RESEARCH ARTICLE

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The IRF2-INPP4B Pathway Aggravates Acute Myeloid Leukemia

IRF2-INPP4B Yolağı Akut Miyeloid Lösemide Olumsuz Etkilidir

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Abstract

Objective: Interferon-regulatory factor 2 (IRF2) and inositol polyphosphate 4-phosphatase B (INPP4B) are indispensable for differentiating immune T-cells, but the regulatory principle of the IRF2-INPP4B signaling channel in the apoptosis of acute myeloid leukemia (AML) cells remains unclear. This work investigates the function and regulatory principle of IRF2-INPP4B signaling in the progression of AML.

Materials and Methods: $CD4^+$ T-cells were extracted from peripheral blood and characterized via flow cytometry. Flow cytometry was used to estimate apoptosis in the HL60 AML cell line and determine the Th1/Th2 cell ratio. Quantitative real-time polymerase chain reaction was used to measure *IRF2* mRNA. Western blotting was performed to evaluate the protein levels of IRF2, INPP4B, JAK2, p-JAK2, STAT3, p-STAT3, and caspase 3. Interleukin-4 and interferon gamma concentrations were determined using enzyme-linked immunoadsorption assay kits.

Results: We discovered that levels of IRF2 and INPP4B were high in AML-derived $CD4^+$ T-cells. Furthermore, $CD4^+$ T-cells encouraged HL60 cell apoptosis. Downregulation of IRF2 encouraged HL60 cell apoptosis via alterations in the Th1/Th2 ratio while the overexpression of IRF2 stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3.

Conclusion: We revealed that IRF2-INPP4B signaling in $CD4^+$ T-cells stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3, reducing AML cell apoptosis and aggravating AML progression. This work highlights an important regulatory principle concerning AML progression, as the IRF2-INPP4B pathway might impact the JAK2-STAT3 signaling channel. The findings contribute to our knowledge of the complicated interplay of these pathways in AML.

Keywords: Acute myeloid leukemia, Apoptosis, IRF2-INPP4B, JAK2-STAT3 signaling channel

Öz

Amaç: İnterferon düzenleyici faktör 2 (IRF2) ve inositol polifosfat 4-fosfataz B (INPP4B), bağışıklık T-hücrelerinin farklılaşmasında kritik rol oynar. Ancak, IRF2-INPP4B sinyal yolunun akut miyeloid lösemi (AML) hücrelerinin apoptozundaki düzenleyici mekanizması net değildir. Bu çalışma, IRF2-INPP4B sinyalinin AML ilerlemesindeki işlevini ve düzenleyici prensiplerini araştırmaktadır.

Gereç ve Yöntemler: Periferik kandan izole edilen $CD4^+$ T-hücreleri akım sitometri ile tanımlandı. HL60 AML hücre hattında apoptozun hesaplanması ve Th1/Th2 hücre oranı belirlenmesi için akım sitometrisi kullanıldı. *IRF2* mRNA ölçümü kantitatif gerçek zamanlı polimeraz zincir reaksiyonu ile yapıldı. IRF2, INPP4B, JAK2, p-JAK2, STAT3, p-STAT3 ve kaspaz 3 protein düzeyleri, Western blot ile değerlendirildi. İnterlökin-4 ve interferon gama konsantrasyonları ELISA kitleri ile ölçüldü.

Bulgular: AML kaynaklı $CD4^+$ T-hücrelerinde IRF2 ve INPP4B seviyeleri yüksek bulundu. Ayrıca, $CD4^+$ T-hücreleri, HL60 hücre apoptozunu artırdı. IRF2 baskılanması, Th1/Th2 oranını değiştirerek HL60 hücre apoptozunu artırırken IRF2 ifadesindeki artış, JAK2-STAT3 sinyal yolunu aktive etti ve kaspaz 3'ü baskıladı.

Sonuç: IRF2-INPP4B sinyal yolu, CD4+ T-hücrelerinde JAK2-STAT3 yolunu aktive ederek kaspaz 3'ü baskılar ve AML hücre apoptozunu engelleyerek AML ilerlemesini artırır. Bu çalışma, AML ilerlemesinde IRF2-INPP4B yolunun önemini ortaya koymakta ve JAK2-STAT3 sinyalizasyonu ile olan etkileşimini vurgulamaktadır. Bulgular, AML'deki bu karmaşık moleküler mekanizmaların anlaşılmasına katkı sağlamaktadır.

Anahtar Sözcükler: Akut miyeloid lösemi, Apoptoz, IRF2-INPP4B, JAK2-STAT3 sinyal yolu

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Introduction

Acute myeloid leukemia (AML) is a hematological malignancy and the most usual type of acute leukemia in adults [1]. It leads to impaired hematopoietic function and bone marrow decline caused by abnormal proliferation of undifferentiated myeloid precursor cells. The disease progresses rapidly and the rates of complete response and long-term survival are low [2]. Chemotherapy and allogeneic and autologous bone marrow transplantation are currently the three primary therapies for AML [3], but bone marrow transplantation not only requires high costs but also entails the possibility of immune rejection. Furthermore, although 50%-75% of AML patients respond to chemotherapy, there is still a high recurrence rate after the initial cure. The poor prognosis and the high recurrence rate of AML are the primary causes of failed treatments [4]. It is of critical importance to further investigate the principles governing the onset and advance of AML, which would support the designing of innovative and effective therapeutic strategies for its management.

