Am J Transl Res 2015;7(10):1838-1849
www.ajtr.org /ISSN:1943-8141/AJTR0014128

*Original Article*

# Matrine increases NKG2D ligand ULBP2 in K562 cells via inhibiting JAK/STAT3 pathway: a potential mechanism underlying the immunotherapy of matrine in leukemia

Xuzhang Lu[1], Zhichao Zhu[2], Lijia Jiang[2], Xiao Sun[3], Zhuxia Jia[1], Sixuan Qian[3], Jianyong Li[3], Lingdi Ma[4]

*[1]Department of Hematology, Changzhou NO. 2 People's Hospital, Affiliated Hospital of Nanjing Medical University, Changzhou 213000, China; [2]Laboratory Center, Changzhou NO. 2 People's Hospital, Affiliated Hospital of Nanjing Medical University, Changzhou 213000, China; [3]Department of Hematology, People Hospital of Jiangsu Province, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China; [4]Department of Laboratory Medicine, Huizhou NO. 3 People's Hospital, Affiliated Hospital of Guangzhou Medical University, Huizhou 615000, China*

Received August 7, 2015; Accepted October 11, 2015; Epub October 15, 2015; Published October 30, 2015

**Abstract:** Purpose: The study aimed to investigate the role of the JAK/STAT3 pathway in the matrine induced ULBP2 expression on the human chronic myelogenous leukemia K562 cells. Methods: K562 cells were cultured, and the relevant mRNA expressions were detected. Results: Matrine induced the expression of four NKG2D ligands on K562 cells, of which ULBP2 had the highest increase. After treatment with 0.8 mg/mL matrine for 24 h, the mean fluorescence intensity (MFI) of ULBP2 increased. After matrine treatment, the sensitivity of K562 cells to NK cell-mediated killing increased significantly. After treatment with 0.2, 0.5 and 0.8 mg/ mL matrine, the percentage of K562 cells killed by NK cells was significantly higher than that of untreated cells (29.2%) ($P<0.05$). Matrine significantly inhibit the protein expression of phosphorylated STAT 3 and JAK2. Matrine markedly inhibited the IL-6 expression of K562 cells, and antagonized the IL-6 mediated STAT3 and JAK2 phosphorylation. In addition, matrine enhanced the inhibitory effect of STAT 3 inhibitor on STAT 3 activity. The silencing of STAT expression and inhibition of STAT3 activity significantly up-regulated the ULPB2 expression. Matrine had no effect on the expression of IL-6R and gp130 on K562 cells, the mRNA expression of IL-6R and gp130 increased slightly and the sgp 130 in cell supernatant significantly increased. Conclusions: Our findings reveal IL-6 and IL-6 receptor-mediated JAK/STAT3 pathway is involved in the matrine induced up-regulation of NKG2D ligands ULBP2 on K562 cells. Matrine might inhibit IL-6 expression and then suppress the activation of IL-6 receptor-mediated JAK/STAT3 pathway.

**Keywords:** Matrine, K562 cells, NKG2D ligand, STAT3, NK cells, IL-6

## Introduction

Leukemia is a malignancy of the hematopoietic system and one of the top ten malignancies in China, ranking the leading cause of cancer related death in the juvenile. In the past decades, investigators are engaged in developing new molecule-targeted drugs for the leukemia treatment. Tumor biotherapies targeting the biological characteristics of leukemic cells have unique advantages on the clearance of residual leukemic cells, the leukemia relapse prevention and the improvement of survival rate, and have been a focus in the studies on the leukemia therapy [1, 2].

In the immunotherapy of cancers, human natural killer (NK) cells constitute the first line of anti-tumor immune defense because they has the advantages of non-MHC restricted killing of the tumor cells [3]. The cytotoxic activity of NK cells depends on the balance between inhibitory receptors (inhibitory killer immunoglobulin-like receptors, iKIRs) and activating receptors on them. The activating killer cell lectin-like receptor NKG2D is closely related to the recognition and killing of NK cells against target cells, and plays a key role in the regulation of NK cells activity. NKG2D-mediated activating signals may antagonize the inhibitory signals produced by the binding of killer inhibitory receptors (KIR)

to MHC Class I molecules, leading to the activation of NK cells to kill tumor cells. Any decrease or loss of NKG2D function is able to activate the inhibitory signaling pathway in NK cells which makes NK cells unable to recognize or kill tumor cells, resulting in tumorigenesis [4-6].

Although the interaction between NKG2D and its ligands can activate NKG2D and further initiate the anti-tumor immune response of NK cells, tumor cells are able to escape from the immune surveillance of NK cells through a series of mechanisms. The abnormal or lack of NKG2D ligand expression on the target cells is a main cause of their immune escape [7, 8]. NKG2D ligands mainly include MHC class I chain-related proteins A and B (MICA/B) and UL16 binding protein family (ULBPs). NKG2D ligands on the target cells are important for the NK cell activation, which determines the consequence of NK cell immunity. Down-regulated or loss of NKG2D ligand expression is frequently found in tumor cells, and thus they are unable to be easily recognized and killed by NK cells. Therefore, to increase the expression of NKG2D ligands on tumor cells and to promote the NKG2D-mediated anti-tumor immune response of NK cells may be promising for the leukemia biotherapy [9-11].

In recent years, it has been found that matrine, an extract of Chinese traditional medicine radix sophorae flavescentis, has favorable anti-tumor activity *in vivo* and *in vitro*. Multiple studies have confirmed that matrine is able to induce the differentiation of leukemia cells, inhibit their proliferation, suppress DNA synthesis in tumor cells and induce their apoptosis, and matrine in combination with commonly used chemotherapeutics can effectively reverse the drug resistance of leukemia cells [12, 13]. Our previous study revealed that matrine is able to increase the expression of NKG2D ligands on the human leukemia K562 cells and enhance the killing activity of NK cells against K562 cells but the specific molecular mechanism remains unclear.

STAT3 is a member of intracellular signal transducer and activator of transcription family. Under normal conditions, it plays an important role in the cell survival, proliferation and differentiation as well as the development and differentiation of hematopoietic cells. Constitutive activation or over-expression of STAT3 is often found in tumors, and aberrant expression and activation of STAT3 are closely related to the occurrence of leukemia [14, 15]. Studies have found that STAT3, as a transcription factor, is able to participate in the regulation of MICA (a NKG2D ligand) expression in leukemia cells and plays a role in the negative regulation of MICA transcription and expression. Inhibition of STAT3 expression or activity can induce the up-regulate MICA expression on cells, which promote recognition and killing of NK cells against tumor cells. Further studies confirm that there is a STAT3 binding site in the promoter region of MICA gene in the nucleus and STAT3 can specifically bind to it to inhibit the transcription and expression of MICA [16]. Our previous study indicated that matrine inhibited the phosphorylation of STAT3 and its upstream Janus protein tyrosine kinase (JAKs) in K562 cells, suggesting that STAT3 and STAT3-mediated signaling pathway are involved in regulation of matrine induced expression of ULBP2.

