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PMA exerts anti-leukemia effect in Ph⁺ ALL through activating PKC δ and its down-stream molecules

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Abstract

Background Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph⁺ ALL) is a subtype of precursor B ALL in genetics and is recognized as a subclass with poor prognosis. Tyrosine kinase inhibitors (TKIs) greatly improve the prognosis of Ph⁺ ALL patients. However, the long-term survival rate is still low (with a 3-year survival rate only 55%). Hence, it is urgent to explore new therapies to improve prognosis for Ph⁺ ALL patients. Protein kinase C (PKC) is proven to be a tumor suppressor in recent years. Activation of PKC by its novel agonist PMA reverts the malignancy of many hematological diseases. However, the effect of PMA on Ph⁺ ALL is unclear.

Methods We investigated the biological effects of PMA on Ph⁺ ALL cells and the potential mechanism in this study. In this study, the SUP-B15 and BP190 cell lines were subjected to PMA treatment. Cell proliferation, cycle distribution, apoptosis, protein expression levels, and gene expression changes were assessed using CCK-8 assay, flow cytometry, Western blot analysis, DAPI staining, and quantitative real-time PCR. Additionally, bone marrow mononuclear cells were isolated for further investigation, and RNA sequencing was conducted on SUP-B15 cells. Furthermore, the mouse model was established to evaluate the in vivo biological effects of PMA.

Results We found that PMA could promote apoptosis, inhibit proliferation and promote differentiation of human Ph⁺ ALL cells. More importantly, we demonstrated for the first time that these effects caused by PMA were related to the activation of PKC δ and its down-stream molecules, such as p-ERK, p21 and so on.

Conclusions PMA exerts an anti-leukemia effect in Ph⁺ ALL by activating PKC δ and its downstream molecules, thereby demonstrating its potential as a therapeutic agent for Ph⁺ ALL.

Keywords Philadelphia chromosome, Acute lymphoblastic leukemia, PMA, PKC, p-ERK

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Introduction

Acute lymphoblastic leukemia (ALL) is a malignancy originated from abnormal proliferation of lymphoblasts in bone marrow. Philadelphia chromosome-positive (Ph⁺) ALL is a subtype of precursor B ALL in genetics. Ph chromosome is produced by the translocation between chromosome 9 and 22, and *BCR::ABL1* fusion gene is formed in the fusion site which encodes BCR::ABL1 oncoprotein with 190 kDa molecular weight. Ph chromosome is the most common chromosome abnormality in adult ALL [1], and Ph⁺ ALL is an aggressive subtype characterized by differentiation blockage with rapid progression and poor prognosis [2]. Tyrosine kinase inhibitors (TKIs) target to bind the ATP binding domain of BCR::ABL1 and greatly inhibit its kinase activity. However, the response of Ph⁺ ALL patients to TKIs is poor (with a 3-year survival rate of only 55% for TKIs therapy) [3]. Furthermore, the leukemia cells tend to develop TKI resistance quickly when treated with TKIs due to mutations in the BCR::ABL1 kinase domain, and recurrence is inevitable [4]. Until now there is no consensus on how to best treat Ph⁺ ALL [5, 6]. Therefore, it is necessary to develop new therapeutic methods for Ph⁺ ALL patients for better prognosis.

Protein kinase C (PKC) belongs to the serine-threonine protein kinase family with 9 subtypes, which divided into three subfamilies: classical PKC (cPKC: α , β , γ), novel PKC (nPKC: δ , ϵ , η , θ) and atypical PKC (aPKC: ζ , λ /i) [7]. PKC family is included in a variety of cellular functions, including cell survival, proliferation, apoptosis and migration [8]. Firstly, PKC family are proven to play a very important role in the occurrence and progression of cancer and is considered to be the target of anticancer therapy [9–14]. Then PKC is proven to be a tumor inhibitor rather than a tumor promoter with further research [15–18]. While the function of PKC is generally lost in cancer [19]. There is growing evidence that activation of PKC plays an important role in cancer regression [12, 20–22]. Specifically, the treatment of PKC-related cancer should mainly focus on restoring the activity of PKC [23–27]. For example, PKC is inhibited in chronic myelogenous leukemia (CML) and activation of PKC significantly reverts the malignancy of CML. The pathogenesis of CML is also BCR::ABL1 oncoprotein. While whether activation of PKC could play anticancer effect in Ph⁺ ALL is still unknown.

PMA (phorbol-12-myristate-13-acetate), which is also known as TPA (12-O-tetradecanoylphorbol 13-acetate), is a broad activator of PKC. PMA can bind to the C1B domain of cPKC and nPKC to activate them and then execute anti-tumor effect. It is proved that PMA exhibits strong anti-tumor effect in hematological diseases. For example, PMA promotes CML cells maturation [28, 29]. Meanwhile, PMA can promote the differentiation of

many types of leukemia cells, such as THP-1, K562, HL60 and U937 [30–33]. Taken together, the excellent anti-leukemia effect of PMA is confirmed by series of studies.

Considering the excellent anti-leukemia and differentiation promotion effect of PMA on leukemic cells, and the characteristic of differentiation completely blockage in Ph⁺ ALL, we wonder if PMA could induce cell differentiation and then execute anti-leukemia effect in Ph⁺ ALL. However, little is known about the effect of PMA on Ph⁺ ALL at present. Therefore, we investigated the biological effects of PMA on Ph⁺ ALL cells and the potential mechanism in this study. We tried to clarify the role of PKC in Ph⁺ ALL, and the potentiality of PMA as a therapeutic medicine for Ph⁺ ALL.

Materials and methods

Reagents

PMA, also called TPA, was purchased from Abmole (USA). RPMI 1640 and IMDM medium were purchased from Gibco (USA). CCK8 kit, Go6976 (classical PKC inhibitor) and Go6983 (novel PKC inhibitor) were purchased from TOPSCIENCE (China). Cell cycle and apoptosis detection kit were purchased from Tianjin Sungene Biotech (China). p21, CDK1, CDK2, p-BCR::ABL1, and c-ABL antibodies were obtained from Cell signal technology (USA). PKC α and PKC δ antibodies were purchased from Bimake (USA).

Cell lines

SUP-B15 is a B lymphoblast cell line that was isolated from the marrow of a White, 8-year-old, male patient with ALL, and is confirmed with Philadelphia chromosome. BP190 cell line is constructed by transferring p190 *BCR::ABL1* gene into Ba/F3 which is a precursor B cell line established from Balb/C mice. SUP-B15 cells were cultured in IMDM medium supplemented with 10% FBS. BP190 cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

CCK8 assay to detect cell proliferation

SUP-B15 cells and BP190 cells were plated into 96-well plates with 5000 cells per well. Cells were treated with different concentration of PMA for indicated time. Then 10 μ l of CCK8 solution was added to each well and incubated for 3 h. At last, the absorbance was measured at 450 nm with a microplate analyzer. Proliferation curves were plotted with Graphpad prism 8 based on absorbance value.

Flow cytometry to detect cell cycle and cell apoptosis

SUP-B15 cells and BP190 cells were seeded into 6-well plates with 5×10^5 cells per well. Cells were treated with different concentration of PMA for 48 h. For cell cycle analysis, cells were harvested and stained with PI/RNase

for 30 min at room temperature, then cell cycle distribution was analyzed by flow cytometry. For cell apoptosis analysis, cells were stained with Annexin V-FITC and PI, then cell apoptosis was analyzed by flow cytometry.

Western blot

SUP-B15 and BP190 cells were lysed with protein lysis buffer (RIPA: PMSF: NaVO_3 : NaF = 100: 1:1:1). Proteins were separated via SDS-PAGE and then transferred onto PVDF membrane. The membrane was subsequently blocked, and then incubated with corresponding primary antibody at 4 °C overnight, then incubated with the horseradish peroxidase-conjugated secondary antibody at room temperature for 90 min. Finally, target protein was visualized with chemiluminescence. All experiments were conducted with three biological replicates ($n = 3$).

DAPI staining

Cells were collected and spread on slides, then fixed with paraformaldehyde and stained with DAPI for 15 min at 37 °C. Finally, cells were sealed with glycerin and observed with the confocal microscope.

Quantitative real time PCR

Cells in logarithmic growth phase were treated with 0, 5, 10 or 20 ng/ml of PMA respectively for 48 h. Then cells were collected and mRNA was extracted with Trizol. mRNA was transformed into cDNA by reverse transcription. The cDNA product was as the template for PCR amplification. β -actin was used as an internal reference gene. All experiments were conducted with three biological replicates ($n = 3$).

Isolation of mononuclear cells from bone marrow

Bone marrow specimen was diluted with sample diluent by the ratio of 1:1. The diluted specimen was added to the upper layer of separation solution in a ratio of 1:1, then centrifuged at 900 g for 20 min. The cells in the white layer were sucked up and transferred to another sterile tube. Red blood cell lysis buffer was added, then PBS was added to stop lysis 5 min later. Eventually, bone marrow mononuclear cells were obtained after centrifugation for subsequent experiments.

The information for each bone marrow sample and related assays

We collected 3 bone marrow specimens with iron deficient anemia as the control group and 2 bone marrow specimens with Ph^+ ALL as the test group. The specific information for each sample is listed in Supplementary Table 2.

Bone marrow mononuclear cells (1×10^6 per well) were placed into 6-well plates and treated with 0 or 10 ng/ml PMA for 48 h. The changes of apoptosis were detected

by flow cytometry, and morphological changes were observed with inverted microscope.

Wright's staining

Cells were treated with 0, 5, 10 or 20 ng/ml of PMA for 48 h, then collected and smeared onto a slide. After natural drying, the cell smear was stained with Wright's stain (Baso, China) according to the instruction.

Flow cytometry to detect cell immunophenotype

SUP-B15 cells were plated into a 6-well plate with 5×10^5 cells for each well and treated with 0, 5, 10 or 20 ng/ml of PMA for 48 h respectively. Then cells were collected and resuspended with 100 μl PBS. Cell differentiation antigens were detected by flow cytometry in the Blood Laboratory of the Second Affiliated Hospital of Chongqing Medical University.