Interferon-regulatory factors (IRFs) belong to the larger category of transcription factors and were originally considered to be transcriptional regulators of the type I interferon system [5]. Research has previously shown that IRFs participate in the differentiating of lymphocytes, cell growth, and apoptosis and play indispensable roles 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 growth [7]. IRF2 can stimulate the malignant proliferation of tumor cells and cause malignant transformation of tumors, and it has been classified as an oncogene in fibroblasts and T lymphocytes [8]. Thus, downregulation of IRF2 could be a potential goal for cancer treatments [9]. Notably, previous works have shown that IRF2 participates in AML progression and may thus be a viable and specific therapeutic factor in treating AML. INPP4B, a newly identified lipid phosphatase, has been ascertained to have indispensable functions in various kinds of cancers [10,11,12]. INPP4B increases proliferation and tumor progression in *PIK3CA*-mutant ER breast cancer cells [11]. Another report suggested that INPP4B-mediated mTORC1 signaling stimulation and cap-dependent translation initiation could facilitate the proliferation of colorectal cancer cells [12]. Additionally, INPP4B, serving as a prognostic and diagnostic marker, regulates the growth of pancreatic cancer cells by triggering AKT [13]. The IRF2-INPP4B signaling channel can promote the progression of AML by encouraging the proliferation and continued survival of AML cells [14], indicating that blocking the IRF2-INPP4B signaling channel might be a vital molecular goal in efforts to treat AML. Researchers have also stated that IRF2 is involved with CD8a dendritic cells via an impact on the differentiation of Th2 cells [15], indicating that IRF2 has a vital function in the differentiating of immune T-cells. Nevertheless, the regulatory principle of the IRF2-INPP4B signaling channel in terms of the apoptosis of AML cells remains unclear.

JAK2, belonging to the protein-tyrosine kinase family, acts as a key regulator in diverse physiological and pathological processes such as cell proliferation and differentiation [16]. Other signaling molecules such as STAT1, STAT3, and STAT5 are also regulated by JAK2. Specifically, the phosphorylation of STAT3 can be stimulated by JAK2, and the JAK2-STAT3 pathway has a key function in human tumorigenesis by modulating cell proliferation, survival, immune response, and differentiation [17]. Researchers have also suggested that the JAK2-STAT3 pathway affects AML progression [18]. It was recently revealed that JARID2, a histone demethylase, has a crucial function in breast cancer growth and advancement by modulating the activities of INPP4B and the JAK2-STAT3 pathway [19]. The question of whether IRF2 and INPP4B take part in processes of AML by modulating the JAK2-STAT3 pathway remains unanswered, however. The present work accordingly aimed to investigate the role of IRF2 and INPP4B in the progression of AML.

Materials and Methods

Reagents

JAK2 inhibitor AG-490 was bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). STAT3 inhibitor Stattic was bought from MedChemExpress (Monmouth Junction, NJ, USA).

Clinical Sample Collection and $CD4^+$ T-Cell Isolation

We collected a total of 20 peripheral blood samples at our hospital in 2021 from 10 AML patients and 10 healthy individuals (control group). The diagnostic criteria for AML were based on the guidelines from the 2017 edition of the European LeukemiaNet recommendations for AML in adults [20]. $CD4^+$ T-cells were extracted from peripheral blood via a commercial $CD4^+$ T-cell separation kit (HS-SJ078, Crondabio, Shanghai, China). Briefly, fresh blood samples were collected in collection tubes and peripheral monocytes were subjected to a preparation process based on density gradient centrifugation. Subsequently, magnetic live cell classification and a $CD4^+$ T-cell separation kit were used to separate $CD4^+$ T-cells from peripheral monocytes. The obtained $CD4^+$ T-cells were then maintained in RPMI-1640 medium supplied by Gibco (Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, and 55 μM β-mercaptoethanol. Ethical permission for this work was granted by the First Affiliated Hospital of Bengbu Medical College on June 25, 2021, with permit number [2021] No. 205. All experiments were conducted following the institutional ethical standards and all participants granted their written informed consent.

CD4+ T-Cell Identification

Flow cytometry was used to determine the purity of the CD4+ T-cells. Briefly, the CD4+ T-cells ($1x10^6$ cells) were blocked with 2% FBS for 30 min in phosphate-buffered saline (PBS) and then underwent incubation with fluorochrome-conjugated anti-CD4 (ab133616, 1:100, Abcam, Cambridge, UK) for 30 min. The cells were then evaluated with the BD FACSVerse device (BD Biosciences, Franklin Lakes, NJ, USA) and findings were evaluated in more detail using FlowJo software (BD Biosciences).

Cell Culture

HL60 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS under 5% CO_2 and 95% air at 37 °C.