STAT3 is activated mainly through the interleukin 6 (IL-6) and its receptor (IL6-R)-mediated JAK/STAT3 pathway [17, 18]. After binding to IL-6R, the signals are transmitted via the gp130 into cells, leading to the aggregation and subsequent activation of JAK coupled to IL-6R due to autophosphorylation, which further catalyzes the phosphorylation of intracellular cytokine receptors. These phosphorylated molecules could act as “anchors” to recruit and phosphorylate STAT3 downstream molecules, resulting in their activation. As an important factor for the growth and survival of leukemic cells, IL-6 secreted by leukemiic cells via autocrine or paracrine can directly activate JAK/STAT3 pathway, resulting in the occurrence of leukemia [19, 20].

In this study, the role of IL-6 and IL-6-mediated JAK/STAT3 pathway in the matrine induced expression of NKG2D ligands was investigated in K562 cells, which will provide a basis for the clinical leukemia therapy with matrine.

## Materials and methods

### *Cells and reagents*

Human chronic granulocytic leukemia (CML) cell line K562 cells were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences Institute of Cell Resource Center. Human cell line NK 92 cells were kindly provided by Professor Ji KH , Shin

Kong Wu Ho-Su Memorial Hospital, Taipei. Matrine with 99.5% purity was purchased from the Institute of Plant and Chemical Development, Xi'an Botanical Garden, Shaanxi Provincial Academy of Sciences, and the concentration of matrine storage solution is 10 mg/mL. Fetal bovine serum (FBS), RPMI 1640 and α-MEM were purchased from the American Gibco Company. MICA/B-PE, ULBP1-PE, ULBP 2-APC, ULBP3-PE fluorescently-labeled antibodies and PE labeled isotype control IgG1 were the products of R&D System Company. IL-6R (FAB227F-025) and gp130-PerCP (FAB228C-100) fluorescently-labeled antibodies were the products of R&D System Company. CellTrace™ CFSE Cell Proliferation Kit (Carboxyflurescein diacetate, Succinimidyl ester, hydroxyl fluorescein double acetate, carboxyfluorescein succinimide ester) was purchased from the Hangzhou Lianke Biotechnology Co., Ltd. Annexin V-FITC Apoptosis Detection Kit was the product of American eBioscience Company. Reverse Transcription Kit (RevertAid™ First Strand cDNA Synthesis Kit, K1622) was the product of American Fermentas (MBI) Company. Marker DL2000 (3427Q) and PCR system were products of TaKaRa Company. Trizol Reagent (15596-026) for total RNA extract was the product of Invitrogen Company. Forward and reverse primers for IL-6 and STAT3 upstream and downstream molecules were from Shanghai Shenggong Company. RIPA Cell Lysis buffer (PP1901) was purchased from Beijing Bioteke Corporation. The Protease Inhibitor Cocktail (ab65621) was the product of Abcam, UK. BCA Protein Quantitative Kit was purchased from the Shanghai KangChen Bio-tech. Antibodies against STAT3 (9132), phosphorylated STAT3 (Tyr705) (9138), and STAT3 (Ser727) (9236) were the products of Cell Signaling Company, USA. STAT3 siRNA lentiviral (Lenti-STAT3 SiRNA) and negative control siRNA lentiviral (Lenti-NC) were prepared by the Invitrogen Shanghai. Human IL-6 (96-200-06-5) and STAT3 inhibitors (573103 STAT3 inhibitor VII) were purchased from the PeproTech Inc USA and Calbioche, Germany, respectively. IL-6 (70-E-EK1062), IL-6R (ELH-IL06SR-1) and gp130 (ELH-sgp130-1) ELISA kits were purchased from the MultiSciences Biotech Co., Ltd. China and Raybiotech Inc. USA, respectively. Chemiluminiscence Amersham ECL PlusTM Kit was purchased from the GE Healthcare USA. Reagents used for flow cytometry were the products of BD Company USA.

*K562 cell processing*

K562 cells in the logarithmic phase were collected and trypan blue staining was performed to determine the viable cells. The percentage of viable cells was ≥95%. The cell density was adjusted to 0.5-1.0×$10^5$/ml and cells were re-suspend it in PRMI 1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL reptomycin. Cells were seeded into 6-well plates and incubated. Then, matrine was added to cells at a final concentration of 0.2, 0.5 or 0.8 mg/mL and incubation was performed at 37°C in a humidified environment with 5% $CO_2$ for 24 h or 48 h. In IL-6 inhibitor group and STAT3 inhibitor group, IL-6 inhibitor (50 ng/L) and STAT3 inhibitor (5 μM) were added, respectively, followed by incubation for 6 h. The matrine was added, followed by further incubation. In negative control group, cells were maintained in RPMI 1640 of equal volume; in blank control group, there was mediam alone in each well. Experiemnt was performed in quadruplicate. At pre-designed time points, cells were harvested, total RNA and protein were extracted for RT-PCR and Western blot assay, respectively. The supernatant was collected and frozen at -20°C for further ELISA.

*Flow cytometry of NKG2D ligand*

K562 cells were treated with matrine or transfected with lentivirus. The cell density was adjusted to 1×$10^6$/ml and PE or APC conjugated anti-MICA/B, ULBP1, 2 or 3 were added. Incubation was done in dark for 30 min at 4°C. After washing twice with PBS, cells were re-suspended in 100 μL of PBS, followed by flow cytometry. The isotype IgG1 served as a negative control. Experiment was repeated thrice.

*Detection of NK cells cytotoxicity by CFSE/PI double staining*

K562 cells were washed in serum-free RPMI 1640 twice and the re-suspended in 500 μL of serum-free RPMI 1640. Then, 100 μL of CFSE fluorescent dye (50 μmol/L) was added, followed by incubation at 37°C for 15-20 min. Cells were collected and washed in PBS thrice. The number of cells was determined. NK cells served as effector cells. After CFSE staining, K562 served as target cells. NK cells were mixed with K562 cells at different effector-to-target ratios (5:1 and 10:1), followed by incubation for 4 h at 37°C. Cells were collected, cell density was adjusted to 5×$10^5$ cells/ml and

**Table 1.** Primers used in RT-PCR

| Gene | | Primer sequence | Product length (bp) |
|---|---|---|---|
| IL-6 | Forward | AAATTCGGTACATCCTCGACGG | 112 |
| | Reverse | GGAAGGTTCAGGTTGTTTTCTGC | |
| STAT3 | Forward | GTCAGATGC CAAATGC | 409 |
| | Reverse | CCTGGAGGCTTAGTGC | |
| IL-6R | Forward | AAATTCGGTACATCCTCGACGG | 112 |
| | Reverse | GGAAGGTTCAGGTTGTTTTCTGC | |
| gp130 | Forward | ACAACTCGTGTGGAAGACA | 215 |
| | Reverse | ATTTGCTCTCTGCTAAGTTC | |
| GAPDH | Forward | ACCACAGTCCATGCCATCAC | 450 |
| | Reverse | TCCACCACCCTGTTGCTGTA | |

then, cells were mixed with 5 μl of Annexin V-FITC and 10 μl of PI (20 μlg/mL). After incubation at room temperature for 10 min, flow cytometry was performed to determined the number of K562 cells killed by NK cells to the number of total K562 cells. Untreated K562 cells served as a control. Experiment was repeated thrice.

