

# Inhibition of tumor necrosis factor- $\alpha$ enhances apoptosis induced by nuclear factor- $\kappa$ B inhibition in leukemia cells

QIAO-MEI DONG, CHUN LING, XUAN CHEN and LI ZHAO

Central Laboratory, The First Hospital of Lanzhou University, Lanzhou, Gansu 730000, P.R. China

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**Abstract.** Inhibition of nuclear factor- $\kappa$ B (NF- $\kappa$ B) results in antitumor activity in leukemia cells, and may be a potential therapeutic strategy for the treatment of leukemia. However, a significant limitation of NF- $\kappa$ B inhibition in the treatment of leukemia is the low efficiency of this technique. NF- $\kappa$ B inhibitor treatment induces apoptosis in leukemia cells; however, it additionally causes inflammatory molecules to induce increased sensitivity of healthy hematopoietic cells to cell death signals, therefore limiting its clinical applications. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a key regulator of inflammation, and induces a variety of actions in leukemic and healthy hematopoietic cells. TNF- $\alpha$  induces NF- $\kappa$ B-dependent and -independent survival signals, promoting the proliferation of leukemia cells. However, in healthy hematopoietic cells, TNF- $\alpha$  induces death signaling, an effect which is enhanced by the inhibition of NF- $\kappa$ B. Based on these observations, the present study hypothesized that inhibition of TNF- $\alpha$  signaling may be able to protect healthy hematopoietic cells and other tissue cells, while increasing the anti-leukemia effects of NF- $\kappa$ B inhibition on leukemia cells. The role and underlying molecular mechanisms of TNF- $\alpha$  inhibition in the regulation of NF- $\kappa$ B inhibition-induced apoptosis in leukemia cells was therefore investigated in the present study. The results indicated that inhibition of TNF- $\alpha$  enhanced NF- $\kappa$ B inhibition-induced apoptosis in leukemia cells. It was also revealed that protein kinase B was significant in the regulation of TNF- $\alpha$  and NF- $\kappa$ B inhibition-induced apoptosis. During this process, intrinsic apoptotic pathways were activated. A combination of NF- $\kappa$ B and TNF- $\alpha$  inhibition may be a potential specific and effective novel therapeutic strategy for the treatment of leukemia.

## Introduction

Leukemia is a malignant disease of the hematopoietic system and is one of the ten most common causes of cancer-associated mortality in the Chinese population, particularly amongst individuals aged <35 years old (1). Chemotherapy is the most commonly utilized strategy for the treatment of leukemia (2). Although 50-70% patients achieve remission following treatment with conventional chemotherapy regimens, the majority of patients subsequently relapse and may develop chemoresistance (3-9). An improved understanding of the molecular mechanisms underlying leukemia may aid the identification of novel chemotherapeutic regimens, and improve the efficiency of treatments, therefore prolonging patient survival times. Thus, the mechanisms underlying leukemia require further investigation.

Previously, by comparing the gene expression and molecular activity of leukemic stem cells and healthy hematopoietic stem cells, a number of molecules (interleukin-3R, cluster of differentiation 47, cluster of differentiation 44) and signaling pathways [Nuclear factor- $\kappa$ B (NF- $\kappa$ B) (10), Wnt/ $\beta$ -catenin (11)] have been identified to be highly expressed or activated only in leukemic stem cells. Due to these results, the NF- $\kappa$ B signaling pathway has attracted the attention of researchers. NF- $\kappa$ B activity is detectable in the majority of leukemic cells, and its expression is significantly increased in leukemic cells compared with that of healthy bone marrow cells (12). Furthermore, *in vitro* studies have identified that NF- $\kappa$ B inhibition is capable of inducing apoptosis in leukemic stem cells, and apoptosis levels are significantly increased these cells compared with those of normal hematopoietic stem cells (10). Due to the ability of NF- $\kappa$ B inhibitor to induce apoptosis of leukemic cells, specifically leukemic stem cells, NF- $\kappa$ B inhibitors may present a potential anti-leukemic therapy (13-15). However, there are a number of potential side effects of NF- $\kappa$ B inhibition in the treatment of leukemia, which may limit its clinical application (16). NF- $\kappa$ B inhibition not only induces apoptosis in leukemia cells, it additionally acts on normal cells, inducing inflammatory molecules, particularly tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), to increase sensitivity to cell death signals (17). It has been reported that the toxicity induced by NF- $\kappa$ B inhibition was significantly suppressed in TNF- $\alpha$  or TNF- $\alpha$  receptor (Tnfr) knockout mice (18). Therefore, a combination of NF- $\kappa$ B and TNF- $\alpha$  inhibition may attenuate the side-effects associated with inhibition of NF- $\kappa$ B alone (18-24).