Treatment and Transfection of Cells

Cells were seeded at $2x10^5$ cells per 6-well plate and permitted to grow to 70%-90% confluency before transfection. The cells were then transfected with 20 μM pcDNA3.1-IRF2 overexpression vectors (Geneseed Biotech, Guangzhou, China) or 20 μM IRF2 siRNA (Genechem, Shanghai, China) using Lipofectamine 2000 Reagent (11668-027, Invitrogen, Carlsbad, CA, USA) following the manufacturer's directions. For transfection, four units (15 μL) of Lipofectamine were diluted in 150 μL of Opti-MEM medium (Gibco). Meanwhile, the specified DNA amount (14 μg) was mixed with 70 μL of Opti-MEM medium. The diluted DNA and Lipofectamine were combined at a 1:1 ratio and incubated at room temperature for 5 min, and the resulting DNA-lipid mixture was gently added to the cells. Culturing continued for 48 h. A negative control (NC) siRNA sequence or pcDNA3.1 plasmid served as the control group. For the CD4+ T-cell co-culture, HL60 cells were maintained in CD4+ T-cell medium with different co-culture conditions. The siRNA sequences targeting IRF2 were: si-IRF2-1, sense: 5′-AGUUAAGCACAUCAAGCAAGA-3′, antisense: 5′-UUGCUUGAUGUGCUUAACUUU-3′; si-IRF2-2, sense: 5′-GGUGAACAUCAUAGUUGUAGG-3′, antisense: 5′-UACAACUAUGAUGUUCACCGU-3′; si-IRF2-3, sense: 5′-GGUCCUGACUUCAACUAUAAA-3′, antisense: 5′-UAUAGUUGAAGUCAGGACCGC-3′.

Flow Cytometry

For HL60 cell apoptosis analysis, the HL60 cells were cultured with CD4+ T-cell medium and then the apoptosis of the HL60 cells was determined by flow cytometry [21]. HL60 cells cultured in RPMI-1640 medium served as the control. Briefly, the HL60 cells were collected and washed three times with PBS and then stained with the FITC Annexin V Apoptosis Detection Kit (HS-SJ069, Crondabio) at 4 °C in darkness according to the manufacturer's directions. Apoptosis was detected using the FACSVerse (BD Biosciences) and the findings were evaluated in detail with FlowJo software (BD Biosciences). The ratio of Th1 cells positive for IFN-γ to Th2 cells positive for IL-4 among the cells was determined by staining with the INF-γ-PerCP-Cy5.5 antibody (BioLegend, San Diego, CA, USA) and PE-IL-4 antibody (BioLegend), respectively.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from the cells using TRIzol reagent (HS-SJ012, Crondabio) following the manufacturer's directions. The cDNA was then obtained via reverse transcription using the PrimeScript RT Master Mix Kit (R223-01, Vazyme, Nanjing, China). Subsequently, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted with the LightCycler 480II instrument (Roche, Basel, Switzerland) using ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme). The PCR protocol consisted of 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 20 s. Gene expression levels were determined using the $2^{-\triangle\triangle Ct}$ method [22], with β-actin mRNA serving as the internal control. The primer sequences utilized 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

Proteins were extracted from the cells using RIPA buffer (HS-SJ011, Crondabio) and then separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime, Shanghai, China), followed by transfer to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were then blocked with Tris-buffered saline with Tween (TBST; 1.5 mM Tris, 5 mM NaCl, 0.1% Tween-20) containing 5% skim milk at room temperature for 1 h and subjected to incubation with specific primary antibodies anti-IRF2 (ab124744, 1:1000, Abcam), anti-INPP4B (ab81269, 1:2000, Abcam), anti-JAK2 (ab108596, 1:5000, Abcam), anti-STAT3 (ab68153, 1:1000, Abcam), anti-p-JAK2 (ab32101, 1:5000, Abcam), anti-p-STAT3 (ab267373, 1:2000, Abcam), anti-caspase 3 (ab32351, 1:2000, Abcam), and anti-actin (GB12001, 1:2000, Servicebio, Wuhan, China) at 4 °C overnight. The membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (BL003A, 1:4000, Biosharp, Hefei, China) for 1 h at room temperature. Finally, membrane bands were visualized using an enhanced chemiluminescence reagent (WBKLS0100, Millipore, Billerica, MA, USA). Actin served as a loading control and band grayscale values were analyzed using ImageJ software (Bio-Rad). Changes in protein levels were quantified by analyzing the grayscale values of the protein of interest and actin bands.

Cytokine Analysis by ELISA

Supernatants were harvested from CD4+ T-cell cultures and analyzed using IL-4 (E-EL-H0101c, Elabscience, Wuhan, China) and IFN-γ (E-EL-H0108c, Elabscience) ELISA kits following the manufacturer's directions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Inc., San Diego, CA, USA) with a minimum of three repetitions. Data were expressed as mean ± standard deviation values. Statistical significance was estimated by utilizing Student's t-test for two groups or one-way analysis of variance for multiple groups. Values of $p<0.05$ were deemed statistically significant.

Results

IRF2 and INPP4B Levels Were High in CD4+ T-Cells from Acute Myeloid Leukemia Patients

To investigate the function of IRF2 and INPP4B in CD4+ T-cells, CD4+ T-cells were obtained from the peripheral blood of healthy individuals and patients with AML. As displayed in Figure 1A, the CD4+ T-cells accounted for 94.6% of all obtained cells. The levels of IRF2 and INPP4B in CD4+ T-cells obtained from healthy individuals and patients with AML were subsequently comparatively evaluated and qRT-PCR revealed a marked increase in the expression of IRF2 and INPP4B mRNA in CD4+ T-cells from AML patients compared to those from the healthy control group (Figure 1B). Furthermore, western blotting indicated significant increases in IRF2 and INPP4B in CD4+ T-cells obtained from AML patients compared to the healthy control group. Normalized to the control group, the IRF2 and INPP4B protein levels in the CD4+ T-cells of the patient group were respectively 2.06±0.1 and 2.11±0.13 (Figure 1C). These findings revealed that IRF2 and INPP4B were expressed in CD4+ T-cells isolated from AML patients at significantly high levels, indicating that IRF2 and INPP4B may be involved in the progression of AML.