RNA sequencing

RNA sequencing was performed on SUP-B15 cells either treated with 10 ng/ml PMA for 48 h (PMA group) or left untreated (control group). Each group included three biological replicates. Total RNA was extracted using Trizol reagent. Sequencing was conducted by Shanghai Lifegenes Company (Shanghai, China). For bioinformatics analysis, raw sequencing data were first filtered to remove low-quality reads, adapter sequences, and reads with excessive unknown bases ($N > 5\%$) using Trimmomatic v0.39 to obtain clean reads. Clean reads were then aligned to the human reference genome (GRCh38/hg38) via HISAT2 v2.2.1, and gene expression levels were quantified as Fragments Per Kilobase of transcript per Million mapped fragments (FPKM) using StringTie v2.2.1. Differentially expressed genes (DEGs) between two groups were identified with DESeq2 v1.34.0, using P -value < 0.05 and absolute Fold Change ($|FC|$) ≥ 2 as the cutoff criteria. Functional annotation of DEGs was conducted via Gene Ontology (GO) enrichment analysis using the clusterProfiler R package (v3.12.0), while Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed with KOBAS v3.0 software.

Animal experiments

NOD/SCID mice with 5–6 weeks age were selected. Fifteen mice were randomly divided into 3 groups (Control, 10 $\mu\text{g/kg}$ of PMA, 50 $\mu\text{g/kg}$ of PMA) and received 250 cGy irradiation. Then 5×10^6 of SUP-B15 cells in 200 μl PBS were injected into each mouse intravenously. 7 days later, mice were injected with DMSO, 10 $\mu\text{g/kg}$ or 50 $\mu\text{g/kg}$ of PMA for each group respectively by tail vein. Subsequently, body weight and white blood cells were monitored weekly. The symptoms of acute lymphoblastic leukemia in mice are weight loss, hair opacity and claudication. When mice developed cachexia, we euthanized

and dissected them. All animal experiments were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No.8023, revised 1978) and were conducted with the approval of the Biomedical Ethics Committee of Chongqing Medical University.

White blood cell count assay

The peripheral blood of mice was collected by tail vein and lysed with 3% glacial acetic acid. The mixed cell suspension was pooled into a Bovine Bowie counting plate, and then counted under microscope.

Immunofluorescence (IF) assay

Cells were collected and smeared on slides, followed by fixation with 4% paraformaldehyde. Afterward, cells were permeabilized with 1% Triton X-100, and blocked with goat serum. After that, cells were incubated with anti-BCR::ABL1 (ZEN-BIOSCIENCE, China) primary antibody at 4 °C overnight, subsequently with Cy3-labeled secondary antibody (Invitrogen, USA) at 37 °C for 1 h. The nucleus was stained with DAPI. Finally, the smears were sealed with glycerol and observed with the confocal microscope.

H&E staining

Tissue sections were prepared by a conventional method, then deparaffinized with xylene and rehydrated in a descending series of ethanol solutions. Samples were then stained with hematoxylin for 10 min, differentiated with 0.7% ethanol hydrochloride for a few seconds, rinsed with running water until turning blue. After that, the sections were treated with 95% ethanol for 30 s, stained with alcoholic eosin for 30 s, and then treated with 95% and 100% ethanol for 30 s. Subsequently, the sections were treated with xylene carbamate for 30 s, and with xylene for 30 s. Finally, samples were sealed with neutral gum, and observed with microscope.

Immunohistochemistry assay

Tissue sections were prepared by a conventional method, then deparaffinized with xylene and rehydrated in a descending series of ethanol solutions. Samples retrieval was performed in 10 mmol/l sodium citrate solution (pH 6.0) at 100 °C for 16 min and then cooled for 30 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide and then tissue sections were blocked with 5% goat serum for 30 min. After that, the slides were incubated with primary antibody at 4 °C overnight, subsequently with anti-rabbit secondary IgG antibody at 37 °C for 1 h. Finally, samples were stained with 3, 3-diaminobenzidine and Mayer's hematoxylin. Slides, then observed with microscope.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0 software. The results of quantitative data were presented as mean $\pm$ standard deviation ($\bar{x} \pm s$). The t-test was used for comparison between two groups, and the variance analysis was used for comparison among multiple groups. $P < 0.05$ indicated that the difference was statistically significant.

Results

PMA inhibited cell proliferation in human Ph⁺ ALL cells

Firstly, we examined the 50% inhibitory concentration (IC₅₀) values of PMA in BP190 and SUP-B15 cells by CCK-8 assay (Fig. 1A), and then selected a specific concentration gradient for the subsequent experiment based on the IC₅₀ value. We investigated the effect of PMA on the proliferation of Ph⁺ ALL cells (including SUP-B15 and BP190 cell lines). As shown in Fig. 1B, PMA could significantly inhibit the proliferation of SUP-B15 cells when the concentration was above 2.5 ng/ml and treated for more than 48 h. The proliferation inhibition effect of PMA on BP190 cells was not so significant. The inhibitory effect was significant only at 48 h, and the inhibitory effect was not continual (Fig. 1C). Cell proliferation is inseparable from the regulation of cell cycle, so we tested the effects of PMA on the cell cycle of Ph⁺ ALL cells. As shown in Fig. 1D, the percentage of SUP-B15 cells in S phase decreased significantly (from 62.7% to 22.78%) after treatment with different concentrations of PMA. The percentage of BP190 in S phase decreased slightly (from 52.52% to 38.78%) by PMA (Fig. 1E). S phase is the phase for DNA synthesis, and the decline in S phase indicates that the proliferation of tumor cells is inhibited. Correspondingly, the percentage of BP190 and SUP-B15 cells in G2/M phase significantly increased, especially in SUP-B15 cells. Then we tested the transcription and protein expression of several cell cycle related molecules. Firstly, we detected the transcription level of c-Myc and p21, and found that the mRNA level of c-Myc decreased significantly, while p21 increased significantly in SUP-B15 cells. However, the changes of the mRNA level of c-Myc and p21 were not obvious in BP190 cells (Fig. 1F and G). Secondly, we detected the changes of cell cycle related proteins in Ph⁺ ALL cells treated with PMA for 48 h. As shown in Fig. 1H, the expression of c-Myc, CDK1 and CDK2 decreased significantly, and the expression of CCND1 and p21 increased significantly in SUP-B15 cells by PMA. While these proteins did not change obviously in BP190 cells by PMA. Taken together, PMA could significantly inhibit the proliferation of SUP-B15 cells.

