAT3 may as an important nuclear transcription factor to regulate the NKG2D ligand expression in cells. It has been reported that to inhibit the expression or activity of STAT3 in leukemia cells can enhance the expression of NKG2D ligand, MICA, on cells and

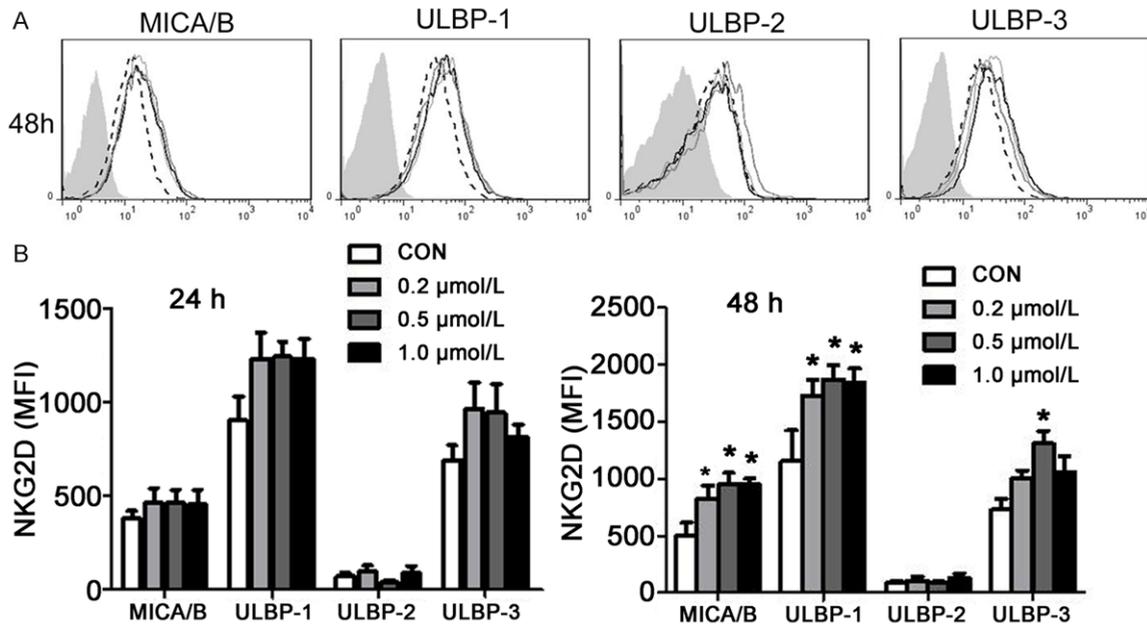
promote the recognition and killing by NK cells [17].

To investigate whether STAT3 is activated following DAC treatment, the expression of STAT3 and its upstream molecules JAKs kinase was detected by Western blot assay in HL-60 cells after DAC treatment for 48 hr. Results showed DAC reduced the protein expression and inhibited the phosphorylation (Tyr705 & Ser727) of STAT3 in HL-60 cells. In addition, DAC inhibited the expression and phosphorylation of JAK1 and JAK2. These findings suggest that DAC significantly suppress the phosphorylation of STAT3 via inhibiting the upstream JAKs kinases of STAT3 (Figure 5).

### DAC increased the SOCSs protein expression

SOCS is a cytokine signal transduction inhibitory protein family and can negatively regulate the signal transduction activated by many cyto-

