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The IRF2-INPP4B pathway is activated in CD4$^+$ T cells and aggravates acute myeloid leukemia development by inhibiting apoptosis

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Abstract

Background: Interferon-regulatory factor 2 (IRF2)/inositol polyphosphate 4-phosphatase B (INPP4B) is essential for the differentiation of immune T cells. However, the regulatory mechanism of IRF2/INPP4B signaling pathway on apoptosis of acute myeloid leukemia (AML) cells remains unclear. Thus, this study intended to investigate the function and mechanism of IRF2/INPP4B in the development of AML.

Methods: CD4$^+$ T cells were isolated from peripheral blood and identified using flow cytometry. Apoptosis of AML cell line HL60 and the ration of Th1/Th2 were analyzed by flow cytometry. The mRNA expression of IRF2 was detected by quantitative real-time PCR method. Western blot was used to detect the protein accumulation of IRF2, INPP4B, JAK2, p-JAK2, STAT3, p-STAT3, and caspase 3. The contents of IL-4 and IFN-γ were measured by enzyme-linked immunosorbent assay (ELISA) kits.

Results: We found that IRF2 and INPP4B were highly expressed in AML-derived CD4$^+$ T cells. Further results indicated that CD4$^+$ T cells promoted HL60 cell apoptosis. Moreover, we found that downregulation of IRF2 promoted HL60 cell apoptosis by regulating Th1/Th2 ratio. In addition, we revealed that overexpression of IRF2 activated JAK2/STAT3 signaling pathway and downregulated caspase 3 expression.

Conclusion: We demonstrated that the IRF2-INPP4B signaling in CD4$^+$ T cells activated the JAK2/STAT3 signaling pathway and downregulated caspase 3 expression, causing inhibition on AML cell apoptosis to aggravate AML development. This study proposes a novel regulatory mechanism in AML development, suggesting that the IRF2/INPP4B pathway might influence the JAK2-STAT3 signaling pathway, adding a new layer to our understanding of the complex interplay of these pathways in AML development.

Key words: Acute myeloid leukemia; Apoptosis, IRF2-INPP4B; JAK2/STAT3 signaling pathway

Introduction

Acute myeloid leukemia (AML) is a hematological malignancy which has become the most common type of acute leukemia (AL) in adults[1]. It leads to an impaired hematopoietic function and bone marrow decline caused by abnormal proliferation of undifferentiated myeloid precursor cells. The disease progresses rapidly, and the rate of complete response and long-term survival is low[2] . Currently, chemotherapy, allogeneic and autologous bone marrow transplantation are three main treatments for AML[3]. However, bone marrow transplantation not only requires high costs but also suffers from immune rejection. In addition, although 50-75% of AML patients respond to chemotherapy, there is still a high recurrence rate after the operation. The poor prognosis and high recurrence rate of AML are the main reasons for treatment failure[4]. Therefore, it remains essential to further investigate the underlying mechanisms governing the onset and progression of AML as well as to identify novel and effective therapeutic strategies for its management.

Interferon-regulatory factors (IRFs) belong to transcription factors which were originally considered to be transcriptional regulators of the type I interferon system[5] . Studies have shown that IRFs can participate in the regulation of lymphocyte differentiation, cell growth, apoptosis and play an essential role in adaptive immunity and tumorigenesis[6]. Interferon-regulatory factor 2 (IRF2) a member of this family, acts as an antagonist of IRF1 and has been linked to tumor development[7] . IRF2 can stimulate the malignant proliferation of tumor cells and cause malignant transformation of tumors and has been classified as an oncogene in fibroblasts and T lymphocytes[8]. Therefore, the negative regulation of IRF2 expression may be a potential target for cancer treatment[9]. Notably, previous studies have indicated that IRF2 is involved in AML development, and may be a viable and specific therapeutic target in AML. Inositol polyphosphate 4-phosphatase B (INPP4B), a newly discovered lipid phosphatase, has been confirmed to play an essential role in various kinds of cancers[10-12]. INPP4B increases the proliferation and tumor growth of PIK3CA-mutant ER breast cancer cells[11]. A report indicated that INPP4B-directed mTORC1 signaling activation and cap-dependent translation could promote the proliferation of colorectal cancer cells[12] . Furthermore, INPP4B, as a prognostic and diagnostic marker, regulates cell growth of pancreatic cancer via activating AKT[13]. It has been indicated that the IRF2/INPP4B signaling pathway can promote the development of AML by promoting the proliferation and survival of AML cells[14], suggesting that inhibition of IRF2/INPP4B signaling pathway might be an important potential molecular target for AML treatment. Studies also confirmed that IRF2 participated in the process of CD8a dendritic cells through regulating Th2 cell differentiation[15], indicating that IRF2 plays an important role in the differentiation of immune T cells. However, the regulatory mechanism of IRF2/INPP4B signaling pathway on apoptosis of AML cells remains unclear.