CD4+ T-Cells Encouraged the Apoptosis of HL60 Cells

To clarify the function of CD4+ T-cells in the apoptosis of AML cells, isolated CD4+ T-cells were co-cultured with HL60 cells. Flow cytometry analysis showed that CD4+ T-cells markedly induced HL60 cell apoptosis compared to the control group, with apoptosis rates of 3.95±0.74% and 20.49±1.13% in the control and CD4+ T-cell groups, respectively (Figure 2A). After confirming the elevated IRF2 and INPP4B expression in CD4+ T-cells isolated from AML patients, we investigated whether CD4+ T-cell-induced apoptosis correlated with the regulation of

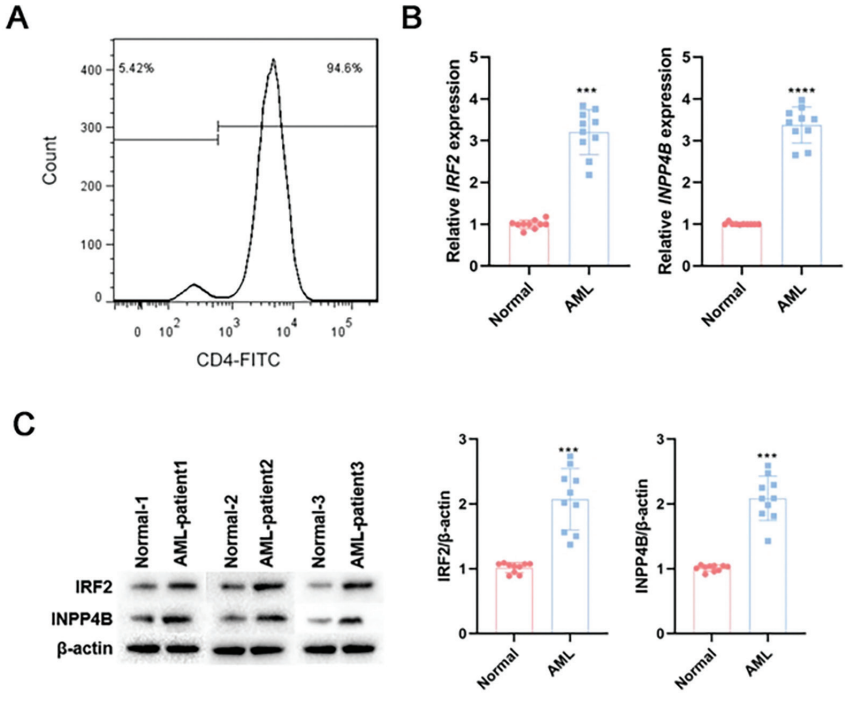

Figure 1. The expression levels of IRF2 and INPP4B were significantly increased in CD4+ T-cells isolated from patients with acute myeloid leukemia (AML). A) CD4+ T-cells were isolated from the peripheral blood of healthy donors and AML patients, followed by flow cytometry analysis. B) Quantitative real-time polymerase chain reaction was used to measure the expression of *IRF2* and *INPP4B* in CD4+ T-cells from healthy individuals and AML patients. C) Western blotting was used to estimate the protein expression levels of IRF2 and INPP4B in CD4+ T-cells from healthy donors and AML patients.

***: $p<0.001$; ****: $p<0.0001$. IRF2: Interferon-regulatory factor 2; INPP4B: inositol polyphosphate 4-phosphatase B.

IRF2 and INPP4B expression. The expression of IRF2 and INPP4B in HL60 cells was evaluated, and both qRT-PCR and western blotting revealed markedly reduced IRF2 and INPP4B expression in the CD4⁺ T-cell group versus the control group at mRNA and protein levels, respectively. When normalized to the control group, the protein expression levels of IRF2 and INPP4B in the patient-derived CD4⁺ T-cell group were respectively 0.57±0.04 and 0.51±0.22 (Figures 2B and 2C). These findings suggest that the CD4⁺ T-cells encouraged HL60 cell apoptosis through the downregulation of IRF2 and INPP4B.

Downregulation of IRF2 Encouraged Apoptosis of HL60 Cells by Influencing the Th1/Th2 Ratio