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**Figure 3.** DAC up-regulates NKG2DL expression on HL-60 cells. The expression of four NKG2DL on HL-60 cells after DAC treatment was determined by flow cytometry. A. After DAC treatment for 48 hr, the expression of MICA/B, ULBP-1 and ULBP-3 increased 189%, 162% and 180% in 0.2  $\mu\text{mol/L}$  group compared with control group, respectively ( $P < 0.05$ ). DAC at a higher concentration (1.0  $\mu\text{mol/L}$ ) failed to further increase the NKG2D ligands expression. Shadow: IgG CON; Black dotted line: vehicle treated cells; Black solid line; 0.2  $\mu\text{M}$  DAC; grey solid line: 0.5  $\mu\text{M}$  DAC. B. The expression of NKG2DL after DAC treatment. After 24-hr DAC treatment (0.2, 0.5, and 1.0  $\mu\text{mol/L}$ ), the expression of NKG2D ligands remained unchanged. After treatment for 48 hr, the expression of NKG2D ligands remained unchanged in 1.0  $\mu\text{M}$  group, but significantly increased in other groups. \* $P < 0.05$  vs control group; \*\* $P < 0.01$  vs control group.

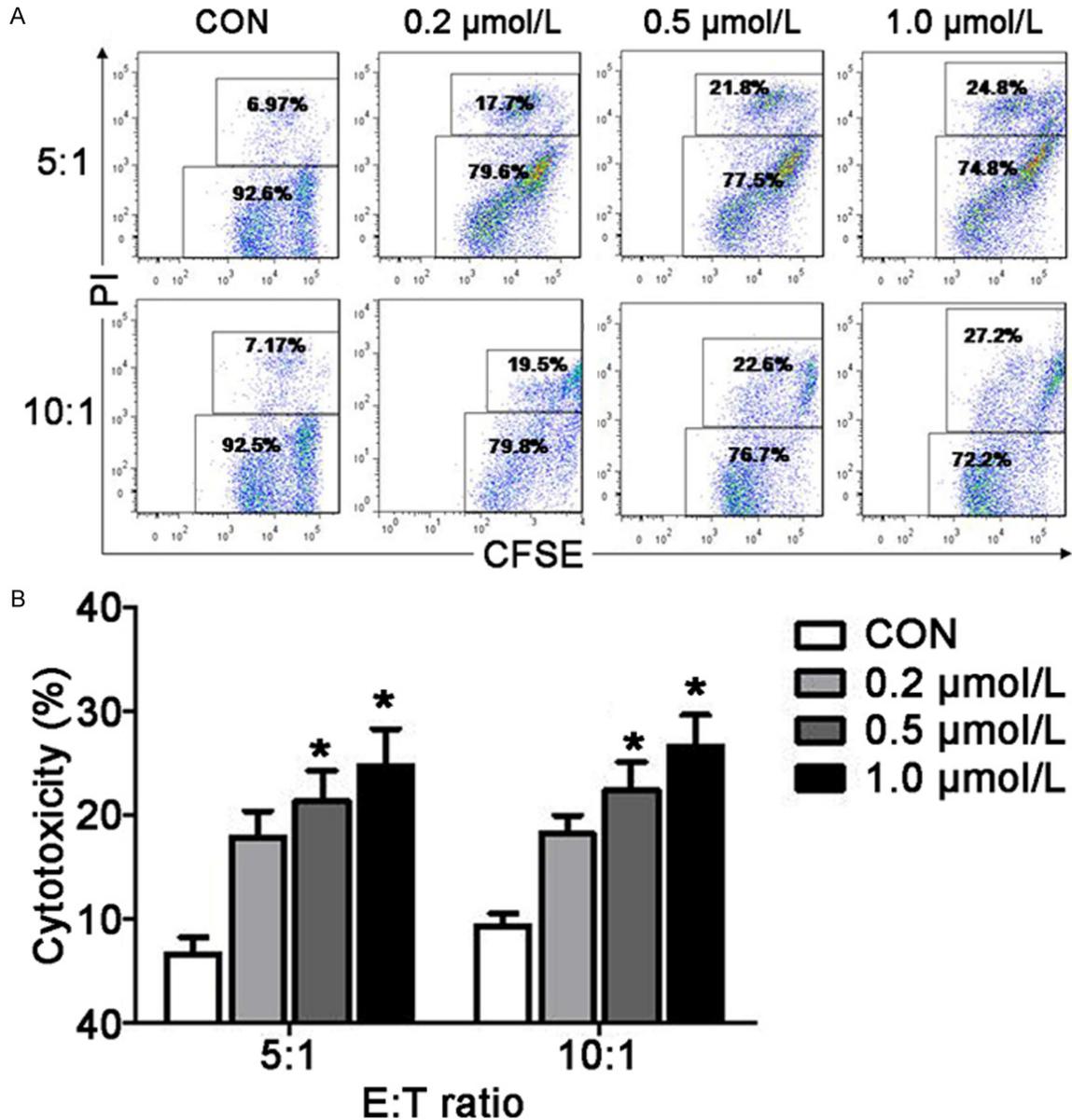
kine or inflammatory factors. SOCSs family includes 8 members, CIS and SOCS-1-7, of which SOCS-1 and SOCS-3 have a specific negative feedback inhibitory effect on STAT3 activity [18]. To further investigate the molecular mechanism of DAC induced inhibition on STAT3 activity, the protein expression of SOCS-1 and SOCS-3 was detected by Western blot assay in HL-60 cells. Results showed, after DAC treatment for 48 hr, the expression of SOCS-1 and SOCS-3 significantly enhanced (**Figure 6**).