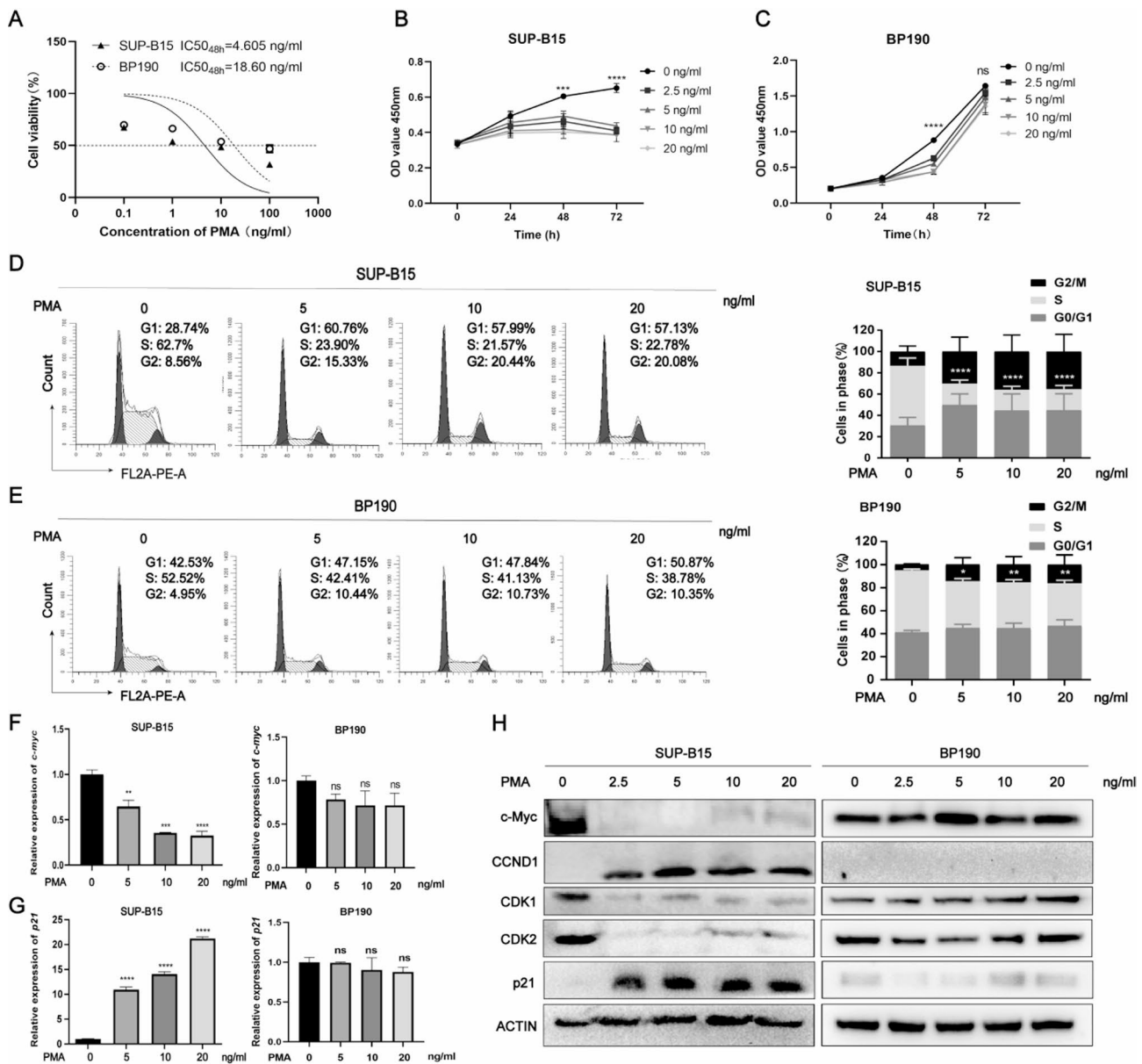


Fig. 1 PMA inhibited cell proliferation in human Ph⁺ ALL cells. **A**, IC₅₀ value for each cell line was calculated by CCK-8 assay. **B**, **C**, SUP-B15 or BP190 cells were treated with the indicated dose of PMA for 0, 24, 48–72 h respectively. Then cell viability was determined with CCK8 assay. The experiment for each group was repeated for three times. **** indicates $P < 0.001$. ***** indicates $P < 0.0001$. **D**, **E**, Cell cycle distribution was determined by flow cytometry. The experiment for each group was repeated for three times. *** indicates $P < 0.05$. **** indicates $P < 0.01$. ***** indicates $P < 0.0001$. (E. 0 ng/ml vs. 5 ng/ml $P = 0.0216$, 0 ng/ml vs. 10 ng/ml $P = 0.0043$, 0 ng/ml vs. 20 ng/ml $P = 0.0048$). **F**, **G**, Cells were treated with PMA for 48 h, after which the transcriptional level of *c-myc* or *p21* was detected by quantitative real-time PCR. The experiment for each group was repeated for three times. **** indicates $P < 0.01$. ***** indicates $P < 0.001$. ***** indicates $P < 0.0001$. **H**, Cells were treated with PMA for 48 h, after which the expression of c-Myc, CCND1, CDK1, CDK2 or p21 was measured by western blot. ACTIN was used as internal reference

PMA promoted cell apoptosis in human Ph⁺ ALL cells

The occurrence of tumor is often related to apoptosis obstruction, so we tested the effect of PMA on the apoptosis of Ph⁺ ALL cells. As shown in Fig. 2A, the percentage of apoptotic cells induced by PMA significantly increased in SUP-B15 cells, even up to 90% when treated with 10 ng/ml or 20 ng/ml of PMA. While PMA had no obvious effect on the apoptosis of BP190 cells

(Fig. 2B). The morphological changes of apoptotic cells were detected by DAPI staining. Fragmentation, marginal chromatin, pyknosis, and inhomogeneity appeared in cell nuclei of SUP-B15 cells after PMA treatment. While there was no obvious change in BP190 cell nuclei (Fig. 2C). Meanwhile, the expression of apoptosis related proteins was detected. We found that PMA could cause PARP cleavage and reduce the expression of BCL-2

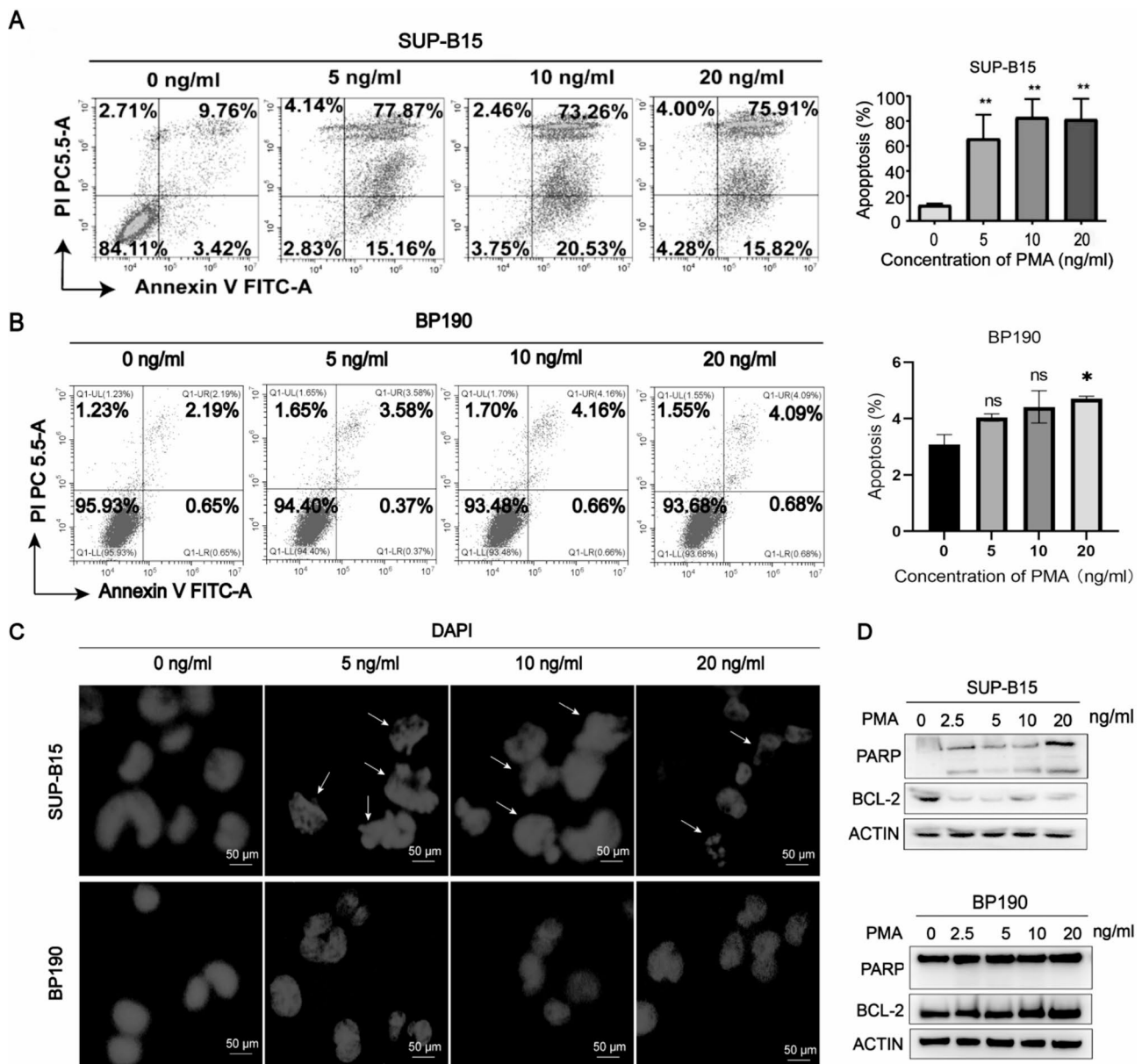


Fig. 2 PMA promoted cell apoptosis in human Ph⁺ ALL cells. SUP-B15 or BP190 cells were treated with the indicated dose of PMA for 48 h. **A, B.** Then cell apoptosis was determined by flow cytometry. The experiment for each group was repeated for three times. ****** indicates $P < 0.05$. ******* indicates $P < 0.01$. (**A.** 0 ng/ml vs. 5 ng/ml $P = 0.0028$, 0 ng/ml vs. 10 ng/ml $P = 0.0075$, 0 ng/ml vs. 20 ng/ml $P = 0.0064$. **B.** 0 ng/ml vs. 20 ng/ml $P = 0.0138$) **C.** The influence of PMA on nucleic morphology of SUP-B15 or BP190 cells was detected by DAPI staining ($\times 400$). **D.** The expression of PARP or BCL-2 was tested by western blot. ACTIN was used as internal reference

which executes anti-apoptotic effect in SUP-B15 cells. The cleavage of PARP is direct evidence that PMA could induce SUP-B15 cell apoptosis. While there was no cleavage band of PARP and little alteration in the expression of BCL-2 in BP190 cells. These results were consistent with the results of flow cytometry and DAPI staining. Taken together, the data in this part proved that PMA could promote cell apoptosis through inhibition of BCL-2 in human Ph⁺ ALL cells.

PMA induced cell differentiation in human Ph⁺ ALL cells

Under the treatment of PMA, we unexpectedly found that PMA could significantly induce morphological changes of SUP-B15 cells but had little effect on BP190 cells. SUP-B15 cells were round, smooth and uniform in size in the control group. While after PMA treatment, a large number of vacuoles appeared in the cells, and the volume of SUP-B15 cells became larger. Meanwhile, some spindle adherent cells appeared (Fig. 3A and B). In order to further ascertain whether the morphological changes