JAK2, a member of the protein-tyrosine kinase family, is a crucial regulator of many physiological and pathological processes, including cell proliferation and differentiation[16]. Other signaling molecules, containing STAT1, STAT3, along with STAT5, are also regulated by JAK2. Specifically, the phosphorylation of STAT3 can be stimulated by JAK2, and the JAK2/STAT3 pathway plays a key role in human tumorigenesis by modulating cell proliferation, survival, differentiation as well as immune response [17]. In addition to tumor progression, several reports have indicated that the JAK2/STAT3 pathway is involved in AML development[18]. Recently, the study demonstrated that JARID2, a histone demethylase, plays a crucial role in breast cancer development and progression via regulating INPP4B and JAK2/STAT3 pathway[19]. However, whether IRF2/INPP4B takes part in AML via regulating the JAK2/STAT3 pathway is obscure. Therefore, this study intended to investigate the mechanism of IRF2/INPP4B in the development of AML.

Materials and methods

Reagents

JAK2 inhibitor AG-490 was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). STAT3 inhibitor Stattic was purchased from MedChemExpress (New Jersey, USA).

Clinical sample collection and $CD4^+$ T cell isolation

We collected a total of 20 peripheral blood samples at the hospital in 2021. Among them AML patients (n=10) and healthy individuals (n=10, normal group) The diagnostic criteria for AML refer to "2017 editions of the European LeukemiaNet (ELN) recommendations for diagnosis and management of acute myeloid leukemia (AML) in adults"[20]. $CD4^+$ T cells were isolated form peripheral blood by using a commercial kit $CD4^+$ T cell isolation kit II (HS-SJ078, Crondabio, Shanghai, China). Briefly, the fresh blood samples were collected in collection tubes, and peripheral monocytes were prepared by density gradient centrifugation. Subsequently, the magnetic-live cell classification and $CD4^+$ T cell separation kit was used to separate $CD4^+$ T cells from peripheral monocytes. And the separated $CD4^+$ T cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium with 10% fetal bovine serum (FBS), 1 mM L-Glutamine and 55 μM β-mercaptoethanol. This study was approved by the First Affiliated Hospital of Bengbu Medical College on June 25, 2021 under the approval number [2021] No.205. All experiments were performed in accordance with guidelines on animal care approved by the College, and all participants signed the informed consent form.

$CD4^+$ T identification

Flow cytometry was used to detect the purity of $CD4^+$ T cells. Briefly, the $CD4^+$ T cells (1×10^6 cells) was blocked with 2% FBS for 30 min in phosphate buffer saline (PBS) and then incubated with fluorochrome-conjugated anti-CD4 (1:100, abcam, Cambridge, UK, ab133616) for 30 min. Then the cells were analyzed by FACSVerse (BD Biosciences, Franklin Lakes, USA) and results were analyzed using FlowJo software (BD Biosciences, Franklin Lakes, USA).

Cell culture

HL60 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Gibco, Rockville, MD, USA) with 5% CO_2 and 95% air at 37 °C.

Cell treatment and transfection

2×10^5 cells were plated in a 6-well plate and seed cells to reach 70-90% confluency prior to transfection. Then the cells were transfected with 20μM of pcDNA3.1-IRF2 overexpressing vectors (Geenseed Biotech, Guangzhou, China) or 20μM of siRNA of IRF2 (Genechem, Shanghai, China) by sing the Lipofectamine 2000 Reagent (11668-027, Invitrogen) according to the manufacturer's protocol. To transfect cells, dilute four different amounts of Lipofectamine® Reagent (15μL) in 150μL of Opti-MEM® Medium. Simultaneously, dilute the desired amount of DNA (14μg) in 70μL of Opti-MEM® Medium. Combine the diluted DNA and Lipofectamine® Reagent at a 1:1 ratio, incubate for 5 minutes at room temperature, and then gently add the DNA-lipid complex to the cells which was further cultured for 48 h. The negative control (NC) sequence of SiRNA or pcDNA3.1 plasmid used as control. For $CD4^+$ T cells treatment, the HL-60 cells were cultured with $CD4^+$ T medium with different treatment. The sequences of SiRNA of IRF2 were as follows: si-IRF2-1, sense sequence: 5'-AGUUAAGCACAUCAAGCAAGA-3', antisense sequence: 5'-UUGCUUGAUGUGCUUAACUUU-3'; si-IRF2-2, sense sequence: 5'-GGUGAACAUCAUAGUUGUAGG-3'; antisense sequence:

5’-UACAACUAUGAUGUUCACCGU-3’; si-IRF2-3, sense sequence:
5’-GGUCCUGACUUCAACUAUAAA-3’ and antisense sequence:
5’-UAUAGUUGAAGUCAGGACCGC-3’.

Flow cytometry

For HL60 cells apoptosis analysis, the HL60 cells was cultured with $CD4^+$ T cells medium and then the apoptosis of HL60 cells was determined by flow cytometry[21]. HL60 cells cultured in RPMI-1640 medium act as control. Briefly, the HL60 cells was collected and washed for three times by PBS and then was staining with FITC Annexin V Apoptosis Detection Kit (HS-SJ069, Crondabio, Shanghai, China) at 4°C under darkness according to the manufacturer’s instruction. The cells apoptosis was detected by FACSVerse (BD Biosciences, Franklin Lakes, USA) and analyzed using FlowJo software (BD Biosciences, Franklin Lakes, USA). In addition, the proportion of Th1 cells ($INF\text{-}\gamma^+$) and Th2 cells ($IL\text{-}4^+$) in cells was determined by staining with INF-γ-PerCP-Cy5.5 antibody (Biolegend, San Diego, USA), PE-IL-4 antibody (Biolegend, San Diego, USA).

Quantitative real-time PCR

Total RNA from cells were separated by using TRIzol reagent (HS-SJ012, Crondabio, Shanghai, China) according to the manufacturer’s protocols. Then the cDNA was reversed transcription by the PrimeScript™ RT Master Mix kit (R223-01, Vazyme, Nanjing, China). And then the qRT-PCR was performed on LightCycler® 480II (Roche, Basel, Switzerland) with the ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme, Nanjing, China). The PCR protocol was as 95°C, 30 s; 40 cycles of 95°C, 10 s; 60°C, 30 s and 72°C, 20 s. The expression of the gens was calculated by $2^{-\Delta\Delta ct}$ methods[22] and β-actin mRNA was used as an internal. The primer sequences in the experiments were as follows: IRF2, forward, 5’-GTTGTAGGACAGTCCCATCT-3’, reverse, 5’-CTATCAGTCGTTTCGCTTT-3’; INPP4B, forward, 5’- GTGTCTGATGCTGACGCTAA-3’, reverse, 5’- AAATCGGAAATGCCAACG-3’; actin, forward, 5’-TGTGACGTGGACATCCGCAAAG-3’, reverse, 5’- TGGAAGGTGGACAGCGAGGC-3’.

Western blotting

The proteins from cells were isolated by RIPA buffer (HS-SJ011, Crondabio). And then the proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) (Beyotime, Shanghai, China) and subsequently were transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, USA). Then, the membrane was blocked with tris buffered saline tween (TBST; 1.5 mM Tris, 5 mM NaCl, 0.1% Tween20) containing 5% skim milk at room temperature for 1 h and incubated with specific primary antibodies anti-IRF2 (1:1000, abcam, Cambridge, UK, ab124744), anti-INPP4B (1:2000, abcam, Cambridge, UK, ab81269), anti-JAK2 (1:5000, abcam, Cambridge, UK, ab108596), anti-STAT3 (1:1000, abcam, Cambridge, UK, ab68153), anti-p-JAK2 (1:5000, abcam, Cambridge, UK, ab32101), anti-p-STAT3 (1:2000, abcam, Cambridge, UK, ab267373), anti-caspase 3 (1:2000, abcam, Cambridge, UK, ab32351) and anti-actin (1:2000, Serivicebio, Wuhan, China, GB12001) at 4°C overnight. And then the membrane was incubated with secondary antibody (1:4,000, biosharp, Hefei, China, BL003A) with horseradish peroxidase-conjugation for 1 h at room temperature. Finally, the bands of membranes were detected using enhanced chemiluminescence regent (WBKLS0100, Millipore, Billerica, USA). Actin was used as an endogenous control and grayscale values of the bands were analyzed by Image J software (Bio-Rad Laboratories, Hercules, USA). Changes in protein expression were quantified by analyzing the grayscale values of the target protein and actin bands.