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*Correspondence to:* Ms. Li Zhao, Central Laboratory, The First Hospital of Lanzhou University, 1 Donggang West Road, Lanzhou, Gansu 730000, P.R. China  
E-mail: dongqm06@126.com

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Furthermore, TNF- $\alpha$  induces NF- $\kappa$ B-dependent and -independent survival signaling, therefore promoting proliferation of leukemia cells (25,26). Based on these previous results, the present study hypothesized that inhibition of TNF- $\alpha$  signaling may be able to protect healthy hematopoietic cells and other healthy tissue cells, while enhancing the anti-leukemic effects of NF- $\kappa$ B inhibition. A combination of NF- $\kappa$ B and TNF- $\alpha$  inhibition may be a potential specific and effective novel therapeutic strategy for the treatment of leukemia. The present study aimed to provide insight into the synergistic effects of NF- $\kappa$ B and TNF- $\alpha$  inhibition on leukemia cells. In addition, the underlying molecular mechanisms were investigated.

## Materials and methods

**Chemicals.** Roswell Park Memorial Institute (RPMI)-1640 and fetal bovine serum (FBS) were purchased from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). Rabbit monoclonal anti-protein kinase B (Akt) primary antibody used at 1:1,000, and rabbit anti-TNF- $\alpha$  antibody (TNF- $\alpha$  inhibitor) were obtained from Abcam (Cambridge, MA, USA). Rabbit polyclonal anti-caspase 9 primary antibody used at 1:1,000 was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). NF- $\kappa$ B inhibitor (MG-132) was purchased from Beyotime Institute of Biotechnology (Beijing, China). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Beijing Baosai Biotechnology Co., Ltd. (Beijing, China).

**Cell culture and drug treatment.** The HL-60, K562 and K562 with doxorubicin resistance (K562-ADM) human leukemia cell lines (Shanghai Institutes for Biological Sciences, Shanghai, China) were grown in RPMI-1640 medium supplemented with 10% FBS, and maintained in humidified 5% CO<sub>2</sub> at 37°C.

For treatment with anti-TNF- $\alpha$  antibody and MG-132, cells were plated at a density of  $1.5 \times 10^5$  cells/well in 2 ml RPMI-1640 in a 6-well plate. Twenty-four hours later, the medium was replaced and anti-TNF- $\alpha$  antibody (10 ng/ml) and MG-132 (3  $\mu$ M) were added, alone or in combination. Cells were incubated at 37°C for 48 h and subsequently used for further experiments.

**Detection of apoptosis by flow cytometry (FCM) and microscopy.** In order to evaluate the apoptotic rate the various cell types, an Annexin V-FITC apoptosis detection kit was used according to the manufacturer's protocol. Briefly, cells were collected and resuspended in 200  $\mu$ l binding buffer (Beijing Baitaike Biology Company (Beijing, China). Subsequently, 10  $\mu$ l Annexin V-FITC and 5  $\mu$ l propidium iodide (PI) were added to the suspension and allowed to react for 15 min at room temperature. Following incubation, 300  $\mu$ l binding buffer was added and the apoptotic rate was determined using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). In addition, cell morphology was observed using a U-TV0 phase contrast microscope (Olympus Corporation, Tokyo, Japan).

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted using the TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) method and reverse transcribed using

an Invitrogen SuperScript III First-Strand Synthesis system (Thermo Fisher Scientific, Inc.) to generate complementary DNA, according to the manufacturer's protocol. Transcription of GAPDH was examined using the following primers (Shanghai Sangong Pharmaceutical Co., Ltd., Shanghai, China): F 5'-AGTCAACGGATTTGGTCGTATT-3' and R 5'-AATGAGCCCCAGCCTTCT-3'. Primers for Akt were, F 5'-AAGACGACGAGGACTGCTATG-3' and R 5'-ACCCTTCCGCTTATCCACTGA-3'. PCR steps were performed using an Applied Biosystems GeneAmp® PCR System 9700 thermocycler (Thermo Fisher Scientific, Inc.).

RT-PCR was performed using a LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Burgess Hill, UK) according to the manufacturer's instructions. Transcription of  $\beta$ -actin was examined with primers, 5'-TGGCACCACAGCACAATGAA-3' and 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'. Primers for Akt were, 5'-CTTCGTCCTCCTCCTCACAC-3' and, 5'-GCCTGCTTCTCCAACAACA-3'; and primers for caspase 9 were, 5'-AACAGGCAAGCAGCAAAGTT-3' and, 5'-CCTC-CAGAACCAATGTCCAC-3'. Following an initial denaturation step at 95°C for 5 min, 40 cycles of amplification for each primer pair were performed. Each cycle included a denaturation step, 10 sec at 95°C; an annealing step, 20 sec at 60°C; and an elongation step, 10 sec at 72°C. A final elongation phase was performed at 65°C for 1 min. Relative gene expression levels were measured using a LightCycler 480 (Roche Diagnostics) according to the manufacturer's protocol. Relative changes in the gene expression levels of caspase 9 and Akt were normalized against the levels of  $\beta$ -actin gene expression in each sample. Experiments were performed at least in duplicate for each data point.

