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ORIGINAL RESEARCH

RETRACTED ARTICLE: Synergistic suppression effect on tumor growth of acute myeloid leukemia by combining cytarabine with an engineered oncolytic vaccinia virus

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Background: In consideration of the drug resistanc nd side effects a oi ed with cytarabine, one of the most effective drugs for the treatment acute yeloid leukemia (AML), there is a need for safer and effective strategies.

Methods: In the present investigation, w bricated a new ncol tic vaccinia virus (oVV-*ING4*), which expresses the inhibitor of gro h fam member 4 (*ING4*) and explored its antitumor activity individually and in combination with cy abine in AML cells.

Results: The experiments c firmed that oVV can ficiently and specifically infect leukemia cells, and augment the *ING* gene expres n. Flow cytometry and western blot demonstrated that oVV-*ING4* enhances ap tosis and G M phase arrest in AML cells, and causes remarkable cancer cell death. In addi the sy rgistic efficiency of oVV-*ING4* and cytarabine was investigated in vi and vivo; the combination significantly inhibited the survival of leukemia cells in vitro and x ogra d l AML tumor growth in vivo.

Conclu : In brie V-*ING4* can increase the sensitivity of leukemia cells to cytarabine and ind cell ap tosis i itro and in vivo. Thus, oVV-*ING4* may be a promising therapeutic candi- e for leu mia and i combination with cytarabine represents a potential antitumor therapy.

Key s: oncolytic vaccinia virus, acute myeloid leukemia, combination therapy, ING4, cytarab

Introduction

Acute myeloid leukemia (AML) is the most frequently diagnosed leukemias in adults (25%) and accounts for 15%–20% of leukemias in children. Despite recent progress in understanding the etiology of the disease, it remains the foremost cause of leukemia-related deaths.[27] AML is a disease that involves the infiltration of the bone marrow, blood, and related tissues by rapidly dividing and poorly differentiated hematopoietic cells. It is difficult to treat owing to patient factors (physiological and coexisting diseases) as well as intrinsic biological factors.[35] High remission rates are achieved using modern chemotherapy treatment (cytarabine and daunorubicin); however, a majority of AML patients relapse, leading to merely 40%–45% and <10% 5-year survival rates in the young and elderly patients, respectively.[26] Cytarabine (cytosine arabinoside, Ara-C) is the most potent drug for the treatment of AML and is frequently prescribed either alone or in combination with other drugs. However, the development of resistance and side effects such as myelosuppression impact negatively on patient survival.[12] Therefore, novel therapies lacking such side effects are immediately required.

Oncolytic viruses are cancer therapeutics which act by selectively replicating in tumor cells, leading to their destruction. Developments in genetic engineering and

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the elucidation of tumor biology targeting each stage of the host–virus interaction have aided the design of novel viruses that selectively target, replicate in, and kill cancer cells. The "designed" viruses exhibit a dual mechanism of action: direct killing of cancer cells as a result of the lytic viral cycle and through the action of the therapeutic gene inserted in the viral genome. Several oncolytic viruses such as the oncolytic vaccinia viruses (oVVs) are promising anticancer agents, with good safety and preliminary evidence of efficacy.[23,32,38] In addition, recent preclinical and clinical results have confirmed the potential of recombinant "armed" oVV in patients with end-stage cancers.[9,11,17]

Inhibitor of growth family, member 4 (ING4) is a tumor suppressor which regulates the cell cycle, chromatin modification, cell proliferation, angiogenesis, and cell migration to inhibit tumor growth, invasiveness, and metastasis through multiple signaling pathways.[4] ING4 also networks with the p65 subunit of nuclear factor kappa B (NF-κB) and inhibits transcriptional activation of target genes of NF-κB.[6] It triggers G2/M arrest in HepG2 cells via upregulation of p21 in a p53-independent way[1] and plays a vital part in tumor suppression.[19] Furthermore, ING4 may hinder phosphorylation activity of cyclin/CDK2 complexes to activate Sp1 degradation through the induction of p21 expression irrespective of p53 status.

It has been reported that adenovirus-mediated ING4 (Ad-ING4) gene transfer enhanced antitumor effects and reduced side effects.[34] However, the cytotoxic effect of oVV-mediated ING4 (oVV-ING4) in AML cells has not been investigated yet. In the present study, we constructed an oVV that expressed ING4 and investigated the effect and mechanism of oVV-ING4 individually and in combination with cytarabine against AML cells.

Materials and methods

Cell cultures and viruses

AML cell lines (THP-1, KG-1, and HL-60) and chronic myeloid leukemia (CML) cell line (K562) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), retained in our laboratory, and cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution in a humidifying environment with 5% CO_2. The vaccinia virus and pCB vector have been received as a friendly gift from the academician, Xinyuan Liu.

Construction and production of homologous recombinant oVV and oVV-ING4 were described below. The cDNA sequence of ING4 gene was amplified by PCR with specific primer pairs: the forward (5′-GGCCTCGAGATGGC TGCGGGGATGTATTTG-3′) and reverse (5′-GGCGGTAC CCTATTTCTTCTTCCGTTCTTGGGAG-3′) primers. The above-obtained DNA has been fragmented with BglII and EcoRI (Takara Bio, Shiga, Japan) and introduced into plasmid pCB, yielding pCB-ING4. pCB vector or pCB-ING4 have been recombined with wild-type vaccinia virus in HEK293A cells utilizing Lipofectamine 3,000 (Invitrogen, Shanghai, China). Subsequent to the observation of the evident cytopathic effect, the medium has been subjected to repeated freezing and thawing four times. To avoid the wild-type virus, mycophenolic acid, dioxopurine, and hypoxanthine have been used. Subsequently, recombinant viruses have been amplified in HEK293A cells (purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and retained in our laboratory) and subjected to ultracentrifugation. Furthermore, viral titers were measured by TCID50 (median tissue culture infective dose).

Cell viability assay and quantitative analysis of synergism in vitro

Leukemia cells have been seeded in 96-well plates (5×10^4 per well) and cultured at 37°C for 12 hours. In different experiments, cells were dealt with respective treatments of indicated concentrations. PBS was used as the cell control. After incubation for 48 or 72 hours, each well was added with 20 µL of MTS Reagent (Promega Corporation, Madison, WI, USA) and cultured for 4 hours at 37°C with 5% CO_2. Finally, the absorbance has been detected at a wavelength of 490 nm by a Microplate Reader Model 550 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Western blot analysis

Leukemia cells have been treated with oVV and oVV-ING4 at an multiplicity of infection (MOI) of 10. After 48 hours, cells have been collected, rinsed two times with PBS, and then lysed in RIPA buffer (Hoffman-La Roche Ltd., Basel, Switzerland). Cell lysates were heated for 10 minutes at 100°C, and BCA kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify protein concentration. About 30 µg of protein samples were purified using SDS-PAGE and moved to polyvinylidene fluoride membrane, which was further blocked in 5% (w/v) nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) for 1 hour. Subsequently, the membrane was incubated with primary antibodies at 4°C using the following dilutions: anti-ING4 (1:1,000; Abcam, Shanghai, China), anti-GAPDH (1:1,000; Abcam), anti-caspase-8 (1:1,000; Bioworld, iow, Dublin, OH, USA), anti-active caspase-3 (1:1,000; Abcam), anti-poly-ADP-ribose polymerase (PARP, 1:1,000; Sino Biological Inc, Beijing, China), anti-NF-κB p52/p100 (1:1,000; Cell Signaling Technology, Danvers, MA, USA), anti-p65 (1:1,000; Immunoway, Plano, TX, USA), anti-phospho-p65

(1:1,000; Immunoway), anti-cyclin D1 (1:10,000; Abcam), anti-cyclin D3 (1:5,000; Abcam), anti-CDK2 (1:5,000; Abcam), anti-CDK4 (1:5,000; Abcam), and anti-p21 (1:1,000; EMD Millipore, Billerica, MA, USA). The membranes were rinsed with TBST and incubated with IgG secondary antibodies (1:5,000) for 1 hour. Finally, the membranes were washed, and the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc.) was used to visualize the results.