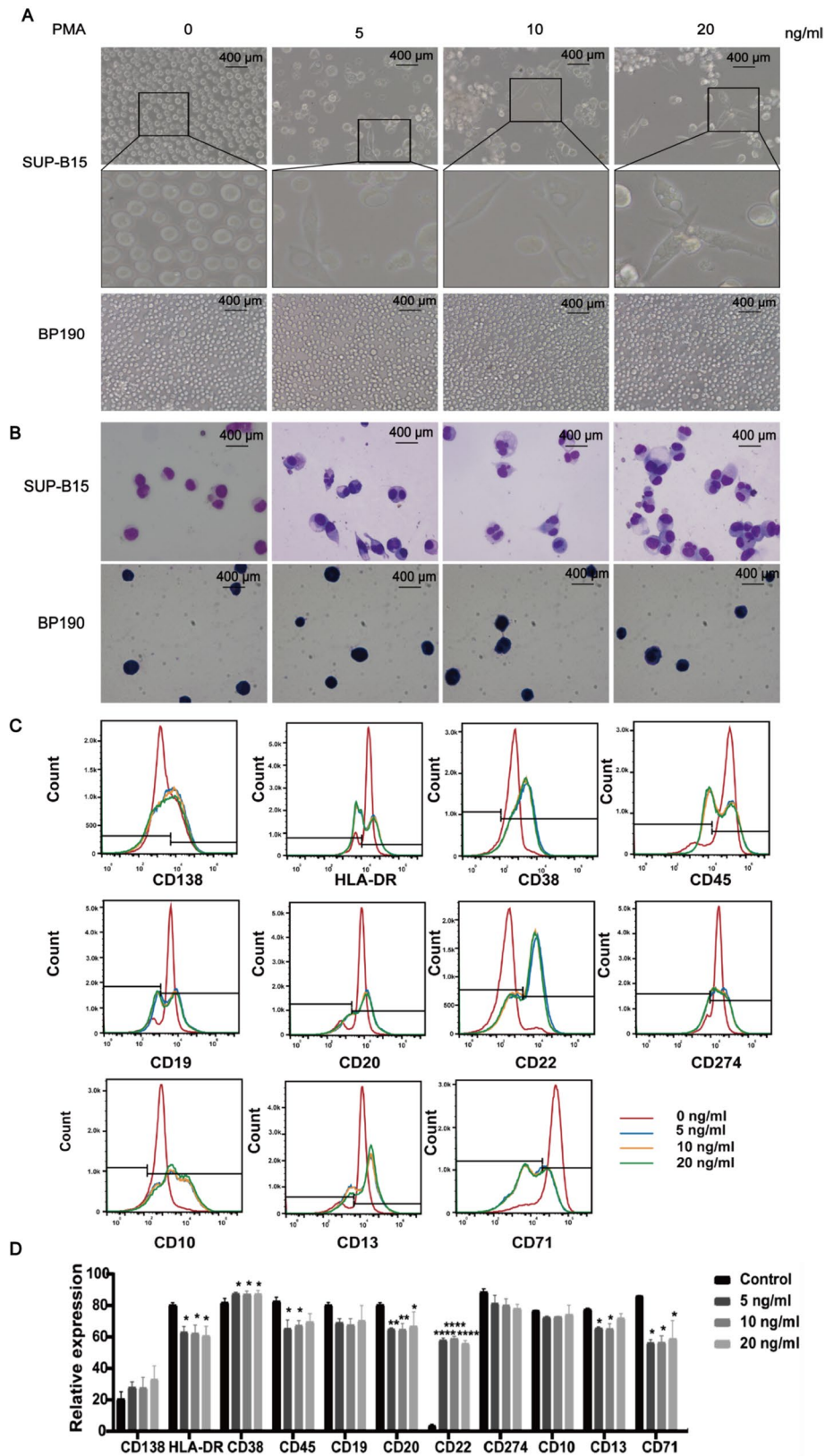


Fig. 3 PMA induced cell differentiation in human Ph⁺ ALL cells. SUP-B15 or BP190 cells were treated with the indicated dose of PMA for 48 h. **(A)** The morphological changes of cells were observed with inverted microscope (×200). **(B)** The morphological changes of cells were analyzed by Wright's staining and observed with microscope (×400). **(C, D)** The surface antigens of SUP-B15 cells were determined by flow cytometry. The experiment for each group was repeated for three times. "*" indicates $P < 0.05$. "**" indicates $P < 0.01$. "***" indicates $P < 0.001$. "****" indicates $P < 0.0001$

of SUP-B15 after PMA treatment were caused by cell differentiation, we detected the immunophenotype of SUP-B15 cells. It is recorded that SUP-B15 cell line is derived from the bone marrow of an 8-year-old child with Ph⁺ B-ALL, therefore we detected some antigens related to different stages of B cells, including Pro-B, Pre-pre-B, Pre-B, immature-B and mature B. As the morphology of SUP-B15 induced by PMA looks like plasma cell, we also selected some antigens related to plasma cell. The specific surface differentiation antigens of SUP-B15, precursor B cell, mature B cell and plasma cell were displayed in Supplementary Table 1. CD274 is known as PDL1 and it can help tumor immune escape [34, 35]. Thus, we tested the expression of CD138, HLA-DR, CD38, CD45, CD19, CD20, CD22, CD10, CD13 and CD71 on cell surface of SUP-B15.

As shown in Fig. 3C and D, the expression of HLA-DR decreased, CD38 was up-regulated, and CD20 decreased after PMA treatment, which is in line with the differentiation phenotype characteristics of B lymphocytes into plasma cells. CD138 was up-regulated slightly, while CD19 and CD274 decreased slightly, but the difference was not statistically significant. Interestingly, the expression of CD22 increased significantly after PMA treatment. Although CD22 increases significantly during the process of B lymphocyte differentiation and maturation, it is not expressed on the surface of plasma cells. Hence the significant increase of CD22 indicated that PMA did induce SUP-B15 cells to differentiate and mature. The expressions of CD71 and CD13 decreased after PMA treatment. CD71 is the transferrin receptor, and it is generally used to label red blood cells. However, studies have shown that it is more likely to express CD71 in childhood acute lymphoblastic leukemia, and patients with high CD71 often have a lower 5-year overall survival and disease free survival [36]. Meanwhile, it is reported that CD13 can enhance tumor invasiveness, promote tumor angiogenesis and proliferation, and inhibit tumor cell apoptosis [37, 38]. Hence it showed excellent anti-cancer function of PMA by the result that PMA made CD71 and CD13 decrease in SUP-B15 cells. Taken together, the results in this part suggested that PMA promoted SUP-B15 cells to differentiate into mature B cells with plasma cell phenotype and attenuated the malignancy of SUP-B15 cells.

PMA activated lymphocyte differentiation pathway, cycle arrest pathway and ERK pathway

To investigate how PMA affects proliferation, apoptosis and differentiation of SUP-B15 cells, we performed transcriptome sequencing and obtained RNA-seq data from the Control and PMA group. It showed that up-regulated differentially expressed genes (DEGs) in the PMA group were particularly enriched in the terms of endoplasmic

reticulum unfolded protein response (UPR), regulation of ERK1 and ERK2 cascade, and lymphocyte differentiation by Gene Ontology (GO) enrichment analysis (Fig. 4A); while down-regulated differentially expressed genes were particularly enriched in the terms of DNA replication, cell cycle G1/S phase transition, and cell cycle G2/M phase transition (Fig. 4B). These results were consistent with our findings that PMA inhibited proliferation and promoted differentiation of SUP-B15 cells. It is reported that UPR pathway takes part in the regulation of terminal B cell differentiation to plasma cells [39] and ERK signaling pathway plays an important role in cell migration and differentiation [40]. Considering the role of ERK on cell differentiation and our findings suggesting that PMA promoted cell differentiation and upregulated ERK, we speculated that PMA promoted differentiation and increased adhesion of Ph⁺ ALL cells by activation of ERK pathway. The volcano plot showed several significantly up-regulated or down-regulated genes, such as CTSL, IGF1R, PRKDC, CDC45, and PCNA which take part in lymphocyte differentiation, MAPK regulation and G1/S phase transition respectively (Fig. 4C). Then we detected the mRNA level of several representative up-regulated or down-regulated genes in SUP-B15 cells after PMA treatment to verify the result of RNA-seq. As shown in Fig. 4E, NABP1, SP6, CTSL, TIMP1, and IGF1R significantly increased, while CENPE, PCNA, CDC45, PRKDC and NASP significantly decreased in concentration dependent manner (Fig. 4F). These results were consistent with the RNA-seq data. We then analyzed the RNA-seq data by combination of STRING (<https://string-db.org>) and Cytoscape software to screen for differential genes. The result suggested that MAPK13 node was enriched (Fig. 4D) which further supported the result of GO enrichment analysis (Fig. 4A).

PMA exerted its function by activating PKC δ

PKC family contains 9 subtypes which are divided into three subfamilies: classical PKC (α , β , γ), novel PKC (δ , ϵ , η , θ) and atypical PKC (ζ , λ , ι). The classical and novel PKC contains C1B domain which is the binding region for the physiological PKC activator DAG. While atypical PKC does not have the C1B domain [41]. PMA shares similar structure with DAG, so it can activate classical and novel PKC, but not atypical PKC. In order to determine the specific subtype on which PMA functions, we applied two PKC inhibitors (Go6976 and Go6983) in combination with PMA to test whether PKC inhibitors could revert the action of PMA. Go6976 is an inhibitor of classical PKC and mainly inhibits PKC α and β . Go6983 is a broad PKC inhibitor which can inhibit the activity of PKC α , β , γ , and δ . SUP-B15 cells were treated with different concentrations of Go6976 in combination with PMA, then cell viability was detected. Go6976 did