*Detection of mRNA expression of IL-6, IL-6R, gp130 and STAT3 in K562 cells by RT-PCR*

K562 cells were harvested and total RNA was extracted with TrIzol Reagent. cDNA was generated by using total RNA via reverse transcription. Two-step qRealtime-PCR was the performed to expand cDNA. $2^{-\Delta\Delta Ct}$ method was employed to calculate the mRNA expression of targeted genes. GAPDH served as an loading reference. Conditions used for PCR were as follows: pre-degeneration at 95°C for 3 min, 40 cycles of denaturation at 95°C for 25 s, annealing at 57°C for 25 s and extension at 72°C for 50 s and a final extension at 72°C for 5 min. Primers were as follows: IL-6: forward, 5'AAATTCGGTACATCCTCGACGG', reverse, 5'GGAAGGTTCAGGTTGTTTTCTGC'3, product length of 112 bp; gp130: forward 5'ACAACTCGTGTGGAAGACA'3 reverse, 5'ATTTGCTCTCTGCTAAGTTC'3 and product length of 215 bp; GAPDH: forward, 5'ACCACAGTCCATGCCATCAC'3, reverse, 5'TCCACCACCCTGTTGCTGTA'3 and product length of 450 bp (**Table 1**).

*Lentivirus mediated transfection of STAT3 siRNA in K562 cells*

The density of K562 cells was adjusted to $5.0\times10^5$/ml and cells were seeded into 12-well plates (1 ml per well). Lenti-STAT3 siRNA was suspended in RPMI 1640 containing 5% FBS at pre-designed concentrations. Cells were transfected with Lenti-STAT3 siRNA) at a multiplicity of infection of 50. Cells in control group were treated with negative control lentivirus (Lenti-NC). At the same time, polyamide was added at a final concentration of 8 μg/ml followed by incubation overnight at 37°C. Untransfected K562 cells served as a blank control. The cell state was observed on the second day after transfection, and cells were further grown for 24 h if there were no significant changes. Then, the medium was refreshed. Cells were collected after 5-day transfection. The transfection efficiency was determined by detecting NKG2D ligands expression via flow cytometry.

*Detection of STAT3 and JAK2 expression by western blot assay*

Cells were harvested at pre-designed time points and lysed with RIPA lysis buffer. Total protein was extracted, and protein concentration was determined by BCA protein assay. For the detection of total proteins and phosphorylated proteins, 20 μg and 50 μg of total proteins were subjected to 12.5% SDS-PAGE, respectively. Proteins were then transferred onto PVDF membrane which was then incubated at 4°C overnight. The membrane was treated with 5% non-fat milk and then incubated with monoclonal antibodies (1:500 or 1:2000) at 4°C incubation overnight. β-actin served as an internal control. After washing in TBST thrice, the membrane was treated with HRP-labeled secondary antibody (1:5000) at 37°C for 4 h. Protein bands were scanned and analyzed with Image J software after visualization with chemiluminiscence ECL kit.

*ELISA*

Contents of IL-6, soluble IL-6R and gp130 in the cell supernatant were determined by double-antibody sandwich ELISA according to the manufacturer's instructions.

*Statistical analysis*

Statistical analysis was performed with SPSS version 13.0. Data are expressed as mean ± standard deviation (SD; x±s). The comparisons of NKG2D ligands expression in K562 cells before and after treatments were performed

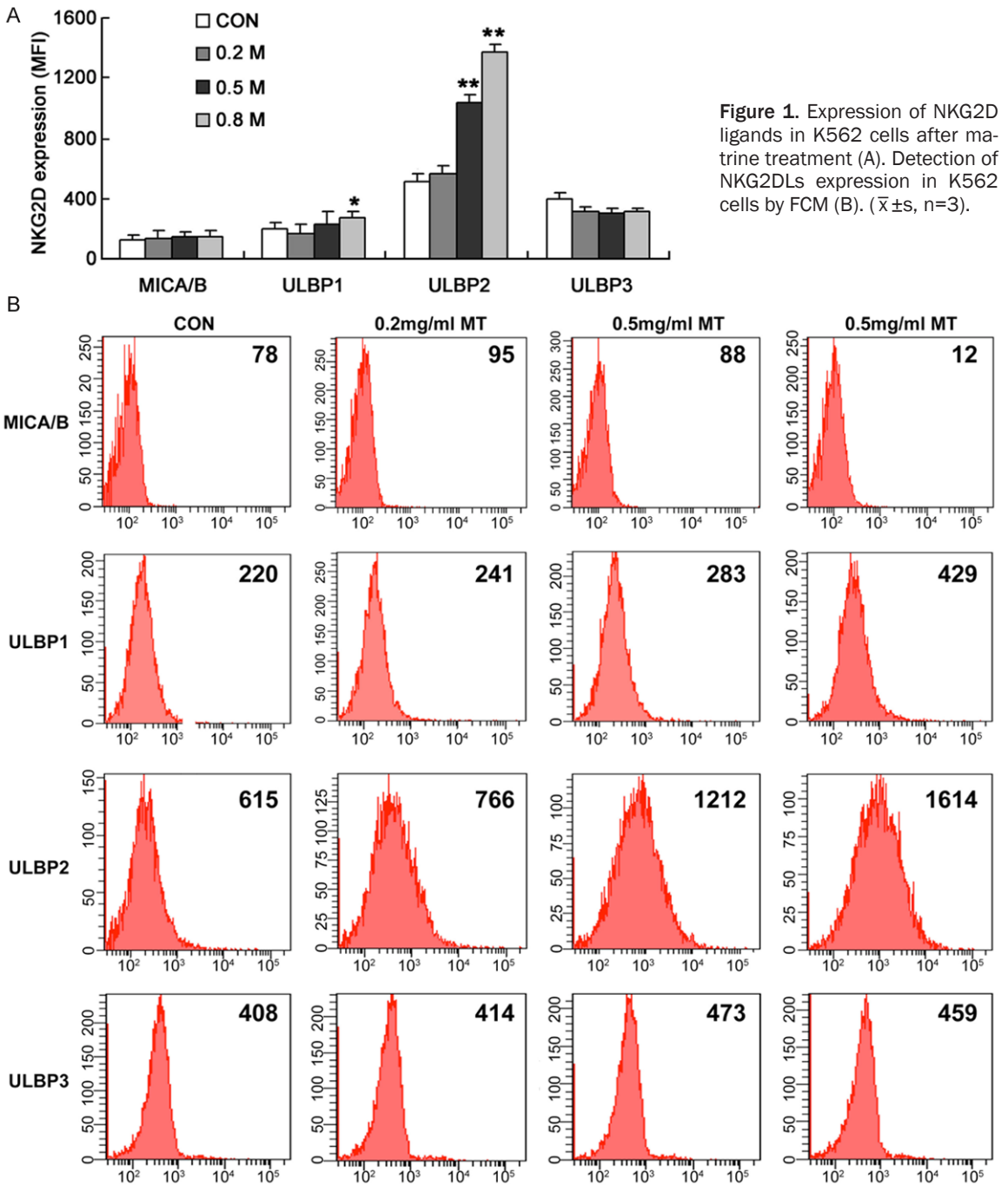


**Figure 1.** Expression of NKG2D ligands in K562 cells after matrine treatment (A). Detection of NKG2DLs expression in K562 cells by FCM (B). ($\bar{x}\pm s$, n=3).

with paired t test. A value of $P<0.05$ was considered statistically significant.