The SOCS family mainly functions to inhibit intracellular cascade reactions mediated by cytokines, and mediate the inhibition of JAK and STAT phosphorylation, through binding to the SH2 domain of protein molecules and JAK kinase in cells or to the phosphorylated tyrosine residues of cytokine receptors. Under normal circumstances, the activation of STAT3 can initiate the SOCS expression which avoids the constitutive activation of STAT3 to a certain extent. However, in malignant tumors, STAT3 is constitutively activated, but SOCS is not activated as a feedback as in normal cells [19]. For instance, STAT3 in multiple myeloma cells can

be constitutively activated under the action of autocrine loop circuit of IL-6/IL-6R. In addition, the expression of SOCS1 and SOCS3 in cells is at a low level, and thus the normal apoptosis may not be initiated. In this study, our results showed that the expression of phosphorylated JAK1, JAK2 and STAT3 in HL-60 cells declined after DAC treatment, but that of SOCS-1 and SOCS-3 significantly increased, which suggest that SOCSs are involved in the effect of DAC on STAT3 activity, and DAC might inhibit the STAT3 upstream JAK activity by inducing SOCS1 and SOCS3 expression, which finally leads to the phosphorylation inhibition of STAT3.

### Discussion

DAC, as a specific DNA methyltransferase inhibitor, is a 2-deoxycytidine analogue, and can be incorporated into genomic DNA and reverse the DNA methylation. This may inhibit the oncogenes that are activated by methylation or activate tumor suppressor genes that are silenced due to methylation, which then induce the normal differentiation of cancer cells or induce the apoptosis of cancer cells [8, 20].