Cytokine analysis by ELISA

$CD4^+$ T cell culture supernatants were collected and was detected by the IL-4 (E-EL-H0101c, Elabscience, Wuhan, China) and IFN-γ (E-EL-H0108c, Elabscience Wuhan, China) ELISA kits according to the manufacturer's protocols.

Statistical analyses

Statistical analysis was conducted by using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA) with at least three repeats. Data were presented as the mean ± standard deviation. Statistical significance was determined by using student's t test for two groups or one-way ANOVA for more than two groups. A p value <0.05 was considered statistically significant.

Results

IRF2 and INPP4B was highly expressed in the $CD4^+$ T cells isolated from AML

To investigate the function of IRF2 and INPP4B on the $CD4^+$ T cells, the $CD4^+$ T was isolated from peripheral blood of normal and AML patients. As shown in Fig. 1A, the $CD4^+$ T cells occupied 94.6% of all the isolated cells. And then the expression of IRF2 and INPP4B in $CD4^+$ T cells isolated from normal and AML patients was detected. Then, qRT-PCR revealed that a marked upregulation of *IRF2* and *INPP4B* mRNA level in $CD4^+$ T cells from AML patients compared to those from the normal group (Fig. 1B). In addition, western blotting confirmed a significant increase in IRF2 and INPP4B protein levels in $CD4^+$ T cells derived from AML patients relative to the normal group (Normalized to the control group, IRF2 and INPP4B protein expression in the $CD4^+$ T group was 2.06±0.1 and 2.11±0.13) (Fig. 1C). These results indicated that the expression of IRF2 and INPP4B was highly expressed in the $CD4^+$ T cells isolated from AML, suggesting that IRF2 and INPP4B might be involved in the development of AML.

$CD4^+$ T cells promoted HL60 cell apoptosis

To elucidate the role of $CD4^+$ T cells in the AML cells apoptosis, the isolated $CD4^+$ T cells was co-incubated with HL60 cells. Flow cytometry assay indicated that $CD4^+$ T cells could significantly promote the HL60 cell apoptosis compared with control group (The apoptosis rate in the control and $CD4^+$ T was 3.95%±0.74 vs 20.49%±1.13) (Fig. 2A). Since we had proved the expression of IRF2 and INPP4B was highly expressed in the $CD4^+$ T cells isolated from AML, we further explored whether the apoptosis induced by CD4+ T cells was associated with the modulation of IRF2 and INPP4B expression. We examined the expression of IRF2 and INPP4B in HL60 cells. Both qRT-PCR and western blot indicated that IRF2 and INPP4B expression was significantly decreased in the $CD4^+$ T group compared with control group at mRNA and protein level, respectively (Normalized to the control group, IRF2 and INPP4B protein expression in the $CD4^+$ T group was 0.57±0.04 and 0.51±0.22) (Fig. 2B and C). These results indicated that $CD4^+$ T cells promoted HL60 cell apoptosis related to the downregulation of IRF2 and INPP4B.

Downregulation of IRF2 promoted HL60 cell apoptosis by regulating Th1/Th2 ratio

We further investigated whether IRF2 regulates Th1/Th2 cell differentiation of $CD4^+$ T cells, loss and gain function of IRF2 on Th1/Th2 cell differentiation of $CD4^+$ T cells was investigated by transfection with overexpressing IRF2 plasmids and siRNA of IRF2. As shown in Fig. 3A, SiRNA-2 of *IRF2* showed the best knockdown effect on IRF2 in the $CD4^+$ T cells. Therefore, SiRNA-2 of IRF2 was used for further study. Further analysis indicated that the contents of IFN-γ were significantly increased in the Si-IRF2 group while decreased in the over-IRF2 group compared with their corresponding NC group. On contrary, the contents of IL-4 showed the opposite results in different group compared with IFN-γ (Fig. 3B). In addition, we found that knockdown of IRF2 significantly elevated that ratio of Th1/Th2 while overexpressing IRF2 reduced the ratio of Th1/Th2 (The ratio of Th1/Th2 in the control,