Flow cytometric analysis of apoptosis and cell cycle

Leukemia cells were cultured in a six-well plate (2×10^5 cells/well) and then infected with oVV or oVV-ING4 at 10 MOI. After incubation for 48 hours at 37°C, the cells were rinsed two times with PBS, suspended in 300 µL of binding buffer, and treated with 5 µL of Annexin V-fluorescein isothiocyanate (FITC) and 10 µL of propidium iodide (PI) (Beyotime, Jiangsu, China) for 15 minutes in the dark at room temperature. Subsequently, the cells were studied using NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA, USA) using an inbuilt software (Novo Express) and by flow cytometry (Accuri C6; BD Biosciences, San Jose, CA, USA). Fluorescence activated cell sorter (FACS) analysis was applied to detect the cell-cycle status of leukemia cells i[n]cubated with oVV or oVV-ING4. Cells were harvested, rinsed tw[i]ce in cold PBS, re-suspended in cold 75% alcohol, and fixed o[v]ernight at –20°C. Then they were centrifuge[d] and r[i]nsed twice in cold PBS. Later, they were incubated [i]n 500 µL of staining buffer (Beyotime) added with 25 µL of 20[×] PI and 10 µL of 50× RNase A in the dark for 30 minutes at 4°C, followed by examination immediately using fluorescence microscope.

Drug-resistance and combination index (CI) analysis

The leukemia cells have been cultured in 96-well plates (5×10^3/well) and treated with several doses of cytarabine. MTS assay was used to evaluate the cytotoxic effect of oVV-ING4 in combination with cytarabine. The cells were treated with cytarabine or oVV-ING4 individually, or in combination. After 48 hours, 20 µL of MTS reagent has been supplemented and incubated for 4 hours at 37°C. Cell viability has been measured using the MTS assay after 48 hours. We used the experimental data of cytotoxicity and analyzed the interaction between cytarabine and oVV-ING4 by CalcuSyn program (Biosoft, Cambridge, UK). For CI plots, CI is denoted as log10 (CI)±1.96 SD, and the algebraic approximation algorithm of the CalcuSyn program was used. In this study, CI values were estimated over a range of growth inhibition percentages (20%–80%).

Animal experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee, Zhejiang Provincial People's Hospital, and all procedures were in accordance with the Guide for the Care and Use of Laboratory Animals. Male BALB/c athymic nude mice were bought from Shanghai Experimental Animal Center. They were divided into five groups (six per group) and injected with 5×10^6 KG-1 AML cells into their right hind limbs. The tumor growth was measured every 3 days and the volume (V, mm^3 was computed using the formula: $V=1/2\times\text{length}\times\text{width}$) was determined.[1,2] When the volume of tumors approximately grew to 200 mm[3], the mice were injected with PBS, cytarabine, oVV, oVV-ING4 or cytarabine, and oVV-ING4. After 1 week, the tumors of two mice from each group were collected and digested with the help of enzymes into cell suspensions, and then subjected to flow cytometry with Annexin V-FITC/PI double staining. Tumor progression and regression were monitored for every 3 days. After 25 days, the mice were sacrificed, and the tumors were excised, treated with 4% paraformaldehyde, and encased in paraffin for hematoxylin & eosin (H&E) staining, immunohistochemistry, and TUNEL assay. For the immunohistochemical (IHC) study, the sections were stained with anti-Caspase-3 or anti-Ki-67 (1:100 dilutions) primary antibodies overnight at 4°C, added with the avidin-biotin-peroxidase complex, and the slides were detected with diaminobenzidine Kit (Thermo Fisher Scientific). Images were photographed by an inverted fluorescence microscope (Nikon E300; Tokyo, Japan). Moreover, the mice were randomly divided into four groups (three per group), and intraperitoneally injected with PBS (100 µL), oVV-ING4 (1×10^7 pfu), a dose of cytarabine (50 mg/Kg), respectively, or combination of oVV-ING4 with cytarabine. After 45 days, the mice were euthanatized, and their hematopoietic stem cells were isolated and cultured in IMDM supplemented with 10% fetal bovine serum and penicillin–streptomycin (100 U/mL) solution in a humidifying environment with 5% CO_2. After 48 hours, add 10 µL anti-CD34 (Biolegend CD34-APC) to 100 µL cell suspension and hemolyze red blood cell. Cell suspension was cultured in the dark for 30 minutes at room temperature and followed by examination immediately using flow cytometric analyses.

Statistical analysis

An ANOVA was applied for the comparison of three or more groups. The analysis of the combined effects was performed with CalcuSyn software 2.0 (Biosoft). Data are expressed as mean ± SD. Statistical analysis was performed with IBM SPSS Statistics software version 20 (SPSS Inc., Chicago, IL, USA). Statistical significance was prescribed at $P<0.05$.

Results

Generation and characterization of a chimeric, ING4-armed oVV

Schematic structure of the recombinant genome of oVV or oVV-ING4 is displayed in Figure 1A and described in detail in the "Materials and methods" section. We initially evaluated the infectious efficiency of the oVV on three AML cell lines: THP-1, KG-1, and HL-60; and one CML cell line: K562. They were infected with oVV-green fluorescent protein (GFP) that is a similar virus without ING-4 but carrying GFP, at multiple doses, and then detected under flow cytometer (ACEA NovoCyte). The percentage of GFP-positive cells was obviously detected at 48 hours and the infectious efficiency augmented in a dose-dependent manner (Figure 1B). To assess the ING4 expression, leukemia cells were treated with oVV or oVV-ING4 for 48 hours. As expected, the results were obtained when ING4 expression was determined at the protein level via Western blotting, suggesting that the expression of ING4 gene has