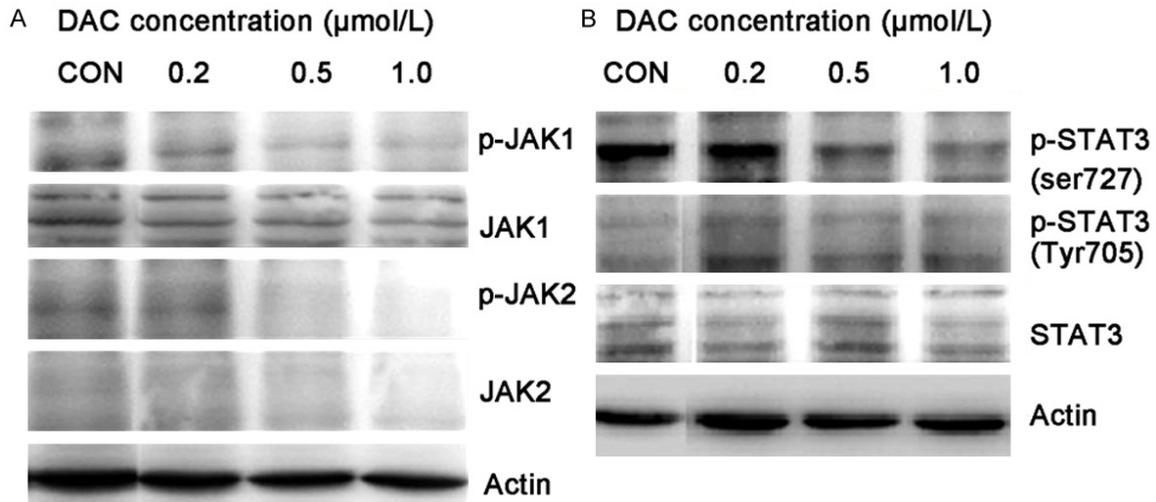


**Figure 4.** DAC enhances the cytotoxic sensitivity of HL-60 cells to NK cells. A. Flow cytometry of cytotoxic sensitivity of HL-60 cells to NK cells. B. Percentage of HL-60 cells killed by NK cells after DAC (0.2-1.0 μM) treatment. After DAC treatment for 48 h, the cytotoxic sensitivity of HL-60 cells to NK cells significantly enhanced. With the increase in E:T ratio (10:1), the cytotoxic sensitivity of HL-60 cells to NK cells remained unchanged. \*P<0.05 vs control group; \*\*P<0.01 vs control group.

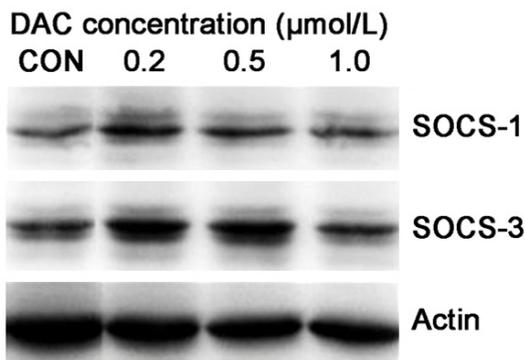
DAC was approved by the FDA in 2006 and has been proven to have a favorable therapeutic effect on adult acute leukemia and multiple myeloma [10-12]. In the present study, results showed that DAC displayed a significant inhibitory effect on the growth of human AML cell line (HL-60 cells) *in vitro*, and could increase the expression of activated receptor NKG2D of NK cell on HL-60 cells and further enhance the kill-

ing activity of NK cells. In addition, DAC was also able to induce the apoptosis of HL-60 cells and up-regulate the expression of NKG2D ligand in a dose-dependent manner. At a low concentration (0.2 and 0.5 μmol/L), DAC increased HL-60 cells apoptosis and NKG2D ligand expression with the increase in DAC concentration, but DAC at a higher concentration (1.0 μmol/L) failed to further enhance HL-60

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**Figure 5.** DAC inhibits the activity of JAK/STAT3 signaling pathway in HL-60 cells. A. HL-60 cells were exposed to 0.2-1.0  $\mu\text{M}$  DAC for 48 h. Cell lysates were prepared and subjected to western blot assay for p-STAT3 (Tyr705 and Ser727) and total STAT3 protein. DAC treatment led to the dephosphorylation of STAT3 both at Tyr705 and Ser727, with moderate effect on total STAT3 protein. B. DAC also down-regulated the expression of p-JAK1 and p-JAK2 as well as total JAK1 and JAK2 in HL-60 cells. The protein expression of STAT3 and JAKs was normalized to that of  $\beta$ -actin and results are representative from three independent experiments.