We further investigated whether IRF2 regulated the differentiation of Th1/Th2 cells in CD4⁺ T-cells. The loss and gain functions of IRF2 for the differentiating of Th1/Th2 cells in CD4⁺ T-cells were investigated by transfection with overexpressing IRF2 plasmids and the siRNA of *IRF2*. As displayed in Figure 3A, the siRNA-2 of *IRF2* exerted the best knockdown effect against *IRF2* in CD4⁺ T-cells. Accordingly, the siRNA-2 of *IRF2* was used for further work. Further analysis demonstrated that IFN-γ was markedly increased in the Si-IRF2 group and decreased in the Over-IRF2 group compared to the corresponding NC group. The opposite result was obtained for IL-4 in the different groups (Figure 3B). We also discovered that the knockdown of IRF2 markedly elevated the ratio of Th1/Th2 while increased expression of IRF2 reduced the ratio of Th1/Th2. The Th1/Th2 ratios in the control, Si-NC, Si-IRF2, Over-NC, and Over-IRF2 groups were 1.07±0.04, 1.08±0.04, 17.14±1.41, 1.07±0.03, and 0.28±0.03, respectively (Figure 3C). To explore the functional effect of the IRF2-regulated Th1/Th2 ratio in CD4⁺ T-cells on HL60 cells, HL60 cells were co-cultured in medium containing CD4⁺ T-cells transfected with either an IRF2 overexpression plasmid or IRF2-targeting siRNA. Subsequently, the CD4⁺ T-cells transfected with the IRF2 overexpression plasmid or IRF2 siRNA were co-cultured with HL60 cells. Flow cytometry analysis revealed that silencing IRF2 further enhanced HL60 cell apoptosis compared to the CD4⁺ T-cell group. The apoptosis rates in the control, CD4⁺ T, Si-NC-CD4⁺ T, Si-IRF2-CD4⁺ T, Over-NC-CD4⁺ T, and Over-IRF2-CD4⁺ T-cell groups were 4.21±0.46%, 20.13±0.48%, 20.68±0.81%, 41.17±1.14%, 20.59±0.65%, and 12.42±1.11%, respectively (Figure 3D). Additionally, qRT-PCR and western blotting revealed that INPP4B and IRF2 mRNA expression and protein levels were significantly decreased in the CD4⁺ T-cell group. Compared to the CD4⁺ T-cell group, the obtained values were further reduced in the Si-IRF2 CD4⁺ T-cell group, whereas they were elevated in the Over-IRF2 CD4⁺ T-cell group compared to the NC-CD4⁺ group. Normalized to the control group, the protein expression levels of IRF2 in the CD4⁺ T, Si-NC-CD4⁺ T, Si-IRF2-CD4⁺ T, Over-NC-CD4⁺ T, and Over-IRF2-CD4⁺ T-cell groups were 0.56±0.03, 0.56±0.05, 0.3±0.03, 0.55±0.02, and 0.83±0.07 while the values for INPP4B were 0.51±0.04, 0.52±0.05, 0.22±0.02, 0.52±0.04, and 0.88±0.04 (Figures 3E and 3F). These findings suggested

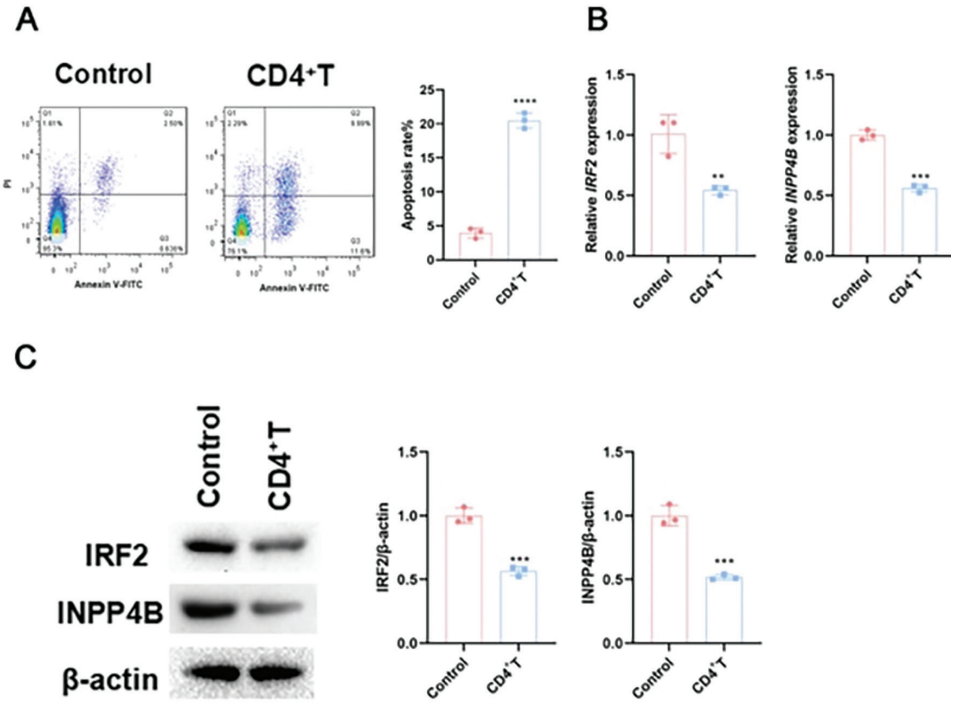

Figure 2. CD4⁺ T-cells induced apoptosis in HL60 cells. A) Flow cytometry was used to identify apoptosis in HL60 cells co-cultured with CD4⁺ T-cells. B, C) Quantitative real-time polymerase chain reaction and western blot analysis were used to measure the expression of IRF2 and INPP4B in HL60 cells, respectively.

**: p<0.01; ***: p<0.001, ****: p<0.0001. IRF2: Interferon-regulatory factor 2; INPP4B: inositol polyphosphate 4-phosphatase B.