## Results

### *NKG2D ligands expression increases in NKG2D following matrine treatment in K562 cells*

The human CML cell line K562 cells were subjected to flow cytometry, and results showed K562 cells had MICA/B and ULBP1-3 expression. ULBPs expression was significantly higher than MICA/B expression. Matrine significantly increased the expression of ULBP1 and ULBP2, and the increase in ULBP2 expression was the highest. After matrine treatment for 24 h, no significant difference was observed in the mean fluorescence intensity (MFI) of MICA/B and ULBP3 on K562 cells as compared to that before treatment. ULBP1 and ULBP2 expression was significantly higher than in untreated

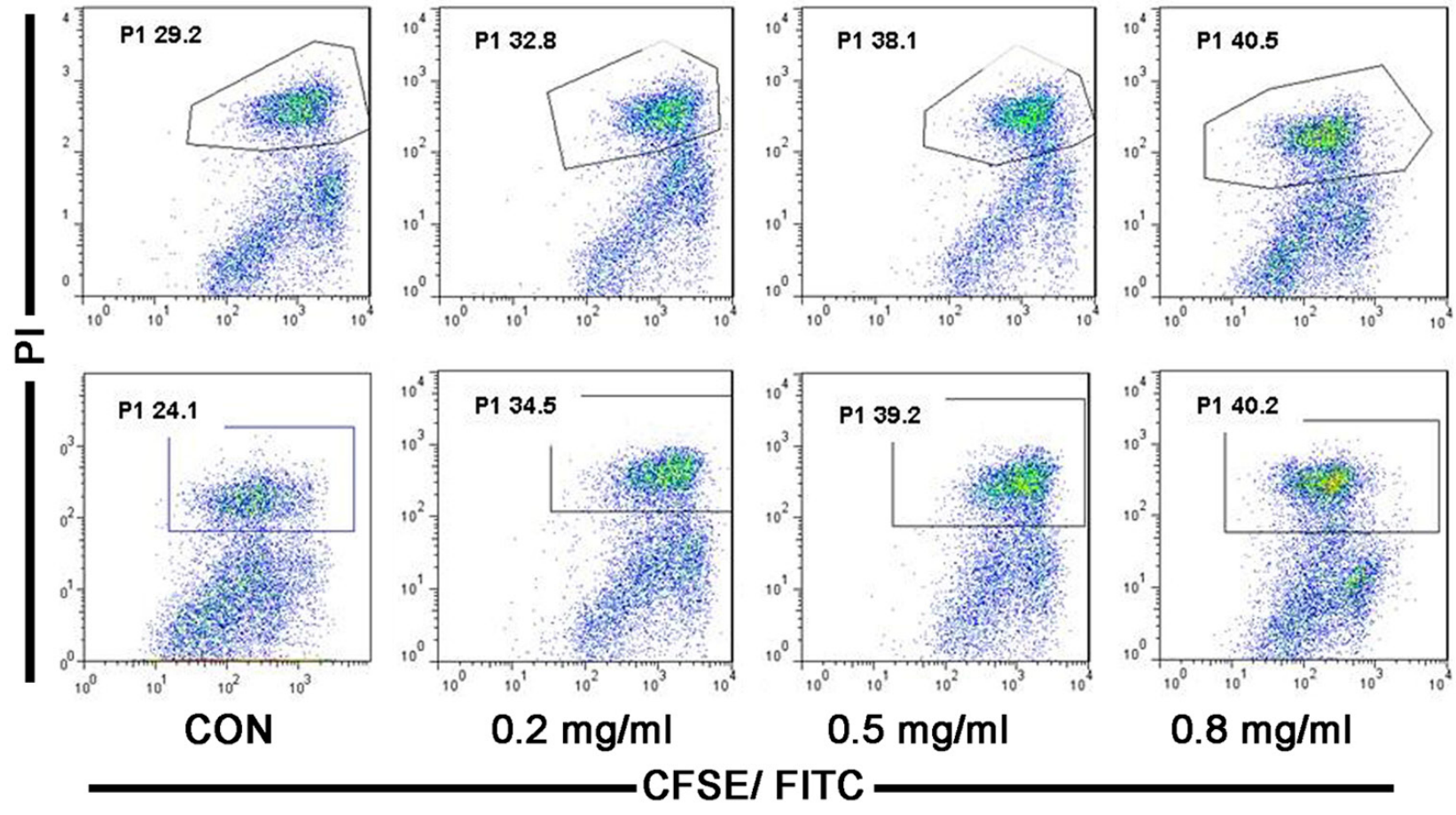


**Figure 2.** Matrine increases the cytotoxicity of NK cells against K562 cells (FCM).

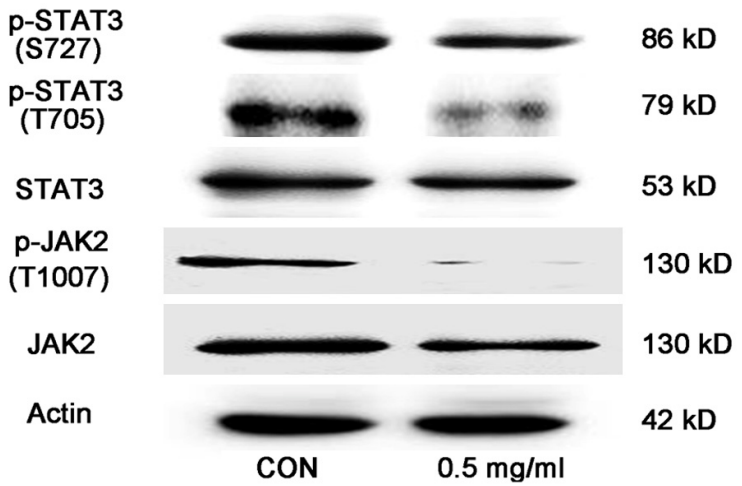


**Figure 3.** Matrine suppressed JAK/STAT3 pathway in K562 cells.

groups (1.95 and 2.65 times increase as compared to those before treatment), and this increase was dependent on matrine concentration (**Figure 1**).

*Matrine increases the cytotoxicity of NK cells against K562 cells*

At the effector-target ratio of 5:1, the percentage of K562 cells killed by NK cells after treatment with 0.2, 0.5 and 0.8 mg/mL matrine was 32.8%, 38.1% and 40.5% respectively, which were higher than that before treatment (29.2%). At the effector-target ratio of 10:1, this percentage increased from 24.1% to 34.5%, 39.2% and 40.2%, respectively. However, there was no significant difference in this percentage at a specific matrine concentration at two effector-target ratios ($p>0.05$) (**Figure 2**).