**Figure 6.** Effects of DAC on the protein expression of SOCS1 & SOCS3. HL-60 cells were treated with DAC at 0.2 or 1.0  $\mu\text{M}$  for 48 h. Cells were collected and SOCS1 & SOCS3 protein expression was detected by Western blot assay. Results showed a significant increase in SOCS3 expression and moderate increase in SOCS1 expression after DAC treatment as compared to RPMI 1640 (vehicle) treated cells. Results are expressed as mean  $\pm$  SD.  $**P < 0.01$  vs control group.

cells apoptosis and NKG2D ligand expression. Some studies have reported that DAC at a high concentration is cytotoxic, and can inhibit DNA synthesis and induce cell death. However, a low concentration, DAC can competitively inhibit the covalent binding of intracellular cytosine and DNA methyltransferase, and inhibit the activity of DNA methyltransferase, an impor-

tant participant in the methylation [21]. Although the effect of DAC on the methylation of relevant genes was not studied in HL-60 cells, it can be concluded that the effect of DAC on HL-60 cells is mainly associated with the methylation of related genes in cells.

In recent years, studies reveal that, besides cytogenetic factors, epigenetic factors are also involved in the pathogenesis of AML [3-6]. Some studies have reported that there are epigenetics abnormalities such as DNA methylation and histone modification in more than 70% of AML patients [22], and 20.5% of patients with mutation of DNA methyltransferase gene (DNMT3A) in AML-M5 patients [23]. The expression of some important genes related to the pathogenesis of leukemia is silenced due to their DNA methylation, resulting in the malignant transformation of normal cells. The inhibition of DNA methylation and the activation of silenced genes may be used for oncotherapy. Consequently, epigenetics drugs, mainly the methylation drugs, are promising for the treatment for leukemia.

Further investigation of mechanisms showed that DAC significantly inhibited the phosphorylation of JAK and STAT3 in HL-60 cells, and increased the expression of SOCS1 and SOCS3, two negative feedback factors of JAK/STAT3

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pathway. These indicate that JAK/STAT3 pathway activated by cytokines may be a target of DAC. STAT3 signaling pathway can be activated by cytokines, growth factors, and other factors to act on specific DNA fragments in the nucleus, regulating the transcriptional expression of many target genes related to cell proliferation, differentiation and apoptosis. STAT3 can be quickly activated under normal conditions. The persistent activation of STAT3 is closely related to the abnormal proliferation and malignant transformation of cells [14, 15]. SOCS is a negative regulatory protein of cytokine signal transduction, and plays an important negative feedback role in the activation of JAK/STAT3 signaling pathway induced by many factors, such as leukemia inhibitory factor, interleukin, tumor necrosis factor, erythropoietin, etc. SOCS can be generated following the treatment of many cellular factors, and in turn, SOCS can inhibit STAT3 signaling pathway activated by these factors. For example, SOCS3 may bind to chemokine receptor to inhibit JAK kinase with coupled-receptor, inhibit the tyrosine phosphorylation of STAT3, and then take part in the regulation of survival, proliferation and apoptosis of tumor cells [18, 24, 25]. Hence, SOCS may act as a tumor suppressor gene.

Many studies show that the regulation of SOCS expression is related to the methylation state of promoter region CpG. Hypermethylation inactivation of SOCS-1 can cause abnormal activation of STAT3 and the related signal pathway, promoting the occurrence and development of tumors [26-28]. Isomoto et al found that there was hypermethylation in the SOCS-3 gene of human cholangiocarcinoma cells, which was not observed in normal cells. The demethylation drugs can significantly induce the protein expression of SOCS-3, inhibit the phosphorylation of STAT3, and finally induce cell apoptosis [29]. In this study, we speculate that, as a methylation inhibitor, DAC is more likely to activate SOCS expression by modifying the methylation of SOCS gene, which enhances the suppression of phosphorylated STAT3 expression. Thus, the reduced STAT3 activity finally inhibits the growth of HL-60 cells. However, other mechanisms are still likely involved in the anticancer activity of DAC besides its inhibitory effect on the specific DNA methyltransferase, and further studies are needed to confirm it.