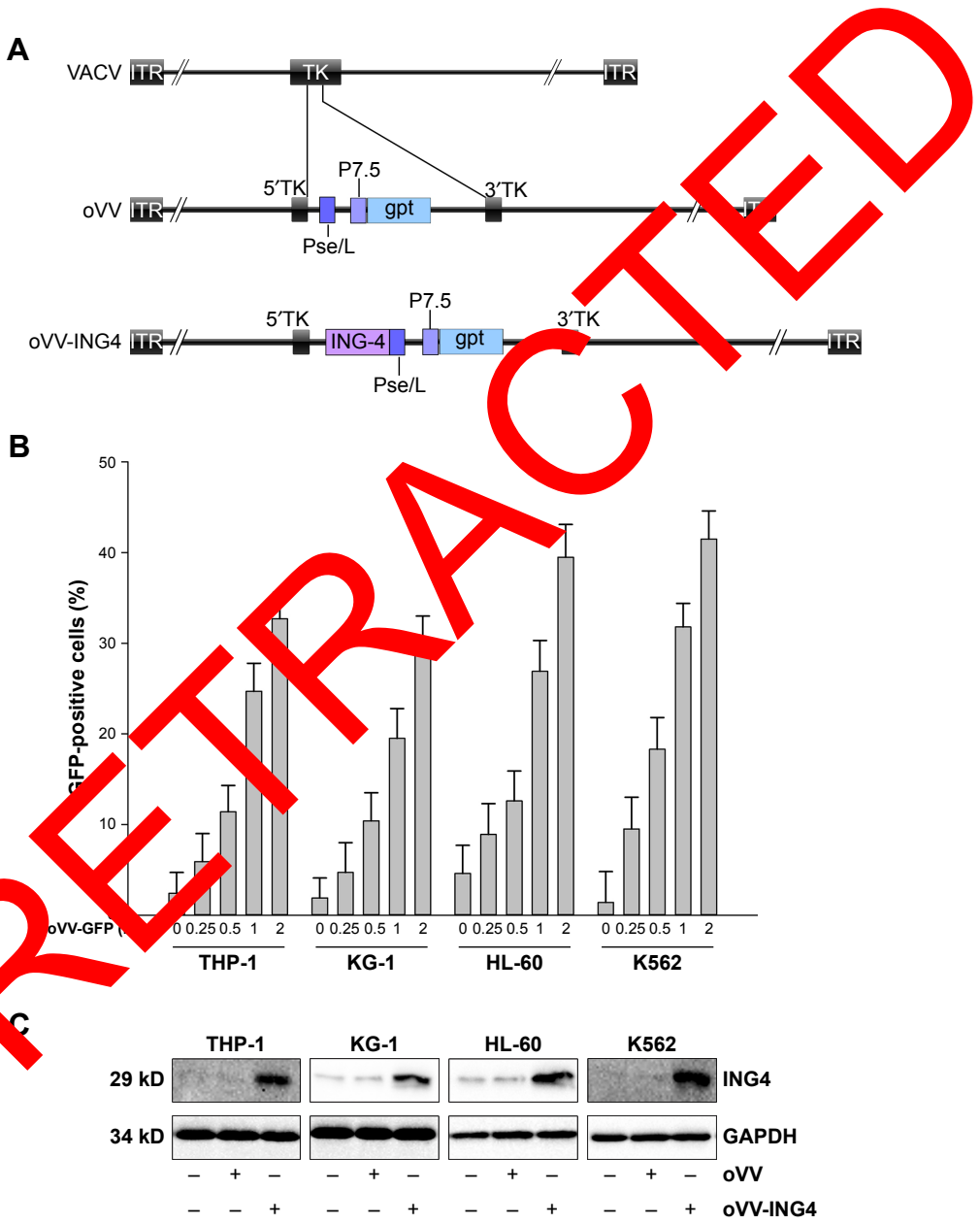


Figure 1 Construction and characterization of oVV-ING4.

Notes: (**A**) Schematic structure of recombinant oVV and oVV-ING4. All viruses were constructed through homologous recombination between pCB-transgene and wild-type vaccinia virus (VACV) in HEK293A cells. T7 promoter and gpt gene work as promoter and screen gene. The ING4 expression cassette was introduced into the TK region of the vaccinia virus. (**B**) Infectious efficiency of oVV by fluorescence microscopy. Leukemia cell lines were treated with oVV-GFP at multiple doses for 48 hours and then observed under the inverted fluorescence microscope. (**C**) ING4 expression was determined by Western blotting 48 hours post-infection (MOI =10). Data shown (mean ± SD) are representative of three experiments. GAPDH was used as a protein-loading control.

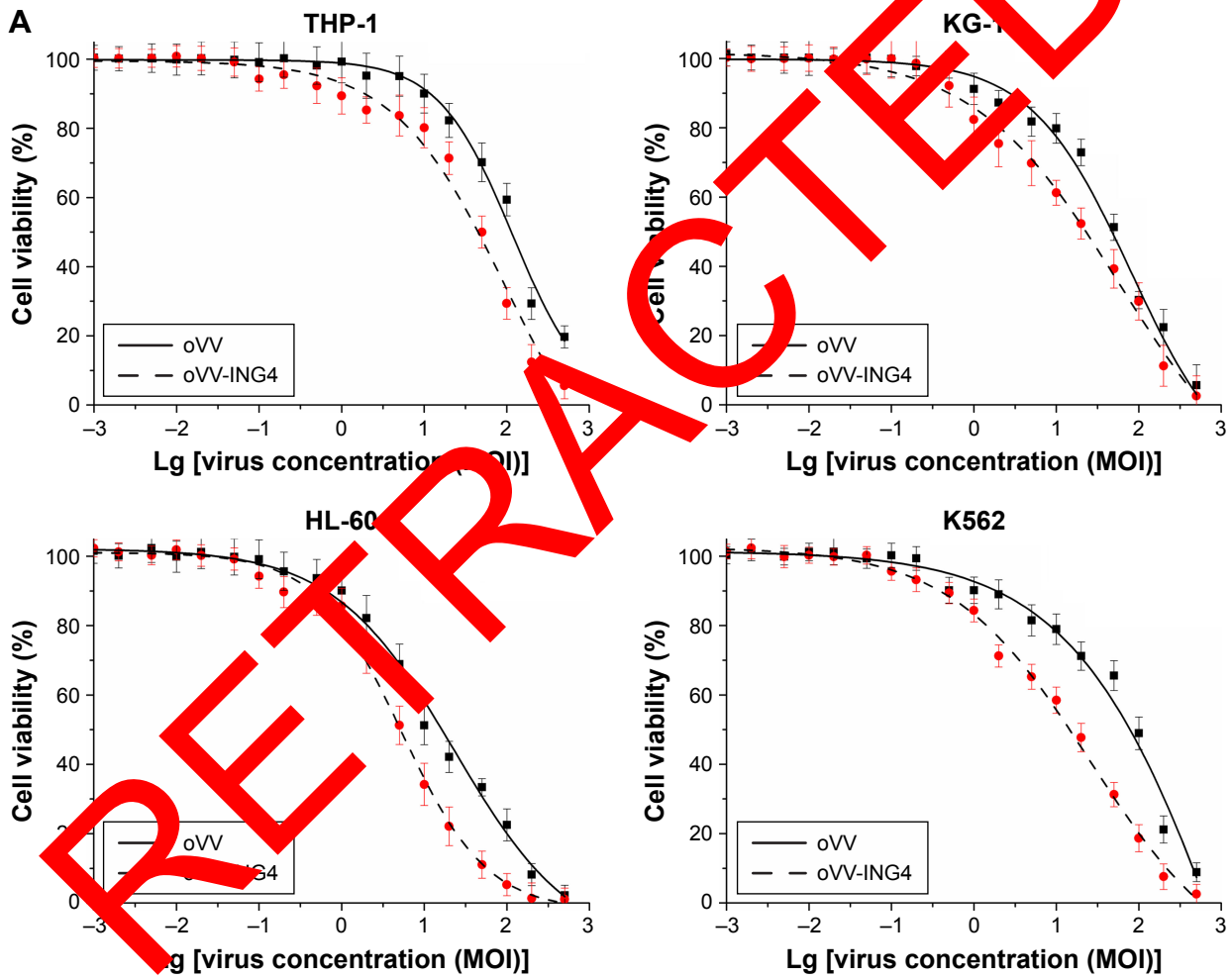
Abbreviations: oVV, oncolytic vaccinia virus; ING4, inhibitor of growth family member 4; TK, thymidine kinase; MOI, multiplicity of infection; GFP, green fluorescent protein.

remarkably increased in all oVV-ING4-transfected leukemia cell lines, but not in oVV- or PBS-treated groups (Figure 1C).

oVV-ING4 induced cell death in leukemia cell lines and was safe in human peripheral blood mononuclear cells (PBMCs)

To investigate the impact of oVV-ING4 on cell viability, the human leukemia cells were infected with oVV or oVV-ING4 at the indicated doses, respectively. MTS viability assay was used to determine the MOI needed to induce 50% cell death (IC_{50}) of leukemia cells; greater apoptosis was observed when MOI increased (Figure 2A). Moreover, IC_{50} values of oVV-ING4 were lower than that of oVV (Figure 2B). The results showed that oVV-ING4 displayed more inhibitory activity on proliferation of leukemia cells, as compared with that induced by oVV. To validate their safety on healthy cells, human PBMCs were infected with the same doses of oVV. In contrast, oVV and oVV-ING4 showed no significant cytotoxic effects on normal PBMCs (Figure 2C). Human PBMCs were donated from volunteers. The Zhejiang Provincial People's Hospital of Medicine Ethics committee approved this study, and all the volunteers [illegible] signed the written informed consent. These findings suggest that oVV-ING4