*STAT3 participates in the regulation of NKG2D ligands ULBP2 expression*

Western blot assay was employed to detect the STAT3 and p-STAT3 expression in K562 cells after matrine treatment. Following treatment with 0.5 and 0.8 mg/ml matrine for 48 h, the total STAT3 expression in the K562 cells remained unchanged as compared to that in untreated cells, but p-STAT3 (Tyr705 and Ser727) expression decreased significantly. The relative p-STAT3 expression in cells treated with 0.5 and 0.8 mg/ml matrine (0.18 and 0.23, respectively), was significantly lower than in control group (0.69). In addition, shows that the expression of p-JAK2, an upstream kinase of STAT, was also significantly inhibited (**Figure 3**).

K562 cells were transfected with lentivirus carrying STAT3 siRNA or K562 cells were treated with an STAT3-specific inhibitor (STAT3 inhibitor VII). The expression of ULPB2 significantly increased, and the MFI of K562 cells transfected with STAT3 siRNA increased by 8.2 times as compared to non-transfected cells. These findings confirm that the effect of matrine on the NKG2D ligands ULBP2 expression is related to the STAT3 inhibition (**Figure 4**).

*Matrine inhibits IL-6 expression in K562 cells*

Our results showed matrine significantly decreased the mRNA expression of IL-6 in K562 cells and IL-6 protein in the supernatant. Moreover, it also up-regulated NKG2D expression in K562 cells and inhibited IL-6 expression significantly. RT-PCR showed that, after treatment with 0.5 mg/mL matrine for 24 h, the IL-6 mRNA expression decreased significantly and the longer the matrine treatment or the higher the matrine concentration, the lower the IL-6 mRNA expression was (**Figure 5A**). ELISA further conformed that after treatment with 0.5 mg/mL matrine for 48 h, the IL-6 protein content decreased significantly in the cell supernatant as compared to that in control group ($P<0.05$) (**Figure 5B**).

*Effect of matrine on the IL-6 receptor expression*

Flow cytometry and ELISA were performed to investigate the effect of matrine on the expression of IL-6R and gp130 on cells and sIL-6R and sgp130 in cell supernatant. Results showed, after treatment with low concentration of matrine (0.5 mg/mL) for 48 hr, the IL-6R expression on cells remained unchanged, and after treatment with high concentration of matrine (0.8 mg/mL), the expression of IL-6R on cells changed significantly ($P<0.05$). After treatment with matrine for 24 or 48 h, the sIL-6R in the supernatant remained unchanged. Matrine had no effect on the gp130 expression on K562 cells, but it increased the sgp130 in the cell

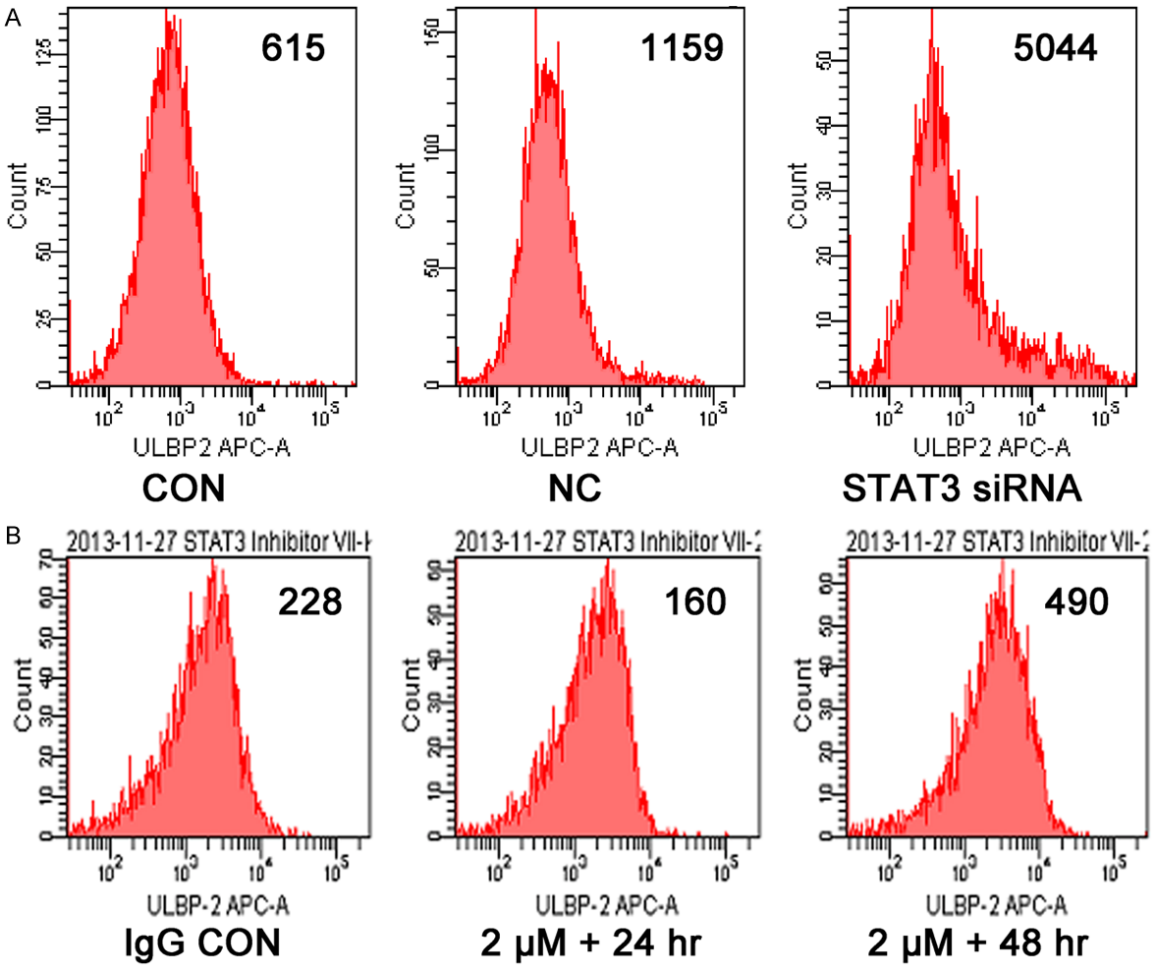


**Figure 4.** ULBP2 expression is significantly up-regulated in K562 cells after transfection with STAT3-siRNA lentivirus (A) or after treatment with 2.0 μM STAT3 inhibitor for 24 hr or 48 hr (B).

supernatant ($P<0.01$) and the higher the matrine concentration (0.8 mg/mL), the higher the sgp130 expression was. Total RNA was extracted And RT-PCR was used to detect the mRNA expression of IL-6R and gp130. Results showed IL-6R increased significantly ($P<0.05$) but gp130 mRNA expression increased slightly in K562 cells treated with matrine (**Figure 6**). These results suggest that the interaction between IL-6 and its receptor may be involved in the matrine-mediated regulation of JAK/STAT3 signaling pathway.