NK cells can recognize cancerous cells and then kill them at initial stage of a tumor. Thus, NK cells play an important role in the anti-tumor immune surveillance [30]. Leukemia cells can escape from immune surveillance by avoiding the recognition and killing of NK cells through a series of mechanisms, which is a major cause of leukemia relapse. NK cells exert their biological activities mainly by regulating the balance between inhibition and activation signals following the binding of a specific receptor of effector cells to its ligand on target cells. The interaction between activated receptor NKG2D of NK cells and its ligand on the target cells is especially crucial. The lack of the function of NKG2D can not activate NK, and thus NK fail to recognize and kill tumor cells [13]. Our results showed that the expression of NKG2D ligand was at a low level in AML cells, thus the NK cells could not be activated, and finally tumor cells escaped from the immune surveillance of NK cells [31, 32]. Hence, to up-regulate the expression of NKG2D and its ligand in NK cell, and to induce the cytotoxic sensitivity of HL-60 cells to NK cells have become important strategies for the therapy of AML.

Some studies have reported that the expression of NK-cell receptor and its ligand is also regulated by methylation. For instance, the demethylation drugs can up-regulate the expression of NKG2D ligand MICB of tumor cells and improve the killing activity of NK cells [33-35]. DAC at a low concentration can up-regulate the expression of NKG2D and NKp44 on peripheral NK cells, and promote the IFN- $\gamma$  secretion of NK cells and their cytotoxic effect on target cells [36, 37]. However, the molecular mechanism by which the epigenetic drugs regulate the expression of related molecules of NK cells is still unclear. As shown in our study, DAC increased the expression of NKG2D ligand on HL-60 cells and improve the cytotoxic activity of NK cells against HL-60 cells, which might be related to the inhibition of STAT3 activity. According to the study of Bedel et al, NKG2D ligand, MICA, is a downstream target gene of STAT3. STAT3 can be region-specifically bind to the intranuclear promoter of MICA to regulate its transcription and expression [17]. As an important nuclear transcription factor in cells, STAT3 might be involved in the regulation of NK cells activity by regulating the transcription and expression of NKG2D ligand. In fact, our previ-

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ous study on CML also showed that, transfection of lentivirus expressing STAT3-siRNA and use of specific STAT3 inhibitor could markedly increase the expression of ULBP2, a NKG2D ligand, on K562 cells. It proved that the up-regulated expression of ULBP2 was associated with the inhibition of STAT3 activity (reported elsewhere). Therefore, STAT3 is speculated as an important molecular target of DAC in HL-60 cells. DAC induced demethylation can induce the expression of SOCS1 and SOCS3, inhibitors of STAT3, which increases the inhibition of STAT3 phosphorylation, promotes the apoptosis of HL-60 cells and up-regulates the expression of NKG2D ligand.

As discussed above, our study show that DAC can significantly inhibit the proliferation of human AML HL-60 cells, and induce their apoptosis. In addition, DAC induces the expression of NKG2D ligand on HL-60 cells, and increases the cytotoxic sensitivity of HL-60 cells to NK cells. The DAC induced apoptosis and up-regulation of NKG2D ligand expression in HL-60 cells are dependent on the DAC concentration. The above effects of DAC on HL-60 cell are found to be related to the inhibition of STAT3 signaling pathway. DAC might induce the expression of SOCS1 and SOCS3 via demethylating the promoter of SOCS1 and SOCS3, two inhibitors of STAT3, in HL-60 cells. Thus, the activity of cytokine mediated STAT3 signaling pathway is inhibited, leading to the HL-60 cells apoptosis and up-regulated expression of NKG2D ligand. It also implies that other mechanism might be also involved in the anti-leukemia effect of DAC besides the specific inhibition of DNA methyltransferase, and the final consequence is as a result of multiple mechanisms. To study molecular mechanisms of DAC related effects from different angles may provide new clues for further studies on its clinical application.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Lingdi Ma, Department of Laboratory Medicine, Huizhou No. 3 People's Hospital, Affiliated Hospital of Guangzhou Medical University, No. 1 Xuebei Street, Qiaodong Road, Huizhou 615000, China. Tel: +86 752 2359773; E-mail: malingdi\_cz@163.com

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