A



B

Cell line	IC_{50} oVV	IC_{50} oVV-ING4	Fold decrease vs oVV
THP-1	111.387	33.584	3.32×
KG-1	41.003	17.948	2.28×
HL-60	14.123	4.8	2.94×
K562	56.339	11.842	4.76×

Figure 2 (*Continued*)

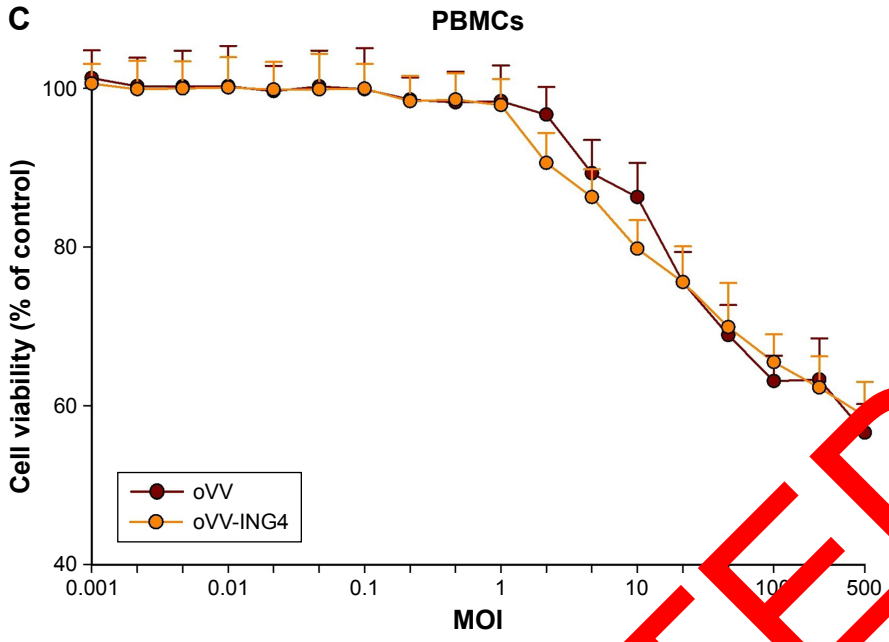


Figure 2 ING4 is specific to AML cells and is safe for normal cells.
Notes: (**A**) Leukemia cells infected with oVV and oVV-ING4 at the indicated MOI (Lg-3 to Lg3) for 48 hours. Then MTS viability assay used to examine the cell viability (real lines indicate the infection with oVV, broken lines indicate the infection with oVV-ING4). (**B**) IC values of different cell lines to oVV or oVV-ING4. (**C**) Human normal peripheral blood mononuclear cells (PBMCs) which treated with similar ways were used as the control to normalize the results.
Abbreviations: oVV, oncolytic vaccinia virus; ING4, inhibitor of growth family member 4; MOI, multiplicity of infection.

has remarkable cytotoxic effects on leukemia cells in vitro, with minimal effect on normal cells. oVV-ING4 induced apoptosis by activating the caspase pathway and depressing the NF-κB pathway in vitro.

To examine whether oVV-ING4 can induce apoptosis in leukemia cells, we used flow cytometry and Western blotting to further investigate the underlying mechanisms. First, Annexin-V-FITC/PI double staining has been employed to quantify the impact of oVV-ING4 on cell apoptosis. As shown in Figure 3A, significant cell apoptosis can be observed in the group treated with oVV-ING4.

Similarly, Western blot analysis proved that oVV-ING4 can activate high expression of caspase-3 and caspase-8 as well as cleavage of PARP by activating caspase-dependent pathways. Additionally, oVV-ING4 has the ability to decrease the expression of p52/p100, p65, and p-p65 by depressing the pathway of NF-κB. The expressions of p52/p100, p65, and p-p65 were reduced in the oVV-ING4 group compared with controls and oVV-treatment groups (Figure 3B). To sum up, these findings indicate that oVV-ING4 killed leukemia cells by inducing apoptosis.

oVV-ING4-induced cell cycle alteration, that is, G2/M cell cycle inhibition in leukemia cells

To explore the impact of oVV-ING4 on cell cycle, all leukemia cells have been infected with oVV-ING4 for 48 hours and harvested for flow cytometry analysis. As seen in Figure 4A, a considerable increase in G2/M phase was observed in the oVV-ING4-treated group, but not in oVV- or PBS-treated groups ($P<0.05$). Western blotting indicated that the expression of p21 has been notably amplified in the oVV-ING4-treated group. On the other hand, cyclin D1, cyclin D3, CDK2, and CDK4 levels were obviously decreased, compared with the group treated with PBS or oVV (Figure 4B). Altogether, our results proved that oVV-ING4 has the ability to enhance the antitumor effect by adjusting protein factors involved in cell cycle alteration.

oVV-ING4 enhanced cytarabine-induced antitumor effect in AML cells

To determine whether oVV-ING4 enhances the cancer cell-killing effect of cytarabine, we analyzed the viability of AML cells by MTS assay following their combined treatment. Cells were treated with cytarabine (0.2, 0.4, 0.8, or 1.6 μM) with or without oVV-ING4 (1, 2, 4, or 8 MOI). The combination significantly inhibited cell growth compared with cytarabine or oVV-ING4 alone (Figure 5). Potential interaction between oVV-ING4 and cytarabine was next evaluated through Chou–Talalay CI method. In KG-1 cells, the CI was found to be <1 (log10 [CI] <0), signifying a synergistic effect of oVV-ING4 and cytarabine. Additionally, the investigation on HL-60 and THP-1 presented similar results (log10 [CI] <0) (Figure 5A–C). Hence, these findings suggested that the

combination treatment of cytarabine and oVV-ING4 has a synergistic repressive effect on AML cell proliferation.

Combined treatment with oVV-ING4 and cytarabine enhances antitumor effect in vivo

To assess the efficacy of the combination therapy of oVV-ING4 and cytarabine in vivo, studies on BALB/c athymic nude mice have been carried out for utilizing an AML tumor xenograft model established by KG-1 cells. The experiment is carried out as described in Figure 6A. As shown in Figure 6B, relative to the individual group, the mean tumor volume of the mice which received combined therapy has obviously decreased. Flow cytometry analysis has been applied to assess the result of combination treatment on apoptosis and confirm the underlying mechanism in vivo. As expected, the combination treatment of oVV-ING4 and cytarabine resulted in an increased percentage of apoptotic KG-1 cells in vivo, compared with PBS ($P<0.001$), oVV ($P<0.001$), Ara-C ($P<0.001$), or oVV-ING4 alone ($P=0.002$) (Figure 6C). In order to understand whether the combination therapy of oVV-ING4 and cytarabine impact normal hematopoietic stem cell ability, we tested the percentage of $CD34^+$ cells in bone marrow by flow cytometry. There is no significant difference in the proportion of CD34-positive cells between the PBS group and the oVV-ING4 group. Similar results can be seen in the Ara-C group and combination group (Figure 6D). These findings proved that oVV-ING4 is