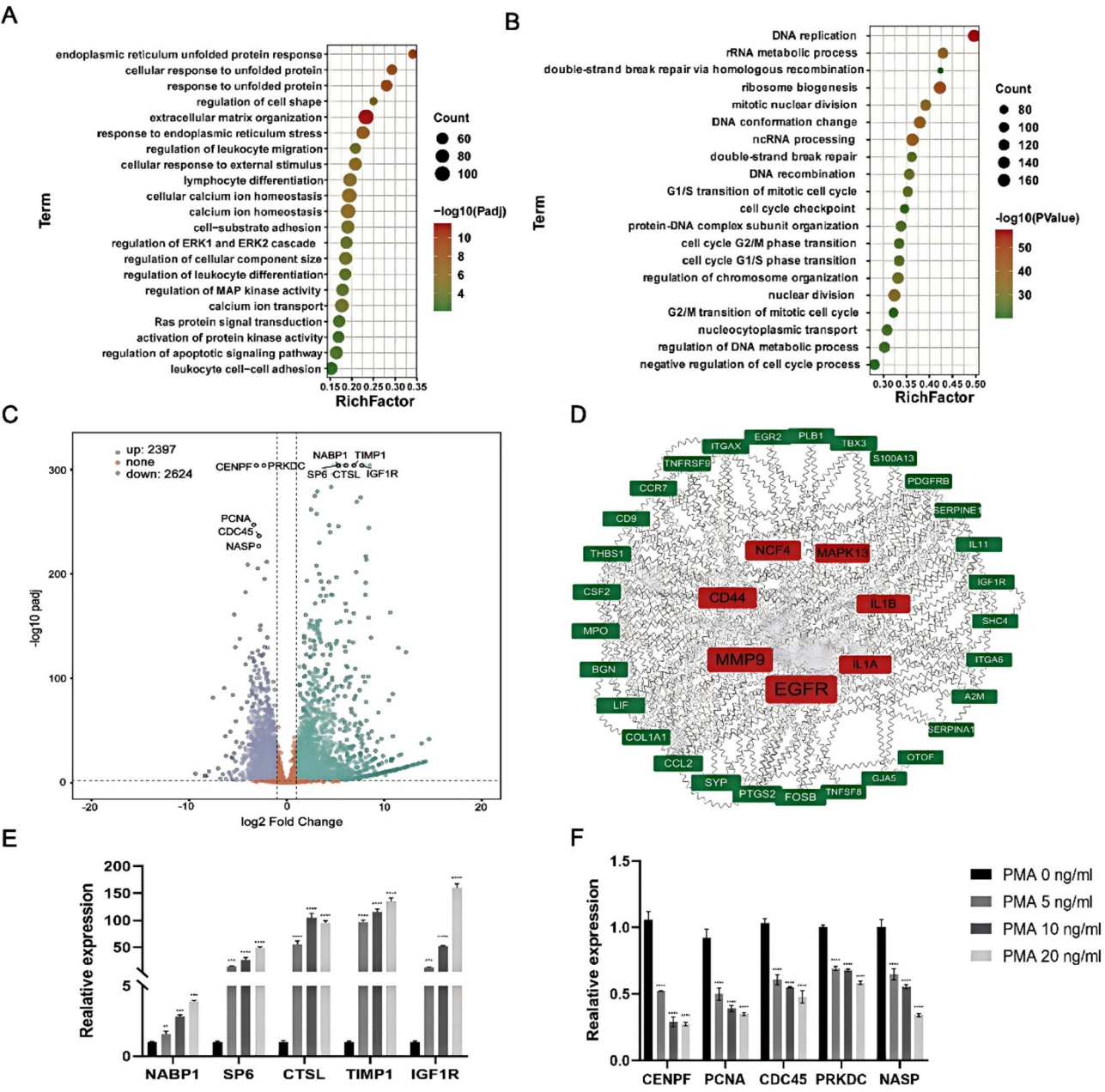


Fig. 4 PMA activated lymphocyte differentiation pathway, cycle arrest pathway and ERK pathway. Transcriptome sequencing was performed and RNA-seq data was analyzed for the Control and PMA group (SUP-B15 cells treated with 10 ng/ml PMA for 48 h). **A, B**. GO enrichment analysis of the up-regulated (**A**) or down-regulated (**B**) differentially expressed genes (DEGs); **C**. The volcano plots of RNA-seq data. p adjust value < 0.05 was regarded as significant. **D**. Critical node Screening analysis by STRING (<https://string-db.org>) with Cytoscape software, which sorted by betweenness centrality. Red and larger boxes and fonts represent higher scores. **E, F**. SUP-B15 cells were treated with the indicated dose of PMA for 48 h. Then the transcriptional level of the top five up-regulated (**E**) or down-regulated (**F**) DEGs was detected by quantitative real-time PCR. The experiment for each group was repeated for three times. *** indicates $P < 0.01$. **** indicates $P < 0.001$. ***** indicates $P < 0.0001$

not restore the effect of PMA on the proliferation (Fig. 5A) and morphology (Fig. 5C) of SUP-B15 cells. These results suggested that PMA did not function by activating classical PKC (α , β , γ). We then treated SUP-B15 cells with Go6983 to test whether it could restore the effect of PMA. As shown in Fig. 5B, 1 μM of Go6983 could almost completely restore the anti-proliferation effect of PMA.

Correspondingly, it was observed that Go6983 could obviously restore the morphological changes of SUP-B15 induced by PMA (Fig. 5C). These results indicated that PMA exerted its function on SUP-B15 through activation of PKC δ . We then detected the expressions of PKC α and PKC δ in order to verify the speculation. As shown in Fig. 5D, the expression of PKC α did not alter obviously,

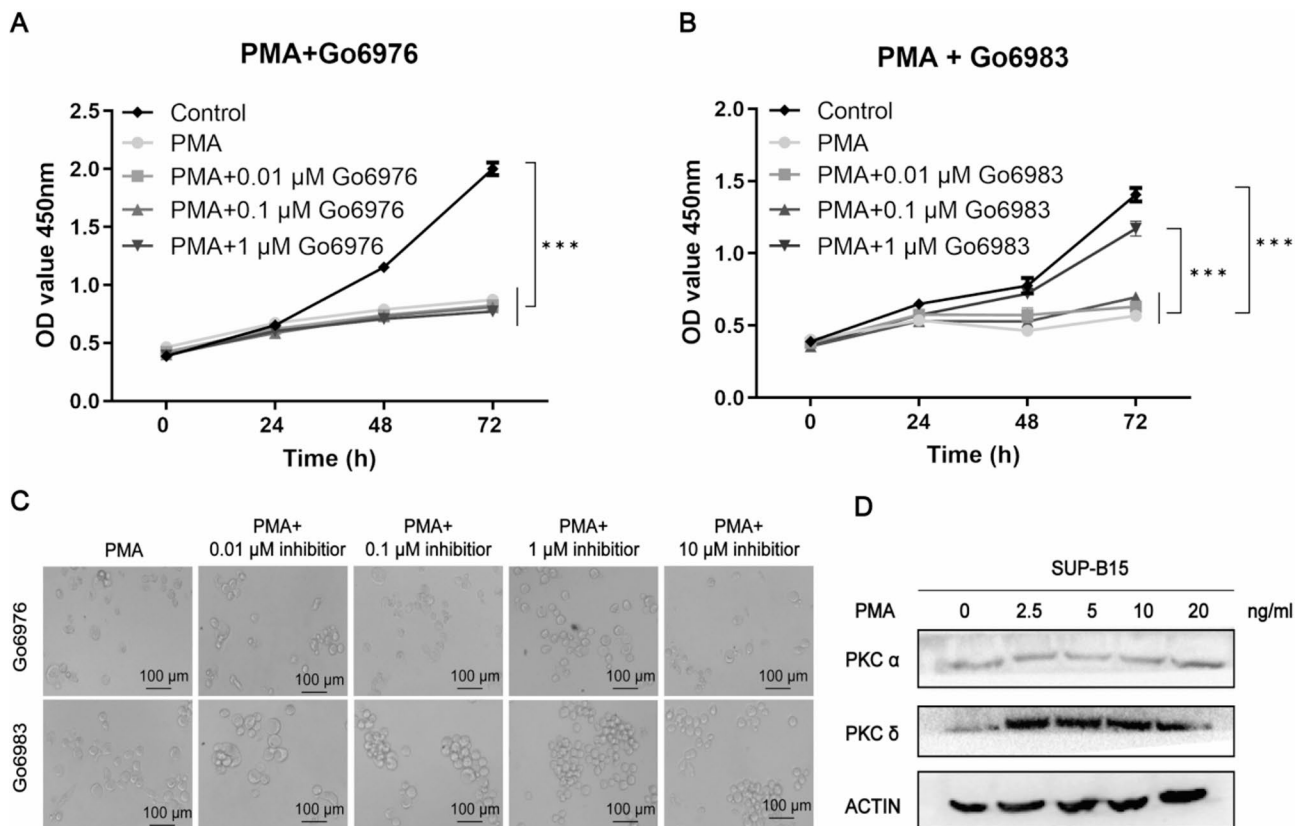


Fig. 5 PMA exerted its function by activating PKC δ . **A, B.** SUP-B15 cells were treated with 10 ng/ml PMA or in combination with Go6976 (**A**) or Go6983 (**B**) for indicated time, then cell viability was determined by CCK8 assay. There were three replicates for each group. "****" indicates $P < 0.001$. (**A.** $P = 0.0007$, **B.** Control vs. PMA $P = 0.0001$, PMA vs. PMA + 1 μ M Go6983 $P = 0.0003$) **C.** The morphological changes of cells were observed with inverted microscope. **D.** SUP-B15 cells were treated with the indicated dose of PMA for 48 h. Then the expression of PKC α or PKC δ was tested by western blot. ACTIN was used as internal reference

but PKC δ was significantly up regulated by PMA. Taken together, PMA exerted its function by activating PKC δ .

PMA activated PKC δ , which in turn activated p-ERK and p21 and down-regulated c-Myc simultaneously

It has been reported that PMA can down-regulate BCR::ABL1 on the transcription level in K562 cells which are typical CML cells driven by p210 BCR::ABL1 [42]. SUP-B15 is a typical Ph⁺ ALL cell line driven by p190 BCR::ABL1, so we detected the effect of PMA on the transcription and protein level of p190 BCR::ABL1 in SUP-B15 cells. The results showed that PMA significantly down-regulated the phosphorylation of p190 BCR::ABL1, but had no obvious effect on the total protein level (Fig. 6A) and no significant effect on the transcription level of p190 BCR::ABL1 (Fig. 6B). Then we wanted to research the downstream molecules or pathways of BCR::ABL1 affected by PMA. It was suggested that the regulation of ERK1 and ERK2 cascade (ERK1 and ERK2 are members of MAPK family) was up-regulated by the GO enrichment analysis (Fig. 4A) and MAPK13 node was enriched by RNA-seq data analysis (Fig. 4D). It has been reported

PMA activates the MAPK pathway in K562, and the MAPK pathway is the downstream of BCR::ABL1 [31, 43]. Therefore, we detected the level of ERK. The results proved that p-ERK was significantly activated by PMA (Fig. 6A). To further confirm the key role of p-ERK in mediating the anti-leukemia effect of PMA, we applied U0126 to selectively inhibit the phosphorylation of ERK1/2, then we tested whether the effect of PMA on SUP-B15 cells could be restored. The results showed U0126 had no obvious cytotoxicity on SUP-B15 alone with the concentration less than 1 μ M (Fig. 6C). When in combination with PMA, U0126 reversed the PMA-induced inhibition of cell proliferation (Fig. 6D). Meanwhile, U0126 could restore the effect of PMA on the morphology change of SUP-B15 (Fig. 6E). These results verified that PMA indeed executed its anti-leukemia effect by activating p-ERK. But U0126 could not fully restore the effect of PMA, so we speculated that PMA exerted its function not only through p-ERK, but also other molecules. It is reported that the ERK pathway is a downstream effector of PKC, so we wondered whether PMA activates p-ERK through PKC δ in SUP-B15 cells.