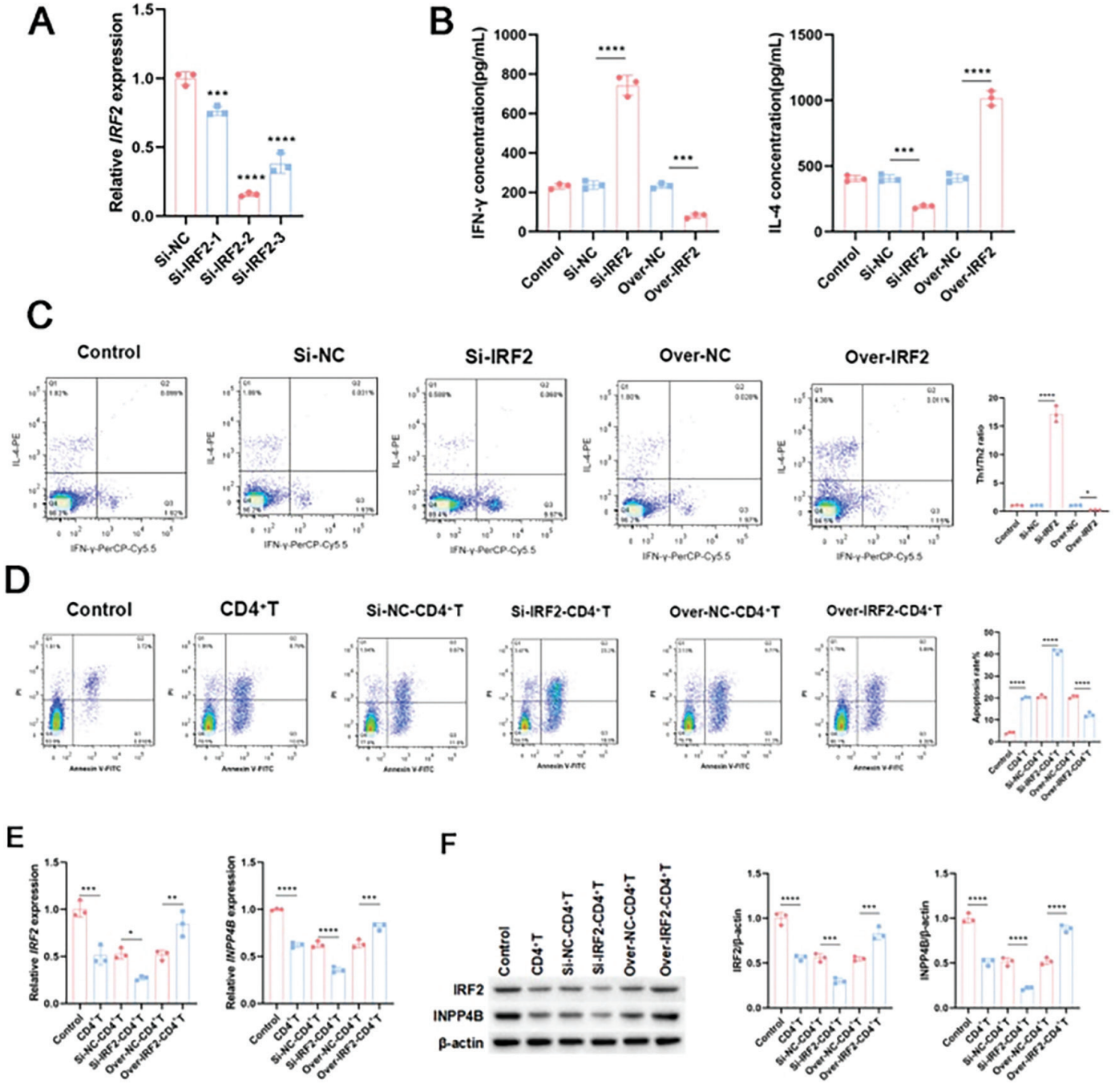

Figure 3. Downregulation of IRF2 induced HL60 cell apoptosis by modulating the Th1/Th2 ratio. A) *IRF2* expression levels in CD4+ T-cells transfected with *IRF2* siRNA. B) Interferon gamma and interleukin-4 levels measured by ELISA in the context of IRF2 manipulation. C) Th1/Th2 ratios analyzed by flow cytometry. D) Flow cytometric assessment of apoptosis in HL60 cells co-cultured with CD4+ T-cells overexpressing or knocking down IRF2, respectively. E, F) INPP4B expression measured by quantitative real-time polymerase chain reaction and western blotting in relation to IRF2, respectively.

*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001. IRF2: Interferon-regulatory factor 2; INPP4B: inositol polyphosphate 4-phosphatase B.

that decreasing the expression of IRF2 encouraged HL60 cell apoptosis by influencing the Th1/Th2 ratio.

Overexpression of IRF2 Stimulated the JAK2-STAT3 Signaling Channel and Downregulated Caspase 3

To explore the impact of IRF2 on the JAK2-STAT3 signaling channel and caspase 3 expression, we examined key proteins of the JAK2-STAT3 pathway, including JAK2, p-JAK2, STAT3, p-STAT3, and caspase 3. Total JAK2 and STAT3 expression remained unchanged by IRF2. In contrast, p-JAK2 and p-STAT3 expression showed significant downregulation in the Si-IRF2 $CD4^+$ T-cell group and upregulation in the Over-IRF2-$CD4^+$ T-cell group compared to the NC-$CD4^+$ T-cell group. Normalized to the control group, the values obtained for p-JAK2 protein expression in the $CD4^+$ T, Si-NC-$CD4^+$ T, Si-IRF2-$CD4^+$ T, Over-NC-$CD4^+$ T, and Over-IRF2-$CD4^+$ T-cell groups were 0.51±0.03, 0.51±0.05, 0.23±0.02, 0.51±0.04, and 0.88±0.04, while the respective values for p-STAT3 were 0.54±0.04, 0.54±0.04, 0.23±0.02, 0.53±0.04, and 0.8±0.03 (Figure 4A). The opposite result was obtained for caspase 3. Normalized to the control group, the values obtained for 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-cell groups were 1.86±0.17, 1.86±0.16, 2.71±0.15, 1.87±0.12, and 1.28±0.08, respectively. To confirm the regulatory effect of the JAK2-STAT3 signaling channel on caspase 3, the HL60 cells were exposed to JAK2 inhibitor AG-490 and STAT3 inhibitor Stattic. This showed that caspase 3 was markedly elevated with both AG-490 and Stattic compared to the $CD4^+$ T-cell group. Normalized to the control group, the protein expression levels of p-JAK2, p-STAT3, and caspase 3 in the $CD4^+$ T, $CD4^+$ T + AG-490, and $CD4^+$ T + Stattic groups were respectively 0.52±0.03, 0.23±0.01, and 0.5±0.04; 0.61±0.03, 0.33±0.03, and 0.19±0.02; and 1.78±0.16, 2.49±0.15, and 2.47±0.15 (Figure 4B). These findings indicated that heightened expression of IRF2 might repress apoptosis in HL60 cells by stimulating the JAK2-STAT3 signaling channel to inhibit caspase 3.