**Western blotting.** Total cells were lysed with buffer (1% sodium dodecyl sulfate, 10 mm Tris-Cl, pH 7.6, 20 g/ml aprotinin, 20 g/ml leupeptin and 1 mm 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; Shanghai Sangong Pharmaceutical Co., Ltd.). Protein concentrations were determined using the Bradford method. Protein (20  $\mu$ g) was separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 10% non-fat milk, membranes were incubated with anti-AKT and anti-caspase 9 antibodies (as mentioned in the Chemicals sections, above) at 4°C overnight. Following washing three times with TBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:200 dilution (Abcam, Cambridge, UK) at room temperature for 1 h. The signals were developed using an enhanced chemiluminescence kit (Applygen Technologies, Inc., Beijing, China) and rabbit polyclonal anti- $\beta$ -actin antibody (dilution, 1:1,000; cat no. ab8227, Abcam) served as an internal control.

**Statistical analysis.** All statistical comparisons were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). Student's t-test was utilized to compare the differences or association between the two groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Inhibition of TNF- $\alpha$  enhances apoptosis induced by NF- $\kappa$ B inhibition in leukemia cells.** K562 cells were treated with

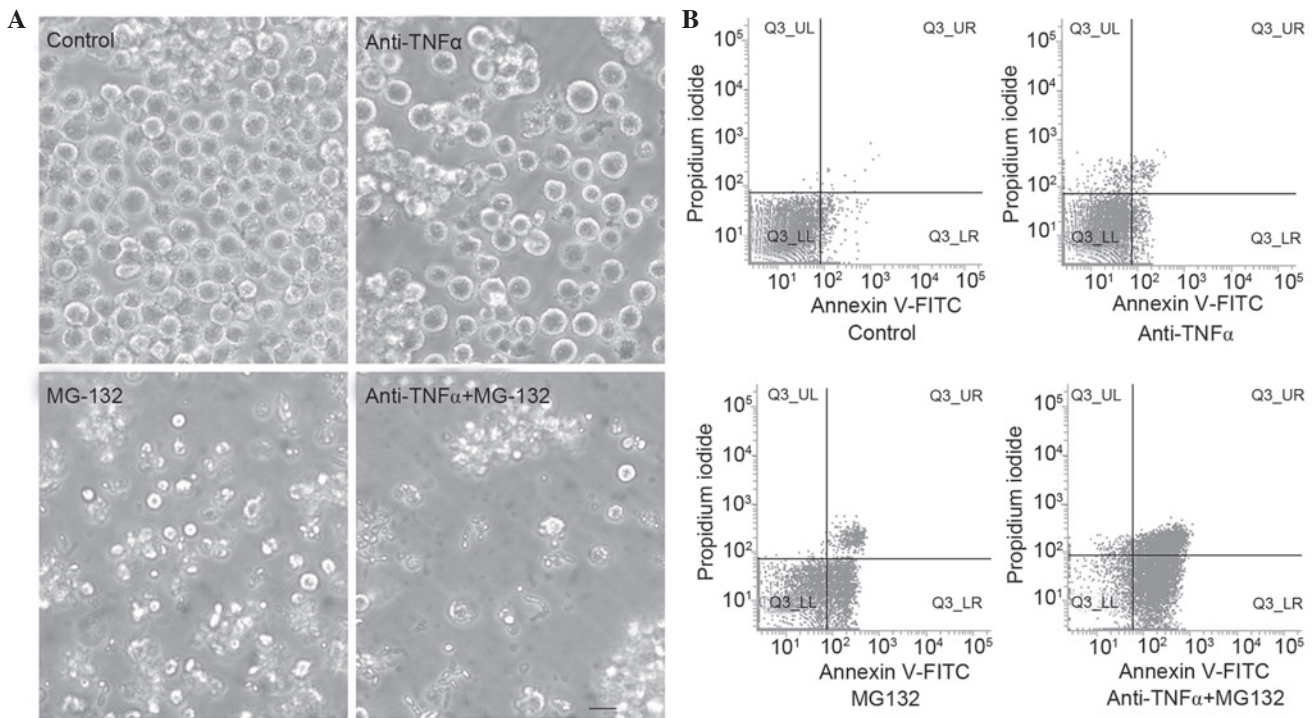


Figure 1. Inhibition of TNF- $\alpha$  enhances apoptosis induced by nuclear factor- $\kappa$ B inhibition in K562 cells. K562 cells were treated with anti-TNF- $\alpha$  antibody and MG-132, respectively or in combination. Following treatment, the apoptotic morphology and rate were compared. (A) Cell morphology was observed by phase contrast microscopy. Scale bar, 10  $\mu$ m; magnification,  $\times$ 400. (B) Cell apoptosis was detected via flow cytometry and Annexin V-FITC and propidium iodide immunofluorescence. FITC, fluorescein isothiocyanate; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