The role of IL-6 in the matrine induced regulation of JAK/STAT3 signaling pathway: STAT3 activation is dependent on the extracellular inflammatory mediator (IL-6)-mediated signal transduction. To investigate whether the inhibitory effects of matrine on JAK2 and STAT3 expression are related to IL-6, RT-PCR and ELISA were performed in K562 cells and results showed matrine could antagonize IL-6-induced STAT3 expression and JAK phosphorylation and enhance the inhibitory effect of STAT3 inhibitor on STAT3 and its upstream JAK2 (**Figure 7**).

## Discussion

Studies have confirmed that tumor cells expressing NKG2D ligands can activate NK cells via NKG2D-mediated signal transduction, leading to the killing of target cells. The cytotoxicity of NK cells is closely related to the NKG2D ligands expression on the target cells [9, 11]. The specific receptors of human NKG2D include MICA/B and ULBPs [4, 21]. Cells with malignant transformation undergoing rapid proliferation and some “stress-induced” cells (such as cells

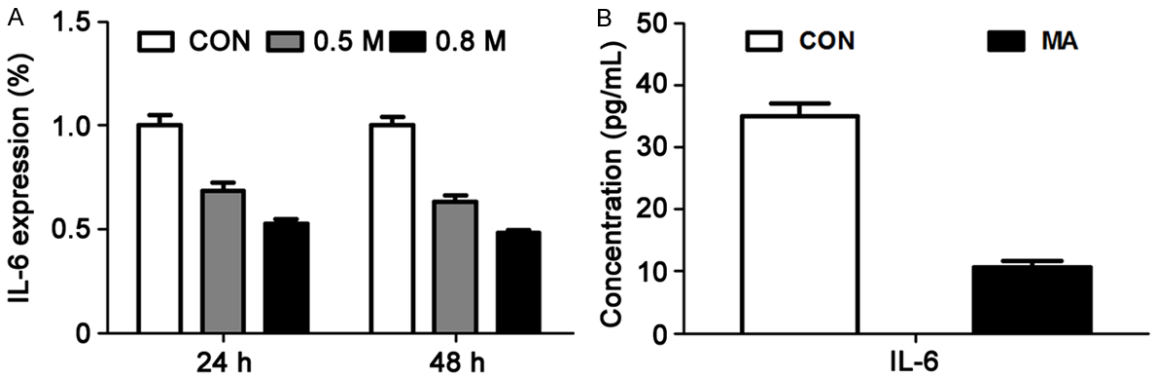


**Figure 5.** Detection of IL-6 expression in K562 cells by RT-PCR and ELISA after matrine treatment. A. Matrine decreases IL-6 mRNA expression in K562 cells. B. Matrine detection IL-6 content of K562 cells supernatant.

undergoing ionizing radiation, heat shock, viral infection and stimulation of other factors) often have a high NKG2D ligand expression. Most of cells in normal tissues have no or a low NKG2D ligand expression [22, 23]. The NKG2D ligand expression reduces in tumor cells, which prevents the recognition and killing by NK cells, and thus tumor cells can escape from the immune surveillance [24, 25]. However, many drugs and small molecules, such as valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, can induce the NKG2D ligand expression on tumor cells and enhance killing activity of NK cells against tumor cells [10, 26, 27].

Although there is no or low NKG2D ligand expression on cells in normal tissues, the transcription of NKG2D ligand genes is still present. However, NKG2D ligand at a low level is unable to activate NK cells. Therefore, it is hypothesized that there is a regulatory mechanism for the transcriptional or post-translational modification of NKG2D ligand, which plays an important role in the regulation of NKG2D ligand on cells, but the specific molecular mechanism is needed to be further studied.

In this study, our results showed that matrine significantly increased the expression of NKG2D ligands ULBP2 on CML K562 cells and enhanced killing activity of human NK cells against leukemia cells, which was related to the inhibition of STAT3 activity in cells. Both transfection of K562 cells with lentivirus expressing STAT3 siRNA and treatment of K562 cells with STAT3 inhibitor were able to increase the ULBP2 expression on these cells. It further confirms that STAT3 participates in the regulation of matrine induced NKG2D ligand expression. Studies have revealed that the inhibitory effect of matrine on the K562 cell proliferation is associated with a wide range of protein tyrosine kinases and tyrosine phosphatases, suggesting that matrine can affect intracellular protein tyrosine kinases [28]. Matrine has significant inhibitory effects on the expression of phosphorylated STAT3 and JAK2, key molecules in the JAK/STAT3 signaling pathway, but it has no significant effect on the total protein expression of STAT3 and JAK2, confirming that matrine plays a role via regulating the expression of phosphorylated STAT3 and JAK2. In addition, our results also showed that matrine significantly up-regulated the expression of ULBP1 and ULBP2, two ULBP family members, but had no significant effect on the MICA/B expression. ULBPs and MICA/B are encoded by different genes and have quite differences in the genetic composition, structure and tissue distribution. That matrine has different effects on different NKG2D ligands suggests that matrine has different biological effects on tumor cells of different sources.

IL-6 receptor plays an important role in the IL-6-mediated JAK/STAT3 pathway activation. IL-6 receptor system consists of IL-6 receptor binding protein (IL-6 binding receptor protein, IL-6R) and signal transduction proteins (signal-transduction protein) gp130 components.

Under physiological conditions, the binding of IL-6 to IL-6R changes the IL-6R conformation rapidly and this complex binds to two gp130 molecules promptly, which transduces cytokine signals to the cytoplasm through the gp130 subunits. Both IL-6R and gp130 have two