Discussion

AML is an extremely heterogeneous disease both biologically and clinically. 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 is still not fully understood, which hinders the identification of new avenues for treatment [3]. Thus, it is vital to explore the key regulators of AML progression and clarify their mechanisms. In this study, we found that IRF2 and INPP4B were expressed in $CD4^+$ T-cells isolated from AML patients at significantly high levels. Mechanistically, we showed that IRF2 overexpression in $CD4^+$ T-cells stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3 expression to promote AML cell survival, thereby facilitating AML progression.

With the advancement of cytogenetic techniques and molecular biology, the pathogenesis of AML has been further explored and the roles of additional leukemia-related genes, such as *TP53*, *TRIM62*, and *EBF3*, have been elucidated. This has yielded new opportunities for early diagnosis, prognostic estimation, and targeted therapies for AML [23]. IRF2, a multifunctional transcription factor, has crucial functions in influencing apoptosis and the cell cycles of cancer cells. For instance, IRF2 suppresses cancer cell proliferation by promoting AMER-1 transcription in human gastric cancer cells [24]. Guo et al. [25] reported that the IRF2-β-catenin axis drives the proliferation of hepatocellular carcinoma (HCC) cells, enhances their resistance to lenvatinib, and blocks HCC cell apoptosis. Additionally, evidence indicates that IRF2 is vital for the differentiation of immune T-cells. Our prior studies similarly showed that the IRF2-INPP4B signaling channel promotes AML progression by facilitating leukemic cell proliferation and survival while inhibiting apoptosis [14,26], indicating that restricting the IRF2-INPP4B signaling channel might be a potential molecular goal for new AML therapies. Further analysis demonstrated that the IRF2-INPP4B axis had a role in the differentiating of Th1/Th2 cells that allowed it to inhibit the apoptosis of AML cells [27]. Nevertheless, the precise regulatory actions by which the IRF2-INPP4B axis impacts that process of differentiation are still not fully elucidated. In the present work, we showed that expression values of IRF2 and INPP4B were significantly higher in $CD4^+$ T-cells obtained from patients with AML. Further analysis revealed that the $CD4^+$ T-cells encouraged the apoptosis of HL60 cells. These outcomes were consistent with those of a previous work reporting that although regulatory $CD4^+$ T-cells were increased in a subset of cultures, in vitro human CD80/IL2 lentivirus-transduced AML cells showed enhanced cytolytic activity [27]. Additionally, we found that reducing the expression of IRF2 encouraged the apoptosis of HL60 cells via an impact on the Th1/Th2 differentiation process, indicating that the IRF2-INPP4B axis inhibited AML cell apoptosis by modulating the differentiating of Th1/Th2, which could lead to AML progression. The JAK/STAT signaling channel is stimulated by cytokines [28,29]. An increasing body of literature confirms that the JAK/STAT signaling channel has vital roles in tumor growth via its influences on various biological processes, such as proliferation, migration, invasion, and drug resistance [28]. Zhao et al. [30] concluded that the viability of myelodysplastic syndrome and AML cells is inhibited by chidamide due to its ability to inhibit the JAK2-STAT3 signaling channel. Mesbahi et al. [18] reported that the antitumor efficacy of arsenic trioxide in AML cells was improved by the induction of reactive oxygen species, which blocked JAK2-STAT3 signaling. Wang et al. [31] showed that miR-146a encouraged AML progression by stimulating JAK2-STAT3 signaling and decreasing the expression of CNFR.