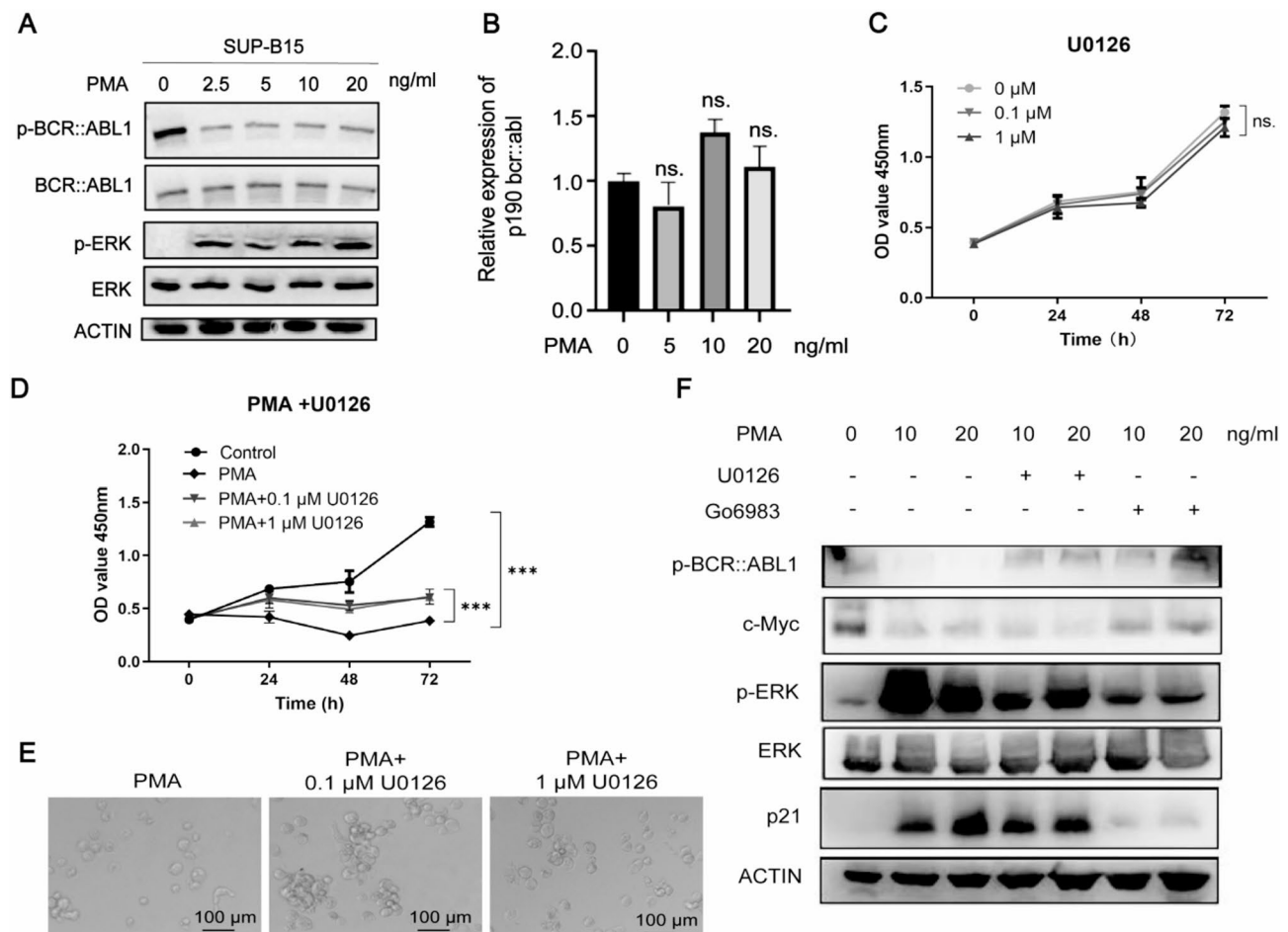


Fig. 6 PMA activated PKC δ , which in turn activated p-ERK and p21 and down-regulated c-Myc simultaneously. **A, B.** SUP-B15 cells were treated with the indicated dose of PMA for 48 h. Then the level of BCR::ABL1, p-BCR::ABL1, ERK, and p-ERK was detected by western blot (**A**), and the transcriptional level of *p190 BCR::ABL* was detected by quantitative real-time PCR (**B**). **C, D.** SUP-B15 cells were treated with U0126 alone (**C**) or in combination with PMA (**D**) for indicated time, and then cell viability was determined with CCK8 assay. **** indicates $P < 0.001$. (**D**, $P = 0.0007$) **E.** SUP-B15 cells were treated with U0126 alone or in combination with PMA for 48 h, then cell morphology was observed with microscope ($\times 200$). **F.** SUP-B15 cells were treated with PMA alone or in combination with U0126 or Go6983 for 48 h, and then the level of p-BCR::ABL1, p-ERK, ERK, c-Myc and p21 was detected by western blot. ACTIN was used as internal reference

Therefore, we pretreated the cells with U0126 or Go6983 for 1 h, then added PMA for 48 h. We found that both U0126 and Go6983 can significantly inhibit the activation of p-ERK caused by PMA (Fig. 6F). This result indicated that the activation of p-ERK was due to the activation of PKC δ . Meanwhile, Go6983 could significantly inhibit the increase of p21 and the decrease of c-Myc caused by PMA, but U0126 could not. These results indicated that p21 and c-Myc were regulated by PKC δ , but not p-ERK. Both U0126 and Go6983 could restore the phosphorylation level of BCR::ABL1 reduced by PMA, indicating that the down-regulation of p-BCR::ABL1 induced by PMA was regulated by p-ERK and PKC δ activation. Taken together, PMA executes its function by activating PKC δ , then PKC δ activates p-ERK and p21 and down-regulates c-Myc simultaneously.

PMA can promote apoptosis of bone marrow cells from Ph⁺ ALL patients

As shown above, PMA could significantly inhibit proliferation and promote apoptosis of SUP-B15 cell line. Then we wanted to explore the anti-leukemia effect of PMA on bone marrow cells from Ph⁺ ALL patients. Therefore, we collected 3 bone marrow samples from iron deficiency anemia (IDA) patients as control and 2 bone marrow samples from Ph⁺ ALL patients and detected the effect of PMA on apoptosis of these bone marrow cells. The results showed that PMA made no statistical difference on apoptosis rate of IDA bone marrow samples (Fig. 7A–C, F), but significantly increased the apoptosis rate of Ph⁺ ALL bone marrow cells (Fig. 7D, E, G). Then we observed the morphology change caused by PMA. We found that there was no obvious morphology change induced by PMA in IDA bone marrow cells. However, cells clumped

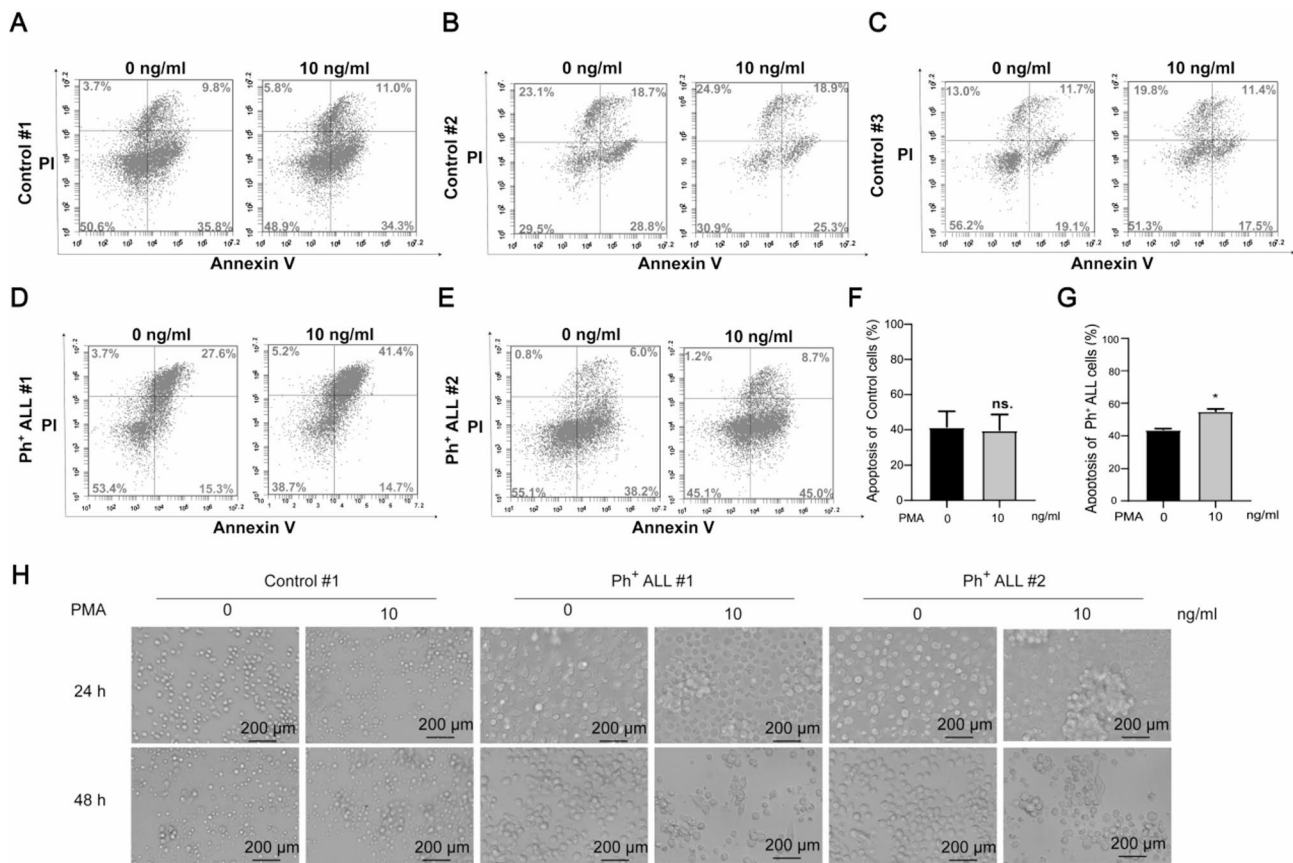


Fig. 7 PMA can promote apoptosis of bone marrow cells from Ph⁺ ALL patients. **A–C.** Bone marrow cells from 3 IDA patients were treated with PMA for 48 h, and then cell apoptosis was detected by flow cytometry. **D, E.** Bone marrow cells from 2 Ph⁺ ALL patients were treated with PMA for 48 h, and then cell apoptosis was detected by flow cytometry. **F, G.** The results of PMA on the apoptosis of bone marrow cells from IDA patients (**F**) or Ph⁺ ALL patients (**G**) were statistically analyzed. “*” indicates $P < 0.05$. **H.** The morphological changes of bone marrow specimens were observed with microscope (x200)

together when treated with 10 ng/ml PMA for 24 h, and fusiform adherent cells appeared when treated for 48 h in Ph⁺ ALL bone marrow cells (Fig. 7H). These results elucidated that PMA could exerted anti-leukemia effect on bone marrow cells from Ph⁺ ALL patients.