Si-NC, Si-IRF2, Over-NC and Over-IRF2 was 1.07±0.04 vs 1.08±0.04 vs 17.14±1.41 vs 1.07±0.03 vs 0.28±0.03) (Fig. 3C). To investigate the functional impact of Th1/Th2 ratio of $CD4^+$ T cells regulated by IRF2 on HL60 cells, HL60 cells were cultured with medium of $CD4^+$ T cells transfected with either an over-expression plasmid for IRF2 or siRNA targeting IRF2. Subsequently, the function of $CD4^+$ T cells transfection with over-IRF2 plasmid or Si-IRF2 was co-incubated with HL60 cell. Flow cytometry assay indicated that knockdown of IRF2 could further promote HL60 cell apoptosis compared with $CD4^+$ T group while overexpression of IRF2 significantly reduced the apoptosis of HL60 cell (The apoptosis rate in the control , $CD4^+$ T , Si-NC-$CD4^+$ T , Si-IRF2-$CD4^+$ T , Over-NC-$CD4^+$ T and Over-IRF2-$CD4^+$ T was 4.21%±0.46 vs 20.13%±0.48 vs 20.68%±0.81 vs 41.17%±1.14 vs 20.59%±0.65 vs 12.42%±1.11) (Fig. 3D). In addition, the results of RT-qPCR and western blot showed that the mRNA expression and protein levels of INPP4B and IRF2 were dramatically downregulated in $CD4^+$ T group. Compared with the $CD4^+$T group, their levels were reduced in the Si-IRF2 $CD4^+$ T group, while upregulated in the Over-IRF2-$CD4^+$ T groups compared with NC-$CD4^+$ T group (Normalized to the control group, IRF2 and INPP4B protein expression in the $CD4^+$ T, Si-NC-$CD4^+$ T, Si-IRF2-$CD4^+$ T, Over-NC-$CD4^+$ T and Over-IRF2-$CD4^+$ T was 0.56±0.03 vs 0.56±0.05 vs 0.3±0.03 vs 0.55±0.02 vs 0.83±0.07 and 0.51±0.04 vs 0.52±0.05 vs 0.22±0.02 vs 0.52±0.04 vs 0.88±0.04) (Fig. 3E-F). These resultssuggested that downregulation of IRF2 promoted HL60 cell apoptosis by regulating Th1/Th2 ratio.

Overexpression of IRF2 activated JAK2/STAT3 signaling pathway and downregulated caspase 3 expression

To investigate the effect of IRF2 on JAK2/STAT3 signaling pathway and caspase 3 expression, we detected the key proteins involved in JAK2/STAT3 signaling pathway, including JAK2, p-JAK2, STAT3 and p-STAT3 in cells as well as caspase 3 expression. The expression of total JAK2 and STAT3 was not affected by IRF2. However, the expression of p-JAK2 and p-STAT3 significantly downregulated in the Si-IRF2 $CD4^+$ T group while upregulated in the Over-IRF2-$CD4^+$ T groups compared with NC-$CD4^+$ T group (Normalized to the control group, p-JAK2 and p-STAT3 protein expression in the $CD4^+$ T, Si-NC-$CD4^+$ T, Si-IRF2-$CD4^+$ T, Over-NC-$CD4^+$ T and Over-IRF2-$CD4^+$ T was 0.51±0.03 vs 0.51±0.05 vs 0.23±0.02 vs 0.51±0.04 vs 0.88±0.04 and 0.54±0.04vs 0.54±0.04 vs 0.23±0.02vs 0.53±0.04 vs 0.8±0.03) (Fig. 4A). On contrast, the expression of caspase 3 showed the opposite results compared with the changes of p-JAK2 and p-STAT3 expression (Normalized to the control group, caspase 3 protein expression in the $CD4^+$ T, Si-NC-$CD4^+$ T, Si-IRF2-$CD4^+$ T, Over-NC-$CD4^+$ T and Over-IRF2-$CD4^+$ T was 1.86±0.17 vs 1.86±0.16 vs 2.71±0.15 vs 1.87±0.12 vs 1.28±0.08). To confirm the regulatory effect of the JAK2/STAT3 signaling pathway on caspase 3 expression, the JAK2 inhibitor AG-490 and STAT3 inhibitor stattic were administrated in the HL60 cells. The results demonstrated that the expression of caspase 3 was significantly elevated in both AG-490 and stattic compared with $CD4^+$ T group (Normalized to the control group, p-JAK2 , p-STAT3 and caspase 3 protein expression in the $CD4^+$ T , $CD4^+$ T+AG490 and $CD4^+$ T+Stattic was 0.52±0.03 vs 0.23±0.01 vs 0.5±0.04, 0.61±0.03 vs 0.33±0.03 vs 0.19±0.02 and 1.78±0.16 vs 2.49±0.15 vs 2.47±0.15) (Fig. 4B). These results indicated that overexpression of IRF2 might repress HL60 cell apoptosis through activating the JAK2/STAT3 signaling pathway to inhibit caspase 3 expression.