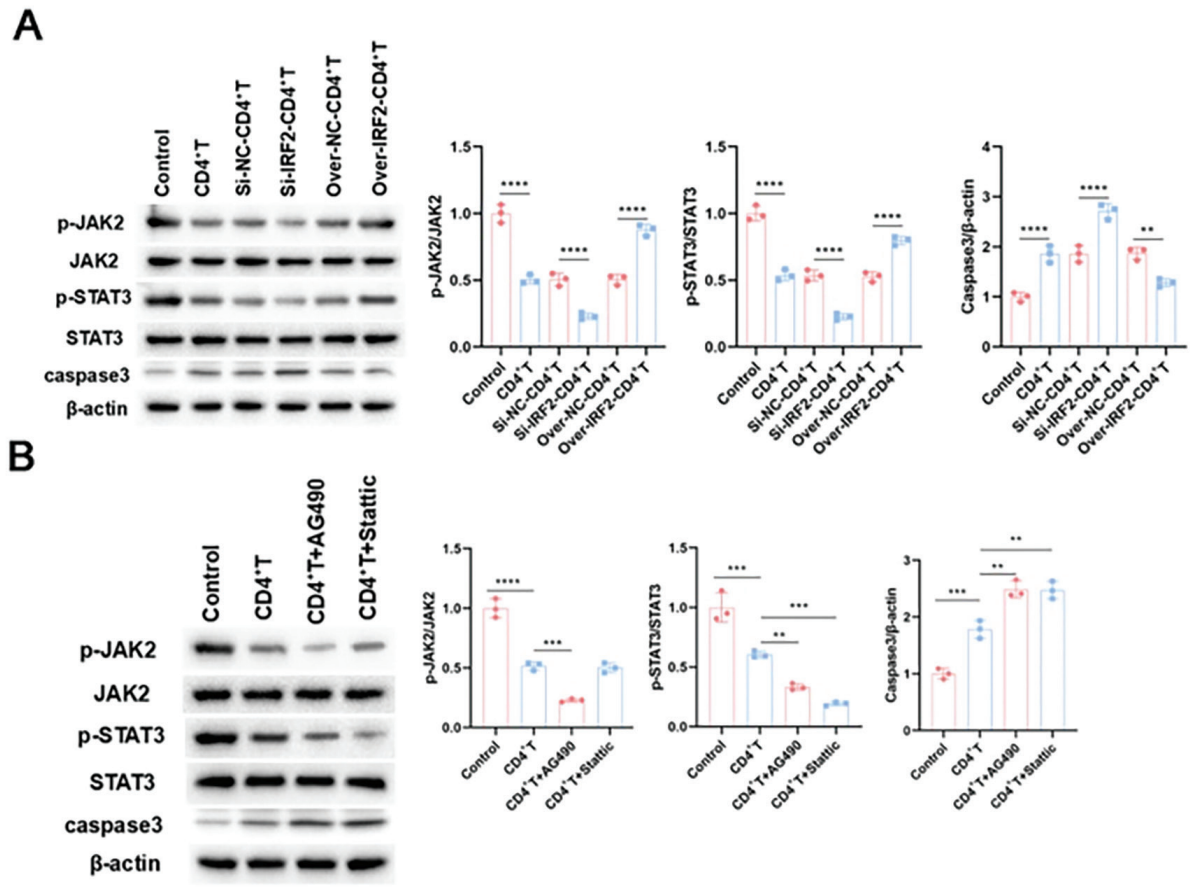

Figure 4. Overexpression of IRF2 stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3 expression. A) Western blotting was used to measure the expression levels of JAK2-STAT3 signaling channel-related proteins and caspase 3 as impacted by IRF2, with the proteins including JAK2, STAT3, p-JAK2, and p-STAT3. B) Effects on the expression of caspase 3 via JAK2 inhibitor AG-490 and STAT3 inhibitor Stattic were detected with western blotting.

**: p<0.01; ***: p<0.001; ****: p<0.0001. IRF2: Interferon-regulatory factor 2.

Nevertheless, it is not yet clear whether the IRF2-INPP4B axis participates in the modulation of JAK2-STAT3 signaling. In the present work, we have offered evidence demonstrating that increasing the expression of IRF2 stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3. Further analysis showed that inhibiting the JAK2-STAT3 signaling channel could elevate caspase 3, implying that JAK2-STAT3 might inhibit AML apoptosis. Thus, this work highlights a previously uncharacterized link between the IRF2-INPP4B and JAK2-STAT3 pathways, offering data on a new potential regulatory mechanism in AML progression. This finding could pave the way for new therapeutic strategies that focus on this interplay.

Study Limitations

Our work has several limitations. First, the sample size of the patient group was relatively small and we could not analyze patient subgroups; thus, the ensuing heterogeneity may have introduced some bias in the interpretation of the obtained data. Furthermore, this work lacked animal experiments to further verify the findings. This work constituted a pilot study and we plan to carry out further large-scale research including animal experiments in the future to further validate our findings.

Conclusion

This work has revealed that IRF2 and INPP4B have significantly high expression levels in the $CD4^+$ T-cells of patients with AML. We also showed that reducing the expression of IRF2 encouraged apoptosis in HL60 cells via an impact on the Th1/Th2 ratio. In contrast, the overexpression of IRF2 stimulated the JAK2-STAT3 signaling channel and downregulated caspase 3. Our work has thus revealed that the activation of the IRF2-INPP4B axis in $CD4^+$ T-cells exacerbated AML progression by stimulating the JAK2-STAT3 signaling channel and inhibiting the apoptosis of AML cells. This might help clarify the factors involved in AML progression while offering innovative guidance for the design of new AML therapies.

Ethics

Ethics Committee Approval: Ethical permission for this work was granted by the First Affiliated Hospital of Bengbu Medical College on June 25, 2021, with permit number [2021] No. 205. All experiments were conducted following the institutional ethical standards.

Informed Consent: Informed consent was obtained from all individual participants included in this study.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: X.X., M.Z., F.Z.; Concept: Y.Y., M.W.; Design: X.X., M.Z., F.Z.; Data Collection or Processing: X.X., M.Z., S.T., F.S.; Analysis or Interpretation: S.T., J.Z., J.L.; Literature Search: J.L., P.Z.; Writing: X.X., M.Z., F.Z.

Conflict of Interest: No conflict of interest was declared by the authors.

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