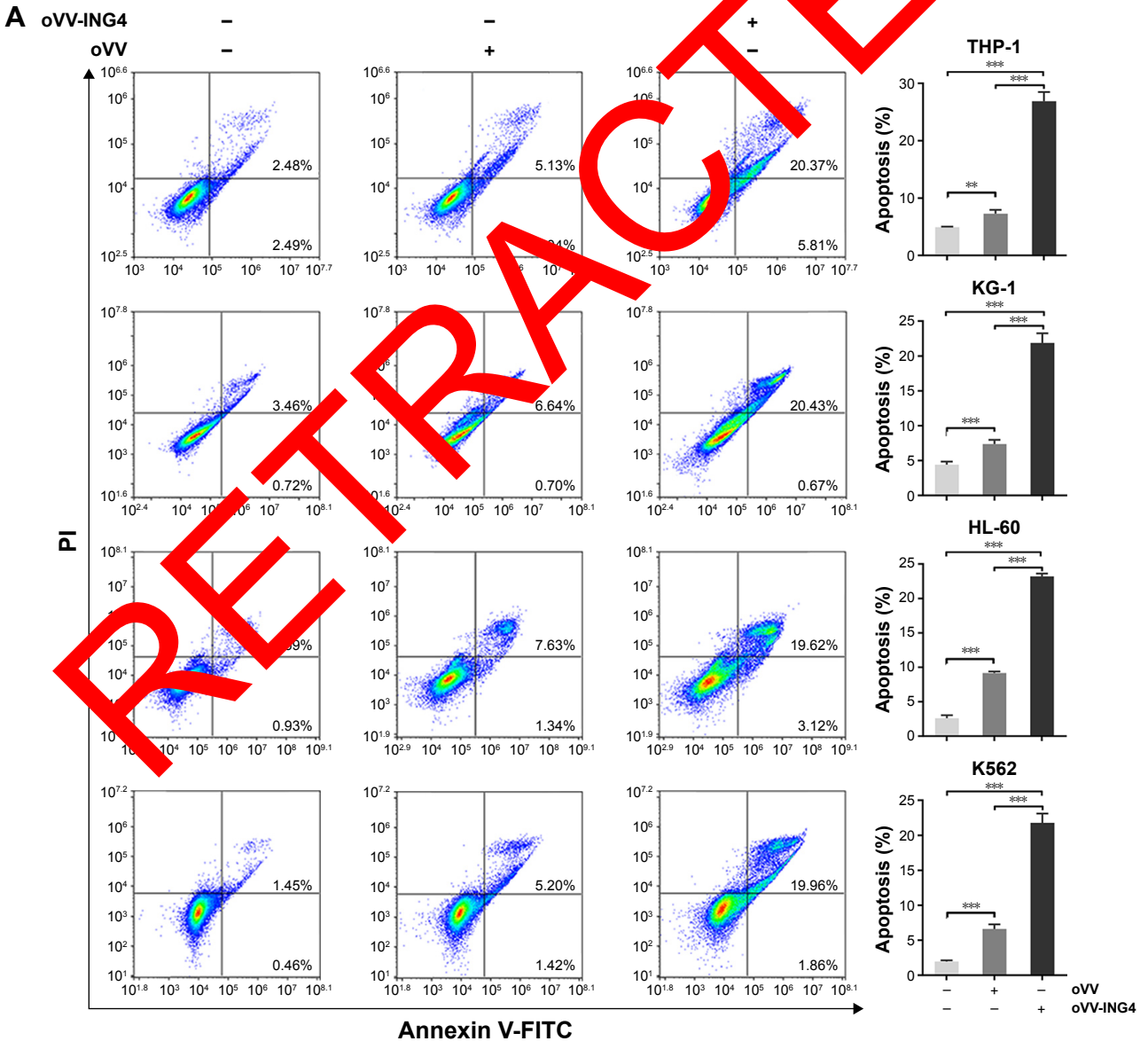


Figure 3 (*Continued*)

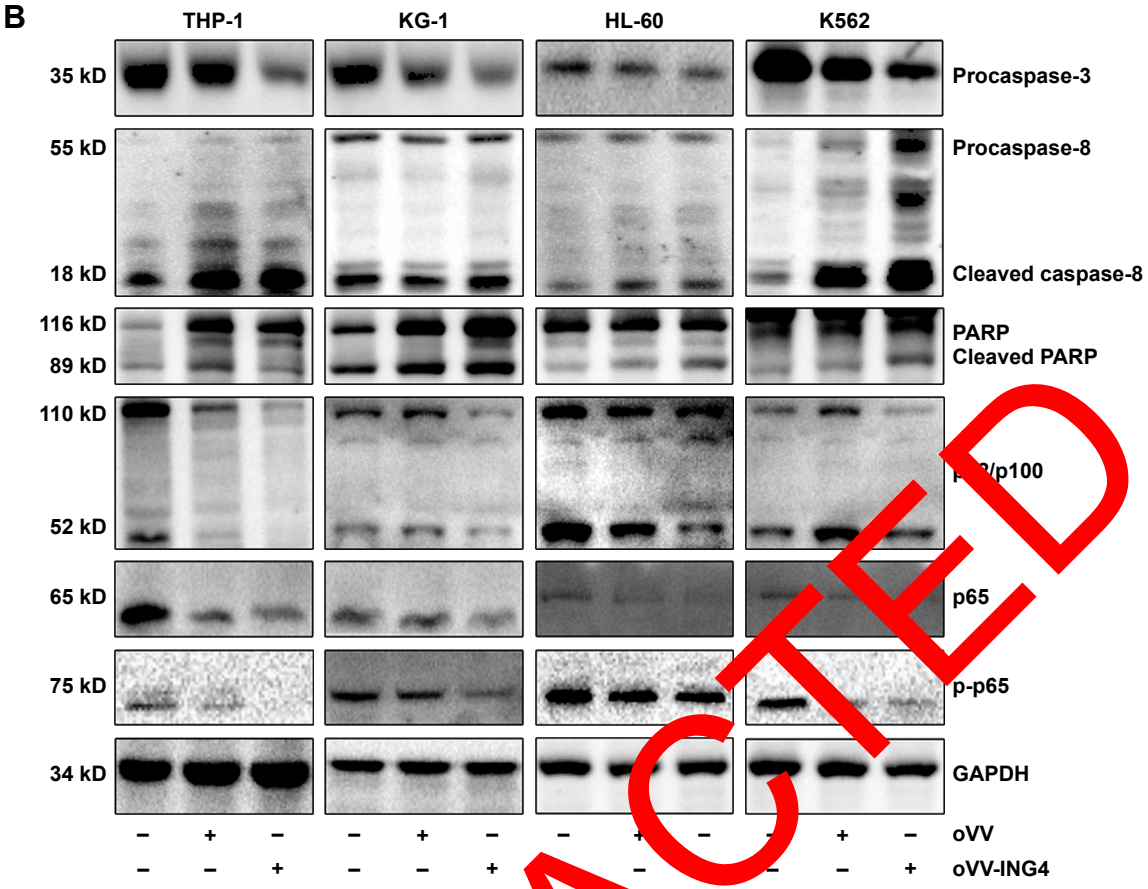


Figure 3 oVV-ING4-induced cell apoptosis in leukemia cells.
Notes: (**A**) Leukemia cells were infected with oVV or oVV-ING4 (10 MOI) for 48 h, cell apoptosis was assessed by FACS analysis of Annexin V-FITC/PI double staining. (columns: apoptotic cell number expressed as the mean ± SD of three separate experiments; bars, SD. $^{**}P<0.01$, $^{***}P<0.001$, one-way ANOVA and multiple comparisons). (**B**) Cells were treated as indicated above, and then cell lysates were immunoblotted for the detection of activation of caspase pathway and inhibition of NF-κB pathway. GAPDH was used as a loading control.
Abbreviations: oVV, oncolytic vaccinia virus; ING4, inhibitor of growth family member 4; MOI, multiplicity of infection; FACS, fluorescence activated cell sorter.

a safe treatment for AML. The side effect of cytarabine on the ability of bone marrow hematopoietic stem cells was not increased by the virus. H&E and IHC staining have been used to explore the histopathological differences in the tumor tissues. As shown in Figure 3B, more severe cytopathic effect can be obviously observed in the combined treatment group by H&E staining. IHC analysis using anti-caspase-3 antibody certified a strong expression of caspase-3 in the tumor tissues treated with oVV-ING4 combined with Ara-C. In addition, the expression of ING4 in mouse tumor tissues has been confirmed by IHC analysis. Furthermore, TUNEL assay indicated that apoptosis during the combination treatment has been significantly increased relative to individual treatment.