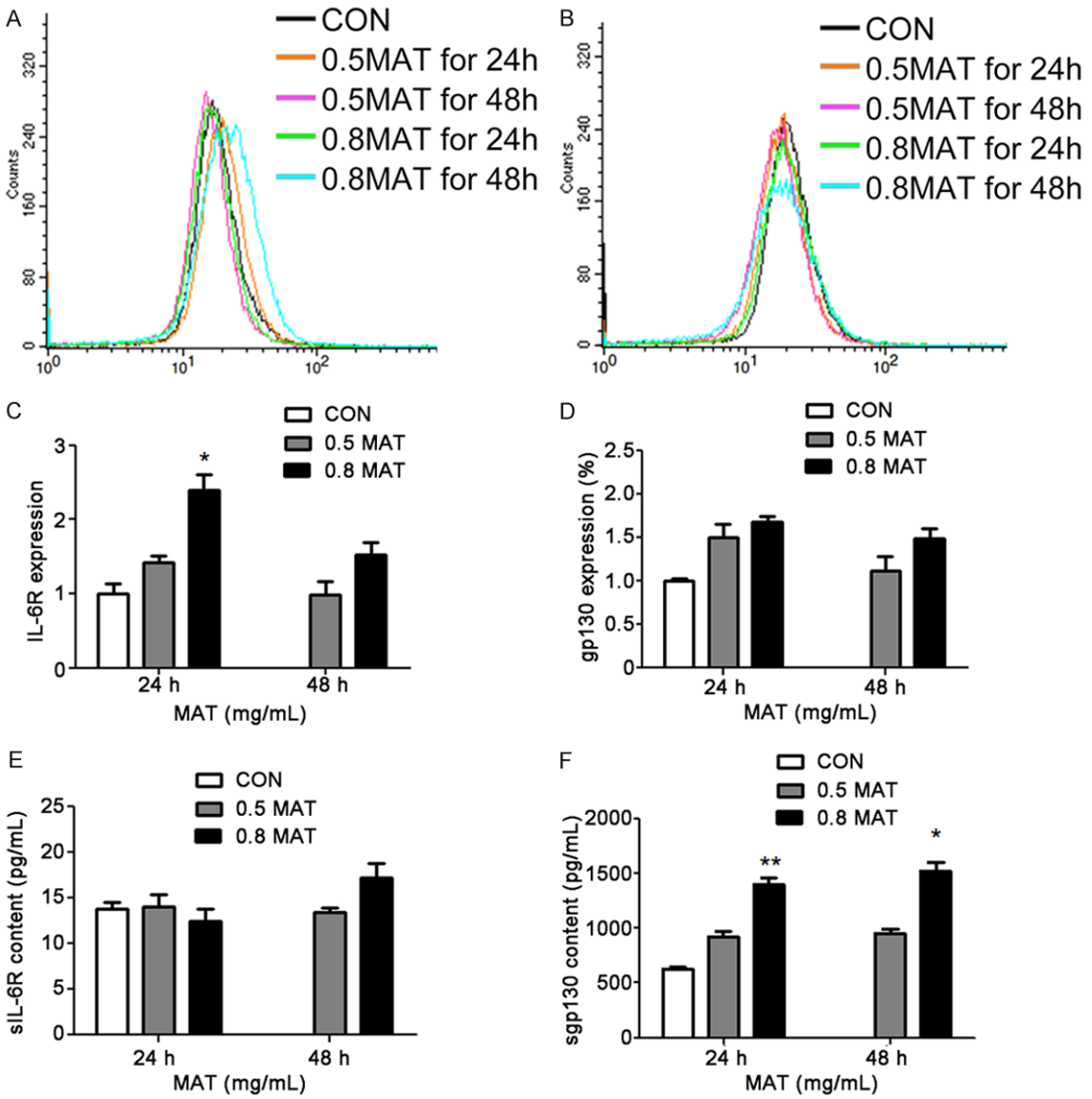


**Figure 6.** Detection of IL-6 receptor IL-6R and gp130 expression in K562 cells after matrine treatment. A, B. Detection of IL-6R and gp130 expression in K562 by FCM. After treatment with 0.5 mg/mL matrine for 48 hr, the gp130 expression on cells remained unchanged, while the expression of IL-6R significantly increased after treated with high concentration of matrine (0.8 mg/mL) ($P<0.05$). C, D. Detection of IL-6R and gp130 mRNA expression by RT-PCR. The IL-6R mRNA expression increased significantly ($P<0.05$) and gp130 mRNA expression increased slightly in K562 cells treated with matrine. E, F. Detection of soluble IL-6R and gp130 expression in the supernatant of K562 cells by ELISA. After treatment with different concentration of matrine for 24 or 48 h, the sIL-6R in the supernatant remained unchanged, while the expression level of sgp130 in the cell supernatant increased ($P<0.05$).

forms: a membrane-bound form and a soluble form. sIL-6R is mainly derived from the membrane IL-6R and can bind to gp130 on cells as the binding of membrane-bound IL-6R, mediating the IL-6R signals. sgp130 acts in a different way It inhibits the formation of IL-6R/IL-6 complexes and blocks IL-6 signal transmission in cells [29, 30].

To further investigate the roles of IL-6 and its receptors in the regulation of STAT activity, the effect of matrine on the expressions of IL-6 and IL-6R was investigated in K562 cells. Results showed that matrine significantly inhibited the expression and secretion of IL-6 in K562 cells, and slightly increase the expression of IL-6R and IL-6R on cells. Matrine has no significant

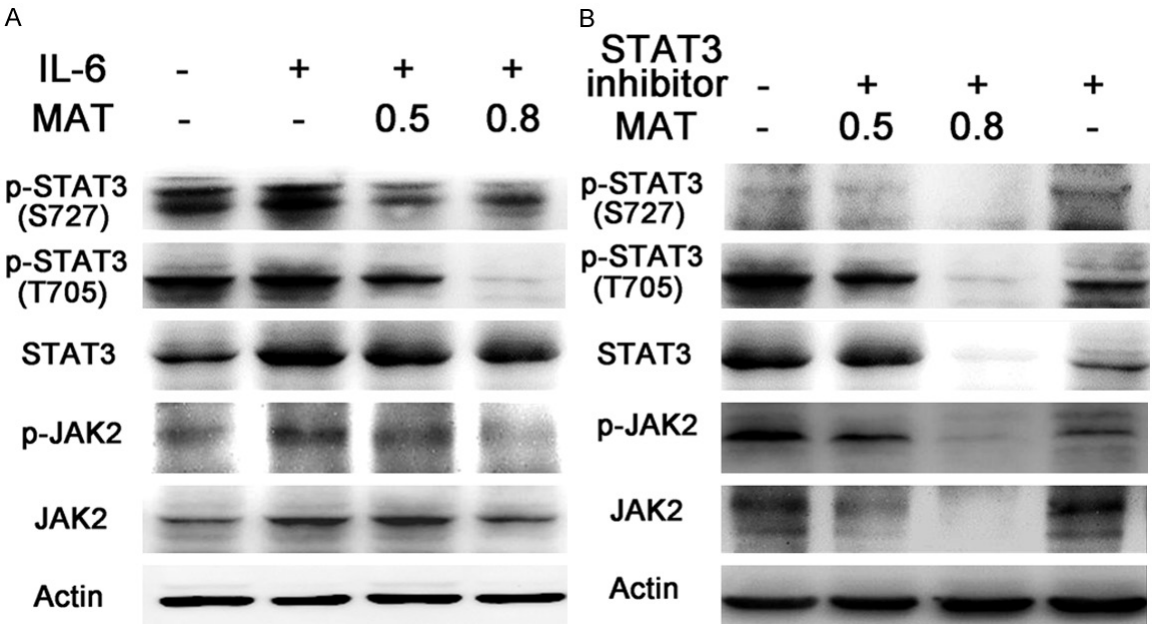


**Figure 7.** Effects of matrine on the expression of JAK2 and STAT3 in K562 cells in the presence of 50 ng/mL IL-6 (A) or 2 μM STAT3 inhibitor VII (B) (Western blot assay).

effect on the expression of gp130 mRNA and gp130 on cells. However, matrine increased the sgp130 in the cell supernatant. These results suggest that IL-6 and its receptor play a role in the matrine mediated regulation of JAK/STAT3 activity.