Discussion

AML is a biologically and clinically highly heterogeneous disease. The incidence of AML in China ranks among the top in the world, and the mortality rate of young AML patients is high [4]. The

pathogenesis of AML remains not fully understood, which hinders the development of new treatment methods for exploring AML[3]. Thus, it is meaningful to explore the key regulators in AML development and clarify the mechanism of key regulators in AML. In the present study, we indicated that IRF2 and INPP4B were highly expressed in the $CD4^+$ T cells isolated from AML. Mechanically, we demonstrated that overexpression of IRF2 in $CD4^+$ T cells activated the JAK2/STAT3 signaling pathway, downregulated caspase 3 expression to inhibit AML cells, thereby aggravating AML development.

With the development of cytogenetics and molecular biology, the pathogenesis of AML has been further studied, and the role of more genes in leukemia has been explained, which provides more potential possibilities for the early warning, prognostic analysis and targeted therapy of AML, such as TP53, TRIM62 and EBF3[23]. IRF2 is a multifunctional transcription factor, which plays a key role in regulating cell apoptosis and cell cycle in cancers. For instance, IRF-2 inhibits cancer proliferation by promoting AMER-1 transcription in human gastric cancer[24]. Guo et al., show that IRF2-β-catenin axis promotes HCC cells proliferation, increases lenvatinib resistance of HCC cells, and inhibits apoptosis of HCC cells[25]. In addition, it has been proved that IRF2 plays an important role in the differentiation of immune T cells. Our previous studies have proved that the IRF2/INPP4B signal pathway promotes AML development by promoting the proliferation and survival and inhibiting the apoptosis of AML cells[14, 26], indicating that inhibition of IRF2/INPP4B signaling pathway might be a potential molecular target for AML treatment. Further analysis has indicated that IRF2-INPP4B axis is involved in regulating T helper 1/2 cell differentiation to inhibit apoptosis of AML cells[27]. However, the precise regulatory mechanisms by which the IRF2-INPP4B axis influences T helper 1/2 cell differentiation remain to be fully elucidated. In the present study, we indicated that IRF2 and INPP4B were highly expressed in the $CD4^+$ T cells from AML. Further analysis proved that $CD4^+$ T cells promoted HL60 cell apoptosis. These results were consistent with the study that, although regulatory $CD4^+$ T cells are increased in a subset of cultures, in vitro human CD80/IL2 lentivirus-transduced AML cells show enhanced cytolytic activity[27]. In addition, we found that downregulation of IRF2 promoted HL60 cell apoptosis by regulating Th1/Th2 differentiation, suggesting that IRF2-INPP4B axis inhibited AML cell apoptosis via regulating Th1/Th2 differentiation which could lead to AML development. JAK/STAT signaling pathway is a signaling pathway activated by cytokines[28, 29]. Increasing numbers of studies have shown that JAK/STAT signaling pathway plays important roles in tumor development via regulating various kinds of biological processes, such as proliferation, migration, invasion, and drug resistance[28]. Zhao et al., indicate that the viability of myelodysplastic syndromes and AML cells is inhibited by chidamide via inhibiting JAK2/STAT3 signaling pathway[30]. Mesbahi et al., report that the anti-tumor efficacy of arsenic trioxide in AML cells is augmented by the induction of reactive oxygen species, which blocks JAK2/STAT3 signaling[18]. Wang et al., shows that miR-146a promotes AML development by activating JAK2/STAT3 signaling and downregulating CNFR[31]. However, it is unclear whether the IRF2-INPP4B axis is involved in the regulation of JAK2/STAT3 signaling. In the present study, we provide evidence that overexpression of IRF2 activated JAK2/STAT3 signaling pathway and downregulated caspase 3 expression. Further analysis indicated that inhibition of JAK2/STAT3 signaling pathway could elevate the expression of caspase 3, suggesting that JAK2/STAT3 might inhibit AML apoptosis. This research highlights a previously uncharacterized link between the IRF2/INPP4B and JAK2-STAT3 pathways, suggesting a potentially novel regulatory mechanism in

AML development. This finding could pave the way for novel therapeutic strategies targeting this interplay.