Discussion

In the recent 30 years, with improved supportive therapies such as anti-infective agents and transfusion, the perspective of the patient with AML has been better.[22] However, ~30%–40% of newly diagnosed patients were not able to achieve complete remission with initial induction treatment. Moreover, approximately half of the patients relapse after achieving complete remission.[28] Among the available treatments, allogeneic hematopoietic stem cell transplantation is the most effective approach for relapsed and refractory AML. Safe and effective therapies are urgently required to improve this situation. Oncolytic viruses were recently developed for the treatment of blood and solid cancers.[2] They can infect and kill malignant cells while sparing their normal counterparts.[5] So far, there has been no study to report that oVV was applied to treat AML, although oncolytic virus therapy has been used for the treatment of other cancers.[15,16,18,36]

In our study, we employed a novel cancer virotherapy, that is, forced expression of ING4 via oVV to treat AML with the view of establishing the synergetic antitumor effect with cytarabine in vitro and in vivo. The experimental results presented in Figure 1 demonstrate that oVV-ING4 could easily

infect the AML cell lines and strongly express ING4 gene. Our results further indicated that oVV-ING4 specifically exhibited cytotoxicity in AML cells but not in healthy cells (Figure 2). Deletion of the thymidine kinase (TK)-encoding gene leads to the dependency of viruses on cellular TK expression. In addition, TK expression is increased in rapidly dividing neoplastic cells but not in healthy ones.

Apoptosis induction plays an important role in the antitumor mechanism of various treatment strategies. Preclinical and clinical investigations have revealed the significance of virus-induced activation of antitumor immunity for optimal therapeutic effectiveness.[25] There are many findings highlighting the fact that oncolytic virotherapy enhanced the activation of a caspase-independent cell apoptosis pathway.[3,21,30] In AML cells, the oVV-ING4 infection triggered significant apoptosis compared with oVV treatment alone. Caspase-8 is engaged in the programmed cell death elicited by Fas and various apoptotic stimuli. Once activated, caspase-8 can

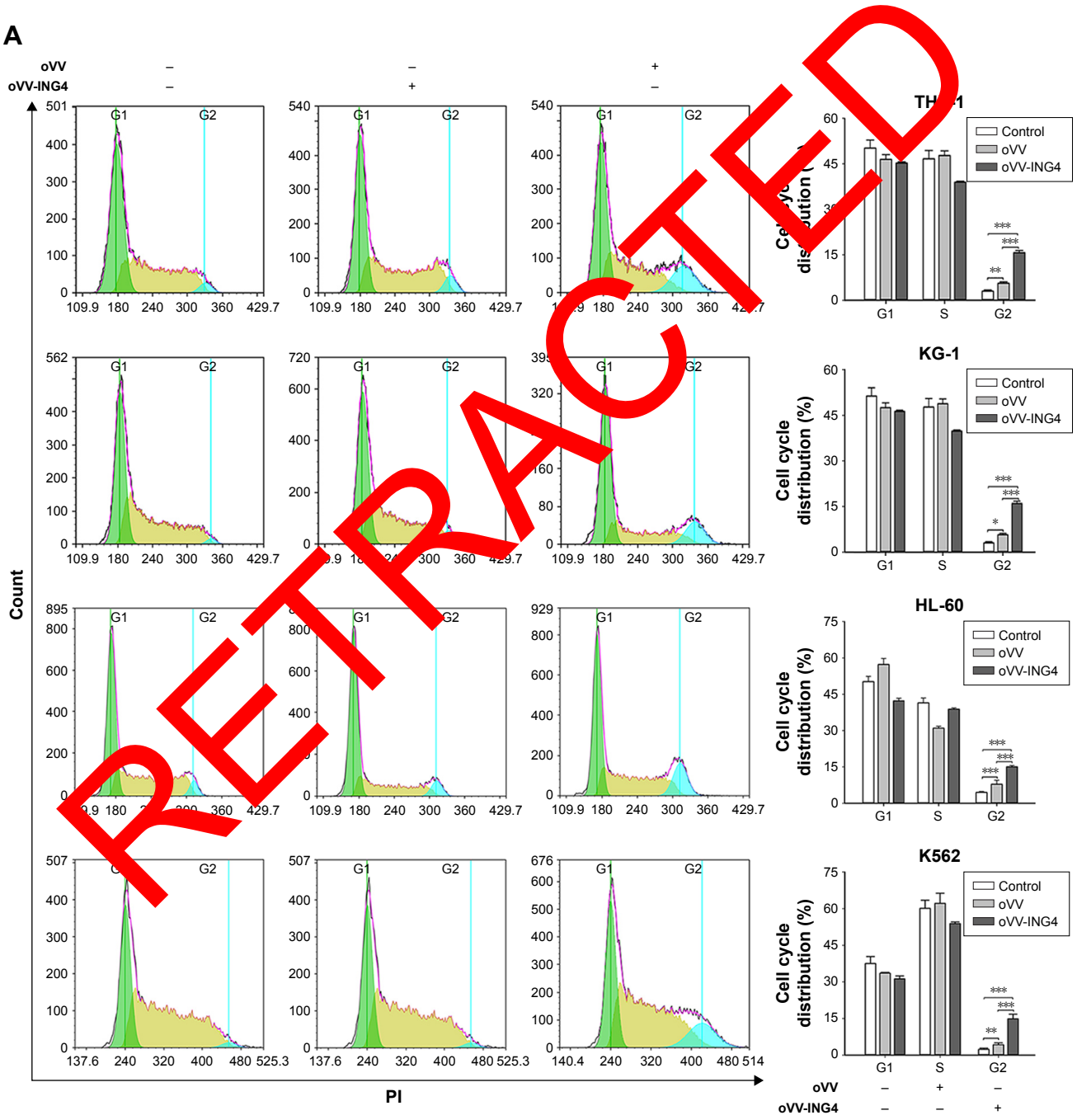


Figure 4 (*Continued*)