PMA exerts anti-leukemia effect in Ph⁺ ALL mice

In order to determine the effects of PMA in vivo, we established Ph⁺ ALL mice model and administrated PMA (Fig. 8A). The body weights of the mice were periodically recorded, and peripheral blood was collected by tail vein for WBC counts detection. As shown in Fig. 8B, the WBC counts of the PMA treatment group were significantly lower than that of the control group. The disease mice were executed and the body weight, liver weight and spleen weight were recorded. As shown in Fig. 8C, the mice body weight exhibited a significant decrease in each group. The liver weight and spleen weight of the PMA treatment group were significantly lower than those of the control group (Fig. 8D, E), which indicated that the hepatosplenomegaly was more severe in the control group. Then the expression of BCR::ABL1 in the

cells from bone marrow, liver and spleen was detected by immunofluorescence assay to assess the infiltration of SUP-B15 cells in these organs. The results showed that BCR::ABL1 fusion protein was detected in the bone marrow, liver and spleen from the control group, but not in the PMA treatment group (Fig. 8F). The liver and spleen sections were dyed by HE staining to analyze the filtration of leukemic cells. The results revealed that spleen samples from the control group displayed severe infiltration of leukemic cells and structural damage. In contrast, samples from PMA group showed little leukemic cells infiltration and slightly structural damage. Similar pathological changes were observed in the liver (Fig. 8G). Meanwhile, we detected the expression of CD22 and CD138 in the liver and spleen to assess differentiation of infiltrated leukemic cells by immunohistochemical assay. The results showed that there was no significant difference in the expression of CD22 and CD138 in the liver tissue between each group, but CD22 and CD138 expressed highly in the spleen tissue from the PMA group compared with the control group (Fig. 8H), which suggested that PMA could promote SUP-B15 cells

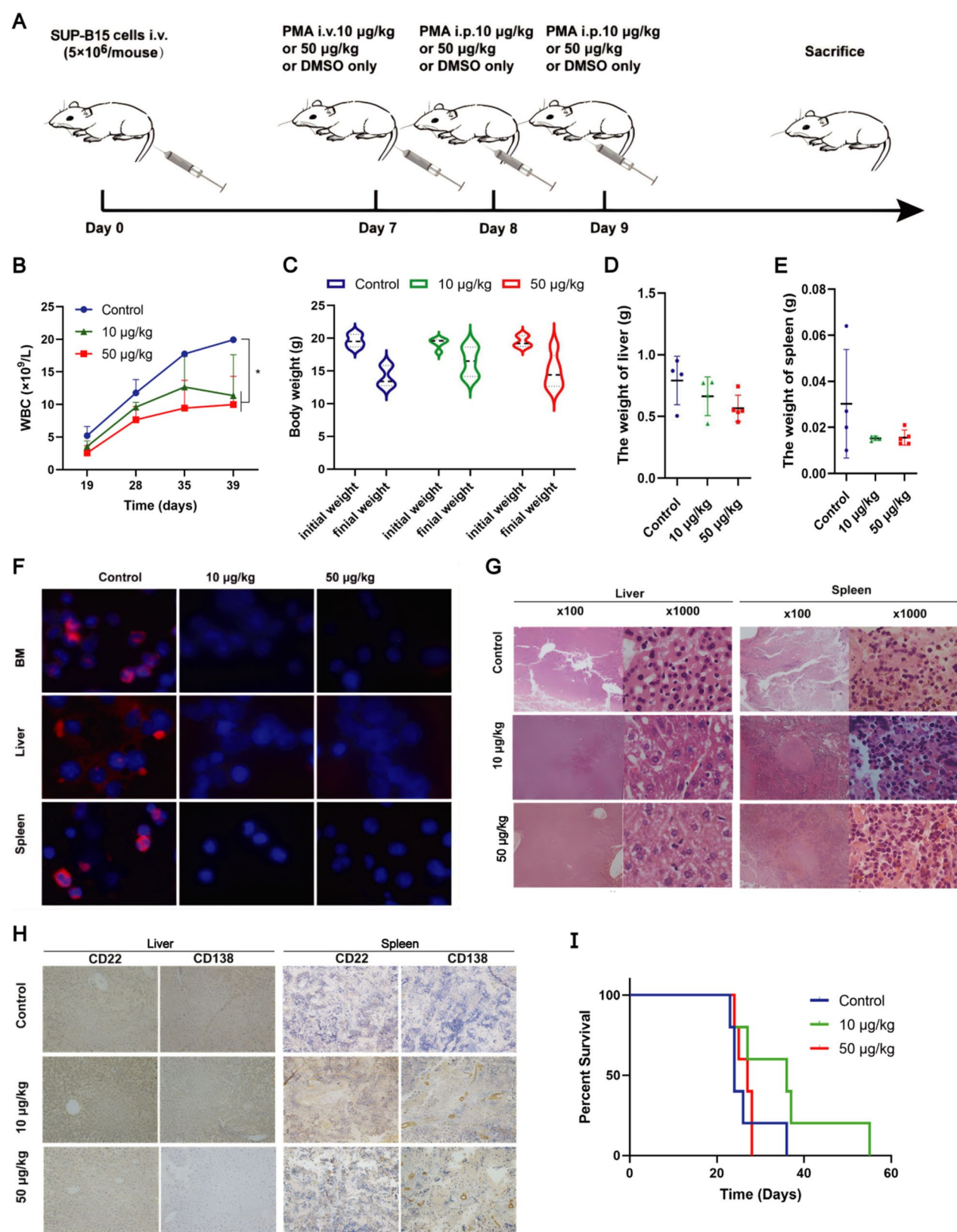


Fig. 8 (See legend on next page.)

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Fig. 8 PMA exerts anti-leukemia effect in Ph⁺ ALL mice. **(A)** The treatment schedule for Ph⁺ ALL model mice. Each group consisted of five mice. **(B)** The peripheral blood was collected by tail vein periodically, and WBC counts were detected for each mouse. *** indicates $P < 0.05$. (Control vs. 10 $\mu\text{g/kg}$ $P = 0.0158$, Control vs. 50 $\mu\text{g/kg}$ $P = 0.0155$) **(C)** The body weight of each mouse was periodically recorded, and the body weight from initial to final was statistically analyzed for each group. **(D, E)** The liver weight (D) and spleen weight (E) of each mouse were recorded and statistically analyzed for each group. **(F)** The expression of BCR::ABL1 in cells from bone marrow, liver, or spleen of each group was detected by immunofluorescence assay. **(G)** The infiltration of leukemic cells in liver and spleen for each group was measured by H&E staining. **(H)** The expression of CD22 and CD138 in liver and spleen was detected by immunohistochemistry. The brown indicated positive signals. **(I)** Kaplan-Meier survival curves of mice in each group

differentiation *in vivo*. The difference in survival of each group was compared, and Kaplan-Meier curves showed that PMA significantly prolonged survival of the mice in 10 $\mu\text{g/kg}$ group ($P < 0.05$) but not in 50 $\mu\text{g/kg}$ group (Fig. 8I). Taken together, the results in this part reveal that PMA can exert anti-leukemia effect in Ph⁺ ALL mice.

Discussion

Currently, the combination of cytotoxic chemotherapy and TKIs can greatly improve the prognosis of Ph⁺ ALL patients, but the overall survival rate is still low. Meanwhile, drug resistance and relapse are still unavoidable, so there is an urgency to explore new therapies. In this study, we explored the biological effects of PMA on Ph⁺ ALL cells and the underlying mechanism. We found that PMA could promote apoptosis, inhibit proliferation and promote differentiation of human Ph⁺ ALL cells. We proved that these effects caused by PMA were related to the activation of PKC δ and its downstream molecules, such as p-ERK, c-Myc, p21, BCL-2 and so on. The aim of this study is to provide therapeutic alternative for Ph⁺ ALL treatment.

Our results showed that PMA executed significant anti-leukemia effect in human SUP-B15 cells, but had little effect in mice BP190 cells. We proved that PMA performed its function through activation of PKC δ . It is reported that the structure of PKC δ in mice is similar with humans, but there are still differences in certain details. We checked the sequence in the NCBI database, and found that there are indeed some differences in the amino acid sequences of the PKC δ between mice and humans, which may affect its function, activity, and response to PMA.

We confirmed that PMA targeted the central pathological feature of “differentiation arrest” in Ph⁺ ALL, specifically promoting the differentiation of leukemic blasts into mature B cells exhibiting plasma cell phenotypes. Based on these findings, PMA demonstrated potential as a candidate agent in differentiation therapy. Currently, the predominant clinical strategies for differentiation therapy involve retinoid-based treatments such as all-trans retinoic acid (ATRA), which are widely used in the management of acute promyelocytic leukemia (APL) and proved successful [44]. Therefore, the primary future direction for developing PMA-based differentiation therapy for Ph⁺ ALL lies in improving its specificity. Strategies could

include developing novel PKC agonists with enhanced selectivity for specific PKC isozymes involved in B-cell differentiation, or utilizing advanced drug delivery systems (e.g., antibody-drug conjugates or nanoparticle-based carriers) to specifically target leukemic blasts.