There are several limitations of our study. First, the sample size of patients is relatively small and We did not refine the patient subgroups, so the heterogeneity may introduce some bias in the interpretation of the results. Second, this study is the lack of animal experiments to further verify our research results. This study is a pilot study, and our study will carry out further large-scale study and animal experiments in the future to further validate our findings.

Conclusion

In conclusion, our study demonstrates that IRF2 and INPP4B are highly expressed in AML-derived $CD4^+$ T cells. Moreover, we indicate that downregulation of IRF2 promoted HL60 cell apoptosis by regulating Th1/Th2 ratio. In addition, we reveal that overexpression of IRF2 activates JAK2/STAT3 signaling pathway and downregulates caspase 3 expression. Our study demonstrated that activation of IRF2-INPP4B axis in $CD4^+$ T cells aggravated AML development by activating JAK2/STAT3 signaling pathway via inhibition of AML cell apoptosis, which might provide clues in better understanding of the mechanisms of AML, and provide a novel sight for AML treatment.

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Author contribution statements

Xiangqin Xing, Mei Zhang and Feng Zhang contributions to idea and design; Shengfen Tan, Junfeng Zhu and YuanYuan carried out the experiment; Xiangqin Xing, Mei Zhang and Feng Zhang wrote the manuscript with input from all authors; Meng Wang and Feng Zhang supervised the project; Junfeng Zhu, Jiajia Li, Pingping Zhang analysed the data; Xiangqin Xing and Feng Zhang conceived the study and were in charge of overall direction and planning.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the 1975 Helsinki declaration and its later amendments or comparable ethical standards. Before collecting samples, written informed consents were acquired from all parents of participants. All animal experiments were approved by the First Affiliated Hospital of Bengbu Medical College.

The 20 peripheral blood samples from patients with AML and the healthy individuals were collected at the First Affiliated Hospital of Bengbu Medical College in 2021.

All experiments were performed in accordance with guidelines on animal care approved by the First Affiliated Hospital of Bengbu Medical College, and all participants signed the informed consent form.

Data Availability

All data generated or analyzed during this study are available from the corresponding author under reasonable requests.

Competing Interests

The authors declare that they have no conflicts of interest.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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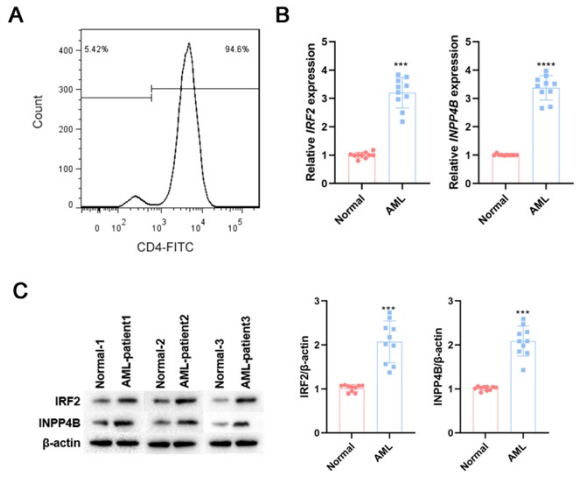

Figure 1. IRF2 and INPP4B was highly expressed in the CD4$^+$ T cells isolated from AML. A, The CD4$^+$ T cells was isolated from peripheral blood of normal and AML patients and detected by flow cytometry; B, qRT-PCR was used to detect the expression of IRF2 and INPP4B in CD4$^+$ T cells from normal and AML patients; C, Western blot was used to detect the protein expression of IRF2 and INPP4B in CD4$^+$ T cells from normal and AML patients. ***, p<0.001, ****, p<0.0001.