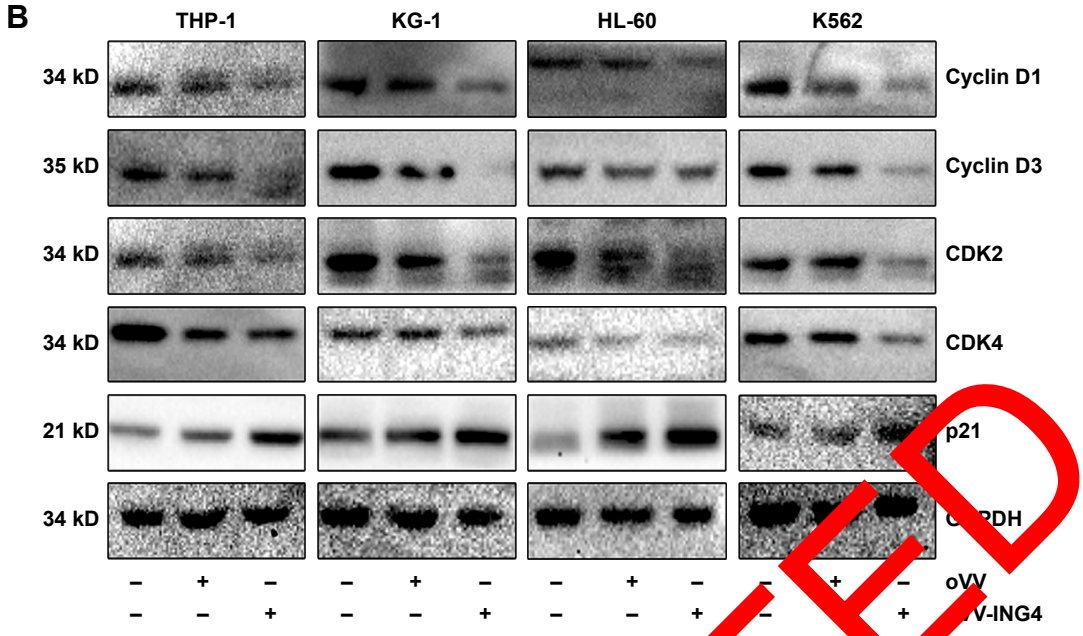


Figure 4 oVV-ING4-induced cell cycle arrest in leukemia cell lines.
Notes: (**A**) Detection of cell cycle distribution. Flow cytometry analysis of PI-stained cells was conducted at 48 hours post-infection at an MOI of 10. The percentages of cells in G1, S, and G2/M phase were scored and presented graphically (*$P<0.05$, **$P<0.01$, and ***$P<0.001$, one-way ANOVA and multiple comparisons. The results are presented as the mean ± SD of three separate experiments). (**B**) The expression of cell cycle-related protein was determined by Western blotting. The cells were treated as same as (**A**), and GAPDH was served as an internal reference.
Abbreviations: oVV, oncolytic vaccinia virus; ING4, inhibitor of growth family member 4; MOI, multiplicity of infection.

autocatalytically process itself, as well as cleave and activate the downstream executioner, caspase-3.[24] Nevertheless, our study established that the caspase apoptotic pathway is associated with the antitumor process, and caspase-8, caspase-3, and PARP cleavage were detected in AML cells (THP-1, KG-1, and HL-60) and CML cells (K562) with oVV-ING4 treatment (Figure 3B).

The activation of NF-κB, a transcription factor, promotes cell survival via induction of target genes, whose products hinder components of the apoptotic pathway.[29,31] In addition, ING4 is a member of the inhibitor of growth proteins and has been regarded as a tumor inhibitor because of its association with the regulation of cell proliferation, apoptosis, and stability in several tumor types.[8] Moreover, ING4 regulates the NF-κB signaling pathway by modifying gene transcription.[10,13,32] Therefore, we investigated whether ING4 could enhance the antitumor effect by inhibiting the NF-κB signaling. Consistent with previous reports, our results indicated that infection with oVV-ING4 downregulated the expression of components of the NF-κB family including p52/p100, p65, and p-p65 (Figure 3B) and induced apoptosis (Figure 3A). Hence, it may be one of the underlying apoptotic mechanisms for the antitumor effect of ING4 in AML.

ING4 in cancer cells can reduce S phase population and act as a potential regulator of p53-mediated cellular processes. It may also act as a tumor suppressor, as proposed for other ING family proteins identified and characterized earlier.[20] ING4 improves the sensitivity of cancer cells to DNA-damaging drugs by increasing the acetylation of p53, interaction with p300, and by negatively modulating the cancer cell proliferation through G2/M phase arrest.[14] Nevertheless, p21 could also be modulated via p53-independent pathways. Zhang et al reported that, in HepG2 cells, the upregulation of p21 was consistent with the expression of ING4 and the degree of G2/M arrest.[1] ING4 might be associated with the p53-independent pathway, and ING4-induced upregulation of p21 has been illustrated in Figure 4B. Our previous studies have demonstrated that oVV-ING4 indeed blocks cell cycle in G2 phase.[33] Despite cytarabine is an S-phase-specific chemotherapeutic drug, its synergistic anticancer effect with oVV-ING4 can be observed in Figure 5. We speculate that this may be related to the complex action mechanism of cytarabine. In this study, we found that oVV-ING4 can suppress AML by multiple ways, such as inducing apoptosis, cell cycle arresting, and inhibiting the NF-κB signaling pathway. Cytarabine may be involved in the above-mentioned mechanism, enhancing the antitumor effect of oVV-ING4. Although high-dose cytarabine (HiDAC) is one of the most popular drugs for the treatment of AML, it shows limited efficacy in monotherapy or combination treatments

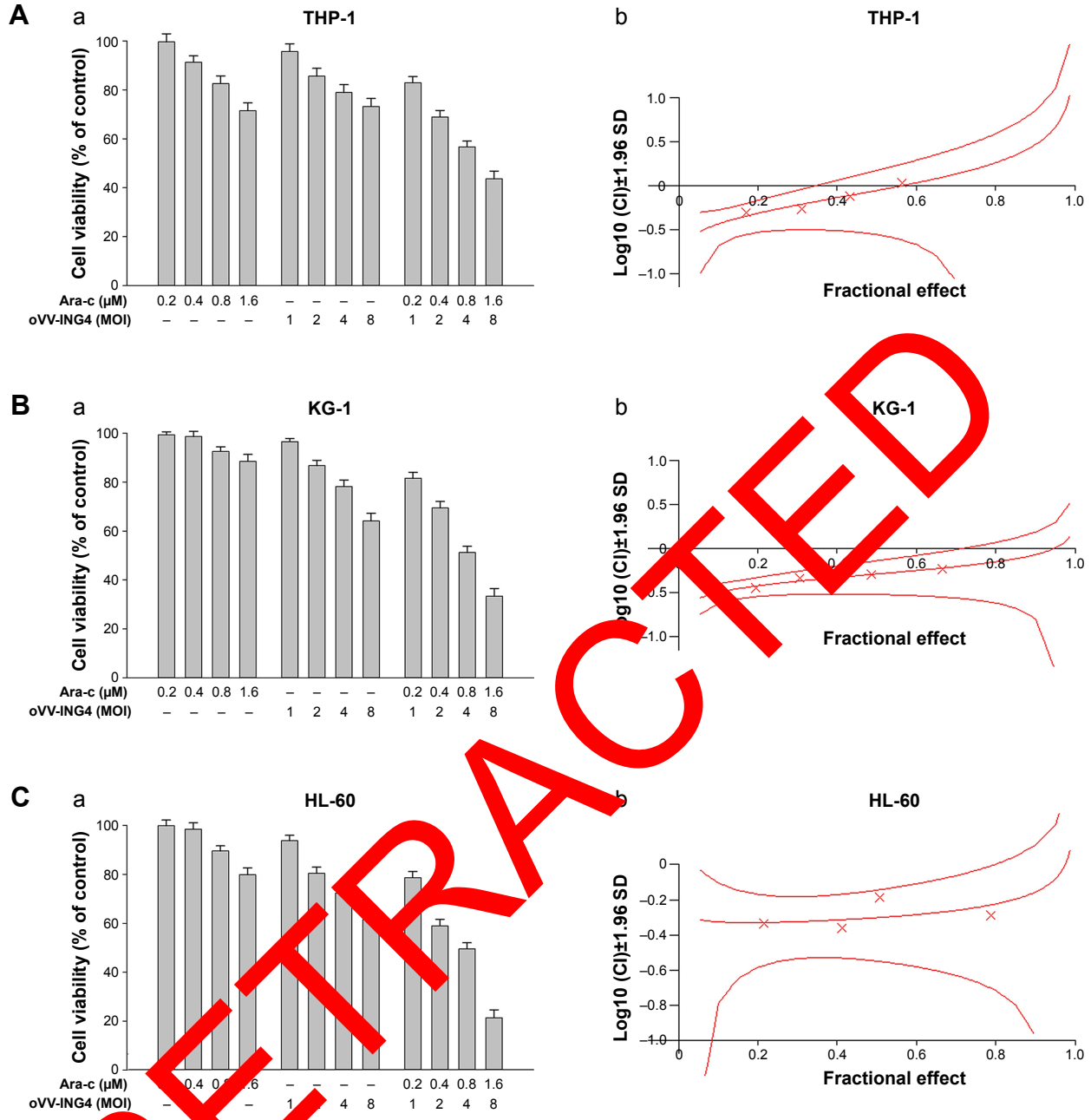


Figure 5 Synergistic effects of cytarabine and oVV-ING4 in leukemia cells.