Our results indicate that matrine can induce the expression of ULBP2, a NKG2D ligand, in CML K562 cells and enhance the cytotoxicity of NK cells, which is related to the inhibition of IL-6 expression, inhibition of IL-6 mediated JAK/STAT3 signaling pathway activation, the up-regulation of ULBP2 expression and the increased cytotoxicity of NK cells. Our results provide useful evidence for further elucidation of mechanism underlying the therapeutic effect of matrine on leukemia and present a basis for the clinical leukemia treatment.

## Acknowledgements

This study was supported by the National Nature Science Foundation of China (No. 30171150) and Postdoctoral Research Program of Nanjing Medical University.

## 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

## References

[1] Chouaib S, Pittari G, Nanbakhsh A, El Ayoubi H, Amsellem S, Bourhis JH and Spanholtz J. Improving the outcome of leukemia by natural killer cell-based immunotherapeutic strategies. Front Immunol 2014; 5: 95.

[2] Shah N, Rezvani K, Hosing C, Kebriaei P, Wierda W, Cooper L and Shpall E. Progress in novel cellular therapy options for chronic lymphocytic leukemia: the M D Anderson perspective. Clin Lymphoma Myeloma Leuk 2014; 14 Suppl: S18-22.

[3] Lanier LL. NK cell recognition. Annu Rev Immunol 2005; 23: 225-274.

[4] Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 2003; 3: 781-790.

[5] Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL and Spies T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999; 285: 727-729.

[6] Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, Nanni M, Biassoni R, Bottino C, Moretta A and Moretta L. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors

of nonepithelial origin. Eur J Immunol 2001; 31: 1076-1086.
[7] Fuertes MB, Girart MV, Molinero LL, Domaica CI, Rossi LE, Barrio MM, Mordoh J, Rabinovich GA and Zwirner NW. Intracellular retention of the NKG2D ligand MHC class I chain-related gene A in human melanomas confers immune privilege and prevents NK cell-mediated cytotoxicity. J Immunol 2008; 180: 4606-4614.
[8] Nowbakht P, Ionescu MC, Rohner A, Kalberer CP, Rossy E, Mori L, Cosman D, De Libero G and Wodnar-Filipowicz A. Ligands for natural killer cell-activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood 2005; 105: 3615-3622.
[9] Champsaur M and Lanier LL. Effect of NKG2D ligand expression on host immune responses. Immunol Rev 2010; 235: 267-285.
[10] Rohner A, Langenkamp U, Siegler U, Kalberer CP and Wodnar-Filipowicz A. Differentiation-promoting drugs up-regulate NKG2D ligand expression and enhance the susceptibility of acute myeloid leukemia cells to natural killer cell-mediated lysis. Leuk Res 2007; 31: 1393-1402.
[11] Huergo-Zapico L, Acebes-Huerta A, Lopez-Soto A, Villa-Alvarez M, Gonzalez-Rodriguez AP and Gonzalez S. Molecular Bases for the Regulation of NKG2D Ligands in Cancer. Front Immunol 2014; 5: 106.
[12] Liu Y, Xu Y, Ji W, Li X, Sun B, Gao Q and Su C. Anti-tumor activities of matrine and oxymatrine: literature review. Tumour Biol 2014; 35: 5111-5119.
[13] Zhang LP, Jiang JK, Tam JW, Zhang Y, Liu XS, Xu XR, Liu BZ and He YJ. Effects of Matrine on proliferation and differentiation in K-562 cells. Leuk Res 2001; 25: 793-800.
[14] Benekli M, Baer MR, Baumann H and Wetzler M. Signal transducer and activator of transcription proteins in leukemias. Blood 2003; 101: 2940-2954.
[15] Lin TS, Mahajan S and Frank DA. STAT signaling in the pathogenesis and treatment of leukemias. Oncogene 2000; 19: 2496-2504.
[16] Bedel R, Thiery-Vuillemin A, Grandclement C, Balland J, Remy-Martin JP, Kantelip B, Pallandre JR, Pivot X, Ferrand C, Tiberghien P and Borg C. Novel role for STAT3 in transcriptional regulation of NK immune cell targeting receptor MICA on cancer cells. Cancer Res 2011; 71: 1615-1626.
[17] Heinrich PC, Behrmann I, Muller-Newen G, Schaper F and Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 1998; 334: 297-314.
[18] Kamimura D, Ishihara K and Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev Physiol Biochem Pharmacol 2003; 149: 1-38.
[19] Yeh HH, Lai WW, Chen HH, Liu HS and Su WC. Autocrine IL-6-induced Stat3 activation contributes to the pathogenesis of lung adenocarcinoma and malignant pleural effusion. Oncogene 2006; 25: 4300-4309.
[20] Grivennikov S and Karin M. Autocrine IL-6 signaling: a key event in tumorigenesis? Cancer Cell 2008; 13: 7-9.
[21] Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G and Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 2003; 374: 1-20.
[22] Eleme K, Taner SB, Onfelt B, Collinson LM, McCann FE, Chalupny NJ, Cosman D, Hopkins C, Magee Al and Davis DM. Cell surface organization of stress-inducible proteins ULBP and MICA that stimulate human NK cells and T cells via NKG2D. J Exp Med 2004; 199: 1005-1010.
[23] Cerwenka A and Lanier LL. NKG2D ligands: unconventional MHC class I-like molecules exploited by viruses and cancer. Tissue Antigens 2003; 61: 335-343.
[24] Watzl C. The NKG2D receptor and its ligands-recognition beyond the "missing self"? Microbes Infect 2003; 5: 31-37.
[25] Ashiru O, Boutet P, Fernandez-Messina L, Aguera-Gonzalez S, Skepper JN, Vales-Gomez M and Reyburn HT. Natural killer cell cytotoxicity is suppressed by exposure to the human NKG2D ligand MICA*008 that is shed by tumor cells in exosomes. Cancer Res 2010; 70: 481-489.
[26] Vales-Gomez M, Chisholm SE, Cassady-Cain RL, Roda-Navarro P and Reyburn HT. Selective induction of expression of a ligand for the NKG2D receptor by proteasome inhibitors. Cancer Res 2008; 68: 1546-1554.
[27] Poggi A, Catellani S, Garuti A, Pierri I, Gobbi M and Zocchi MR. Effective in vivo induction of NKG2D ligands in acute myeloid leukaemias by all-trans-retinoic acid or sodium valproate. Leukemia 2009; 23: 641-648.
[28] Liu BZ, Jiang JK, He YJ, Zhang Y and Liu XS. [Effects of matrine on the activity of protein tyrosine kinase and phosphatase in K562 cells]. Ai Zheng 2002; 21: 1292-1295.
[29] Sutherland CL, Chalupny NJ, Schooley K, VandenBos T, Kubin M and Cosman D. UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. J Immunol 2002; 168: 671-679.
[30] Radtke S, Wuller S, Yang XP, Lippok BE, Mutze B, Mais C, de Leur HS, Bode JG, Gaestel M, Heinrich PC, Behrmann I, Schaper F and Hermanns HM. Cross-regulation of cytokine signalling: pro-inflammatory cytokines restrict IL-6 signalling through receptor internalisation and degradation. J Cell Sci 2010; 123: 947-959.