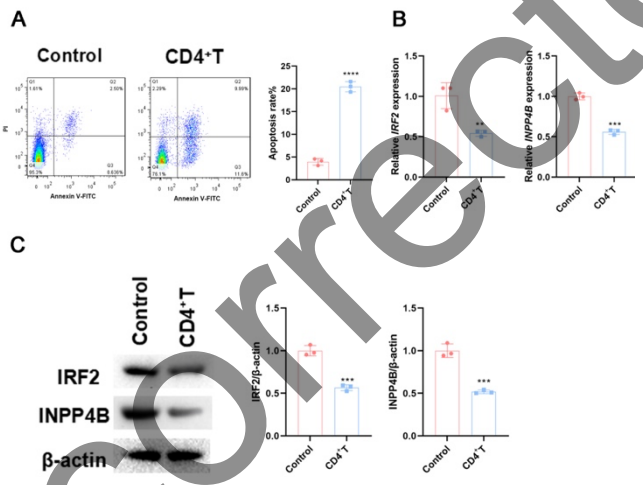

Figure 2. CD4$^+$ T cells promoted HL60 cell apoptosis

A, Flow cytometry assay was used to detect the apoptosis of HL60 cell affected by CD4$^+$ T cells; B and C, qRT-PCR and western blot were used to detect the expression of IRF2 and INPP4B in the HL60 cells, respectively. **, p<0.01, ***, p<0.001, ****, p<0.0001.

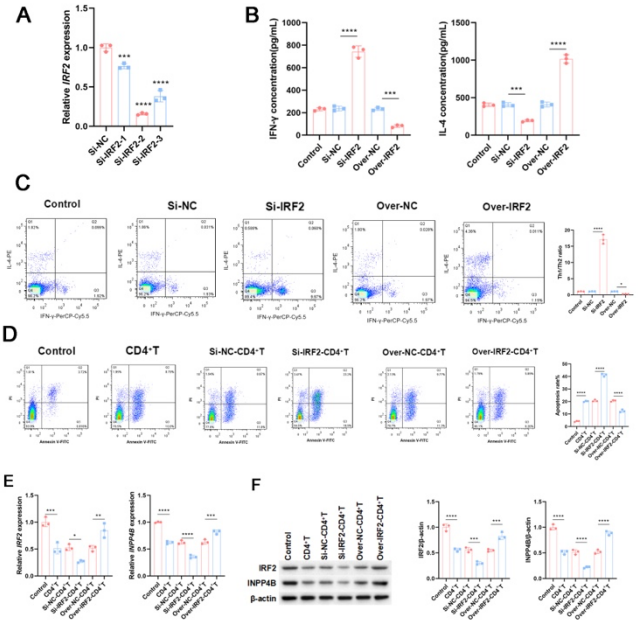

Figure 3. Downregulation of IRF2 promoted HL60 cell apoptosis by regulating Th1/Th2 ratio

A, The expression of IRF2 in the $CD4^+$ T cells transfection with IRF2 siRNA; B, The contents of IFN-γ and IL-4 was detected by ELISA affected by IRF2; C, The ratio of Th1/Th2 was detected by flow cytometry; D, Flow cytometry assay was used to detect the HL60 cell apoptosis cocultured with $CD4^+$ T cell medium with IRF2 overexpression or knockdown, respectively; E and F, The expression of INPP4B was detected by qRT-PCR and western blot affected by IRF2, respectively. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$.

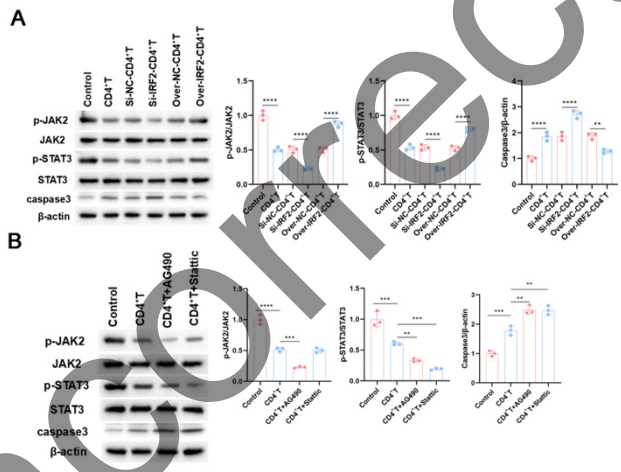

Figure 4. Overexpression of IRF2 activated JAK2/STAT3 signaling pathway and downregulated caspase 3 expression

A, Western blot was used to detect the expression of JAK2/STAT3 signaling pathway related proteins, and caspase 3 affected by IRF2, including JAK2, STAT3, p-JAK2, and p-STAT3; B, The expression of caspase 3 was detected by western blot affected by JAK2 inhibitor AG-490 and STAT3 inhibitor stattic. **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$.