This study demonstrated that PMA induces the differentiation of SUP-B15 cells, which not only alters leukemic cell phenotypes but may also modulate the tumor immune microenvironment through changes in immune-related molecule expression. Previous reports indicate that PMA influences macrophage differentiation into specific subtypes [45]. Our study demonstrated PMA down-regulated CD274 (PD-L1) and suppressed pro-tumorigenic CD71 and CD13, reducing the immune escape and immunosuppressive remodeling capabilities of leukemic blasts *in vitro*. PMA-treated Ph⁺ ALL mice showed elevated CD22 and CD138 expressions on splenic leukemic cells, along with reduced hepatic and splenic infiltration, suggesting improved immune-mediated clearance. RNA-seq data further support that PMA activates lymphocyte differentiation pathways, indicating a dual mechanism of action: dampening immunosuppressive signals and enhancing immunogenicity.

We found that PMA could significantly prolong the survival of Ph⁺ ALL mice with 10 $\mu\text{g/kg}$ but not 50 $\mu\text{g/kg}$. We speculate that the concentration of 50 $\mu\text{g/kg}$ is too high to execute therapeutic effect out of its side effect. The primary cause is likely the acute toxicity that occurs when plasma concentrations exceed the therapeutic window. It is reported that PMA plays its role usually less than 50 ng/ml *in vitro*. And PMA, as a broad-spectrum activator of PKC, can trigger inflammatory responses and induce organ injury through the activation of classical PKC isoforms (PKC α and PKC β) under high-concentration conditions. Too high concentrations of PMA may cause side reaction through induction of inflammatory response, immune inhibition, cardiovascular reaction, visceral injury and so on. Furthermore, PMA possesses inherent toxicity. High-dose PMA may exhibit enhanced non-specific cytotoxicity, leading to unintended damage to normal hematopoietic cells. However, the exact reason for which 50 $\mu\text{g/kg}$ of PMA could not prolong the survival of mice still needs to be confirmed. There is no recommended concentration of PMA for *in vivo* mice experiments until now. Our experiments provide a reference for future research. Given the inherent toxicity of PMA, the realization of its clinical translation necessitates the

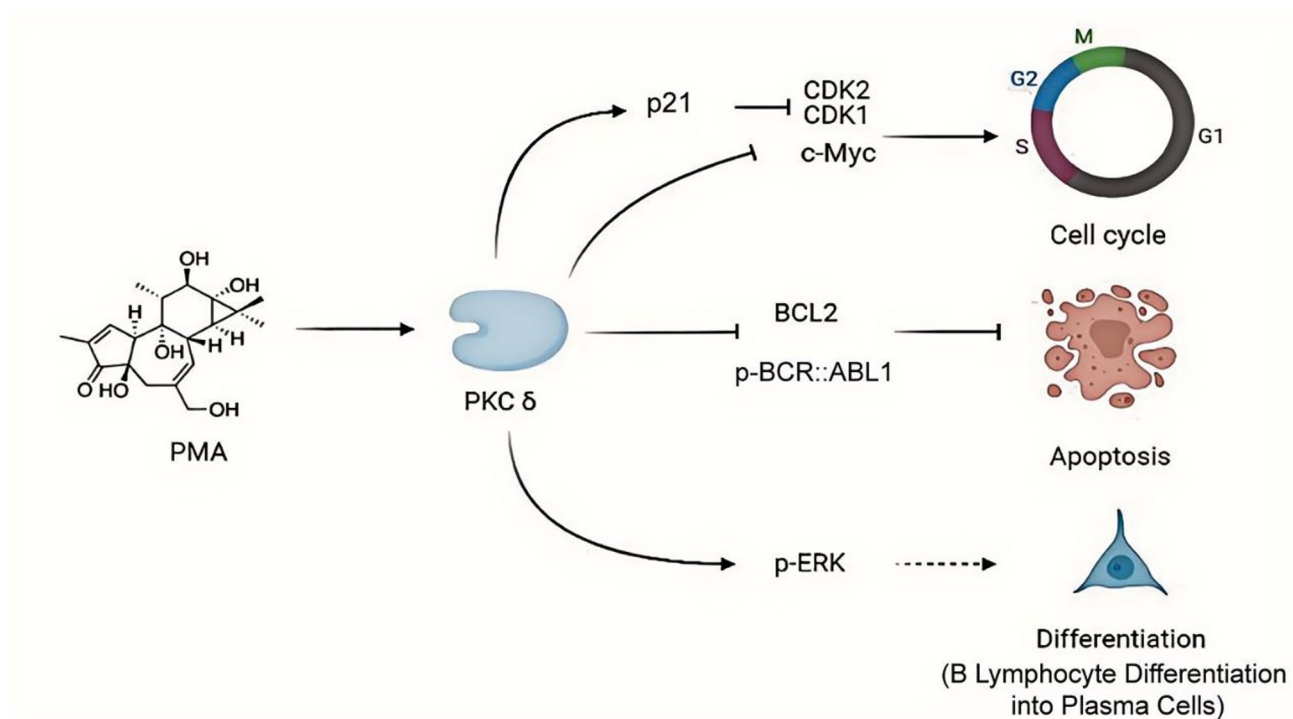


Fig. 9 The schematic diagram for the function of PMA and the potential mechanism. The anti-leukemia effect of PMA on Ph⁺ ALL cells is mediated through the activation of PKC δ. PKC δ activation subsequently induces p21 expression and suppresses c-Myc, leading to cell cycle arrest. Additionally, PKC δ activation contributes to apoptosis induction by downregulating BCL-2 and p-BCR::ABL1, and promotes cell differentiation via activation of p-ERK

implementation of precise strategies to effectively mitigate this challenge. The relevant literatures suggest that the toxicity issue may potentially be addressed through the development of PKC δ-specific activators [46], the synthesis of optimized PMA derivatives [47], and the refinement of PMA delivery methodologies [48, 49].

The S phase is for DNA synthesis in cell cycle. It is reported that the S phase fraction usually reflects the proliferation and malignancy of tumor cells [50]. PMA significantly reduced the percentage of SUP-B15 cells in S phase, and blocked a large number of cells in G1 and G2/M phases. We tried to explain the related mechanism. It is reported that the combination of CDK2 and cyclin E controls the progression from G1 to S phase [51]. p21 can inhibit the activity of CDK2 to phosphorylate downstream substrates and then lead to G1 arrest. c-Myc is an oncogene that makes cells proliferate indefinitely, obtain immortalization function, and promote cell division. Down-regulation of c-Myc can inhibit the growth of tumor cells [52, 53] and block cells in the G1 phase without proliferating. Our results confirmed that PMA significantly increased p21 and decreased CDK2 and c-Myc, which could well explain why SUP-B15 cells were blocked in G1 phase. CDK1-cyclin B controls the progress of G2/M phase. PMA reduced the expression of CDK1, which prevented cells from G2 phase transitioning to M phase leading to G2 phase block [51, 54].

The arrest of G1 and G2 phase resulted in a significant decrease of S phase which was consistent with the result of cell cycle analysis detected by flow cytometry. Usually, CCND1 and CDK4 or CDK6 form a complex to promote cells from G1 phase to S phase. It has been reported that when cells enter S phase, CCND1 initiates DNA synthesis and then the level of CCND1 decreases significantly due to instability. If the level of CCND1 cannot be inhibited in S phase, DNA synthesis will be inhibited [55]. PMA made CCND1 increase significantly in SUP-B15 cells, which might inhibit DNA synthesis and result in cell cycle blockage.

Taken together, we found out the anti-leukemia effect of PMA depended on the activation of PKC δ. We further demonstrated that PKC δ could in turn activate p21 and inhibit c-Myc to influence the progress of cell cycle, inhibit BCL-2 and p-BCR::ABL1 to promote apoptosis, and activate p-ERK to promote cell differentiation (Fig. 9). In summary, this is the first report of PMA in Ph⁺ ALL. We demonstrate that PMA can execute anti-leukemia effect through activating PKC δ in Ph⁺ ALL cells. This study provides the possibility that PMA as a PKC activator could be a potential therapeutic alternative for Ph⁺ ALL patients.

Conclusions

In this study, we prove that PMA can inhibit proliferation and promote apoptosis of human Ph⁺ ALL cells. Moreover, we innovatively elucidate that PMA can promote differentiation of Ph⁺ ALL cells. These effects depend on the activation of PKC δ . We further demonstrate that PKC δ in turn activates p21 and inhibits c-Myc to cause cell cycle blockage, inhibits BCL-2 and p-BCR::ABL1 to promote apoptosis, and activates p-ERK to promote cell differentiation. The results of this study prove that PMA has clinical value for Ph⁺ ALL treatment.

Abbreviations

Ph ⁺ ALL	Philadelphia chromosome-positive acute lymphoblastic leukemia
TKIs	Tyrosine kinase inhibitors
PKC	Protein kinase C
IF	Immunofluorescence

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12935-025-04013-4>.

Supplementary Material 1

Acknowledgements

Not applicable.

Author contributions

XW, ZLH and MG conceived and designed the experiments. XW conducted the experiments, acquired and analyzed the data, and wrote the draft. JFT, JT collected data and conducted the experiments. TW provided reagents. DCZ, STL conducted the experiments. RRZ, WW, JZ and YD provided reagents and analyzed the data. XW, JH obtained funding. ZLH, MG analyzed the results, supervised the whole experiment, and revised the manuscript. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The research conformed to the standard stipulated by Declaration of Helsinki and was performed with the approval of the ethical committee of Chongqing Medical University (Approval No. 2021039). And informed consent was obtained from each subject.

Consent for publication

Not applicable.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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