Notes: AML cells were seeded at 5,000 cells/well into 96-well plates. (**A**–**C**) Cell lines are treated with Ara-C at multiple doses (0.01–50 μM) with or without oVV-ING4 (MOI =1 or 2). Cell viability was measured by MTS after 3 days. Data shown are representative of three independent experiments (a). The potential synergistic effect of cytarabine combining with oVV-ING4 on AML cells was assessed by Chou–Talalay CI analysis using CalcuSyn software. The middle curved line stands for the simulated CI values, which was expressed as the log10 (CI)±1.96 SD, encircled by two lines of algebraic evaluation of the 95% confidence intervals. The log10 (CI) values attained at given fractional affects represent an additive efficiency when equal to 0 and a synergism when <0. It was quantified by CIN analysis and expressed as CIN vs fraction affected. Where calculable, 95% CIs are shown (b).

Abbreviations: oVV, oncolytic vaccinia virus; ING4, inhibitor of growth family member 4; MOI, multiplicity of infection; CIN, combination index.

and toxicity, particularly in patients over 60 years of age, who constitute the majority of the AML population.[7] Our results indicated that oVV-ING4 has the ability to sensitize cytarabine and to reduce contradicting reactions induced by HiDAC through reducing the dosage. In conclusion, introducing the ING4 gene into the oVV backbone, antitumor activity, induce apoptosis, and asset cell cycle of the virus can be significantly improved.

In conclusion, we have successfully demonstrated that co-treatment with oVV-ING4 and cytarabine showed synergistic effect to kill AML cells in vitro and in vivo. Hence, oVV expressing the ING4 gene might possibly be employed as

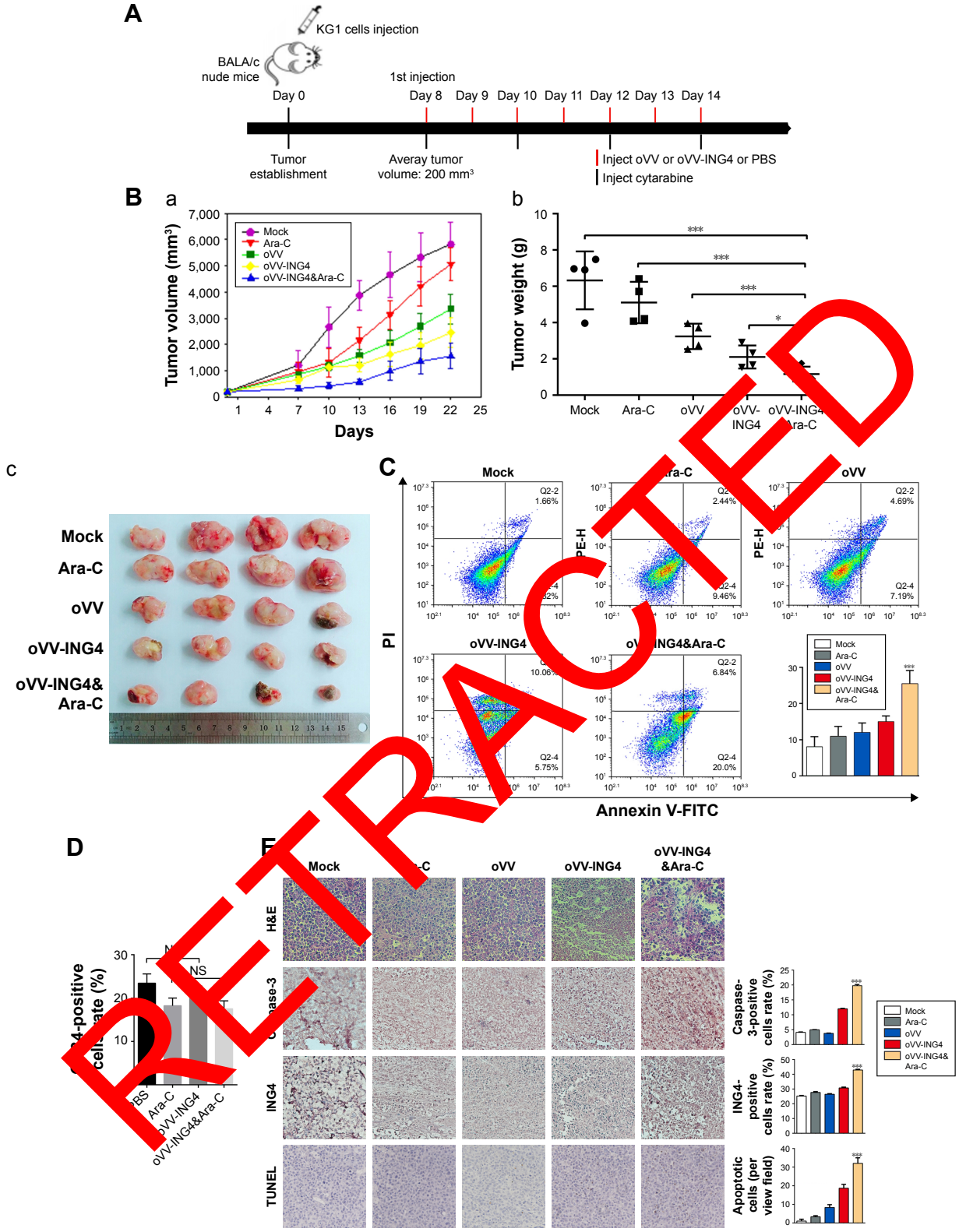


Figure 6 Combined treatments with oVV-ING4 and Ara-C showed an enhanced effect of inhibition tumor growth in vivo.

Notes: (**A**) Schematic representation of the treatment. BALB/c athymic nude mice bearing KG-1 AML tumors (0.2–0.3 cm^3) were intratumorally injected with oVV-ING4 (1×107 pfu), oVV (1×107 pfu), or PBS (100 μL) every day for a total of seven times and intraperitoneally injected with a dose of cytarabine (50 mg/kg) on alternative days for four times or coinjection of cytarabine and oVV-ING4. (**B**) Tumor volume was measured at different times after treatment (a). Twenty-five days after injection, the mice were sacrificed and the tumors were excised to weight (b) and photograph (c). Flow cytometry was used to assess tumor cell apoptosis in vivo (**C** and **D**) the safety of oVV-ING4. The treated hematopoietic stem cells were assessed by FACS analysis of anti-CD34-APC staining. (**E**) Histopathological analysis. The effect of apoptosis induced in tumor tissues was detected by H&E staining, TUNEL assay and immunohistochemical analysis of ING4 and caspase-3 (magnification 400×). (Data are presented as means ± SD [n=6]) (*$P<0.05$, and ***$P<0.001$).

Abbreviations: oVV, oncolytic vaccinia virus; ING4, inhibitor of growth family member 4; MOI, multiplicity of infection; CI, combination index; H&E, hematoxylin & eosin; FACS, fluorescence activated cell sorter; NS, non-significant.

a promising gene-virotherapy for the treatment of leukemia in the near future.

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Disclosure

The authors report no conflicts of interest in this work.

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