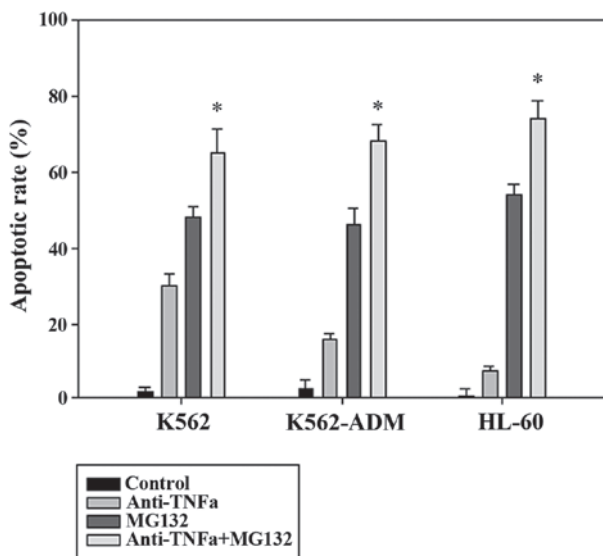


Figure 2. Combined effects of anti-TNF- $\alpha$  antibody and MG-132 treatment in various leukemia cell types. K562, K562-ADM and HL-60 cells were treated with anti-TNF- $\alpha$  antibody and MG-132, respectively or in combination. The control cells were cultured in unsupplemented RPMI-1640 medium. Following treatment, apoptotic rates were detected via flow cytometry. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \* $P$ <0.05 vs. control.

anti-TNF- $\alpha$  antibody (TNF- $\alpha$  inhibitor) and MG-132 (NF- $\kappa$ B inhibitor) (27), respectively or in combination. Following treatment, the apoptotic morphology and rate were compared with those of the control group. Cell morphology was observed by phase contrast microscopy. Compared with the control group, anti-TNF- $\alpha$  antibody or MG-132 treatment

induced a typical apoptotic cell morphology (intense staining, fragmented and condensed nuclei) in K562 cells, and anti-TNF- $\alpha$  antibody and MG-132 co-treated cells displayed more marked apoptotic morphology compared with that of the groups treated with anti-TNF- $\alpha$  antibody or MG-132 separately (Fig. 1A).

Subsequently, the apoptotic rates of the anti-TNF- $\alpha$  antibody and MG-132 combined treatment group, and the separately treated groups, were detected using FCM and Annexin V-FITC and PI immunofluorescence (Fig. 1B). The results indicated that the apoptotic rate of the combined treatment group ( $68.31 \pm 4.15\%$ ) was increased compared with that of the groups that were treated separately (anti-TNF- $\alpha$  antibody treatment,  $30.5 \pm 3.11\%$ ; MG-132 treatment,  $50.53 \pm 2.85\%$ ) and the control group ( $2.23 \pm 1.23\%$ ;  $P$ <0.05).

In order to confirm that the effects of co-treatment with TNF- $\alpha$  inhibitor and NF- $\kappa$ B inhibitor were not restricted to K562 cells, additional types of leukemia cell, including K562-ADM and HL-60, were investigated (Fig. 2). These cells were also treated with anti-TNF- $\alpha$  antibody and MG-132, respectively or in combination. Following treatment, the apoptotic rate was detected via FCM. The apoptotic rates of the combined treatment group in K562-ADM cells ( $72.38 \pm 2.57\%$ ) were increased compared with those of the separately treated groups (anti-TNF- $\alpha$  antibody treatment,  $18.37 \pm 1.35\%$ ; MG-132 treatment,  $46.35 \pm 3.65\%$ ) and the control group ( $3.25 \pm 2.33\%$ ;  $P$ <0.05). The apoptotic rate of the combined treatment group in HL-60 cells ( $78.65 \pm 3.72\%$ ) was also increased compared with the separately treated groups (anti-TNF- $\alpha$  antibody treatment,  $6.85 \pm 1.42\%$ ; MG-132 treatment,  $57.44 \pm 2.46\%$ ) and the control group ( $1.46 \pm 2.28\%$ ;  $P$ <0.05).



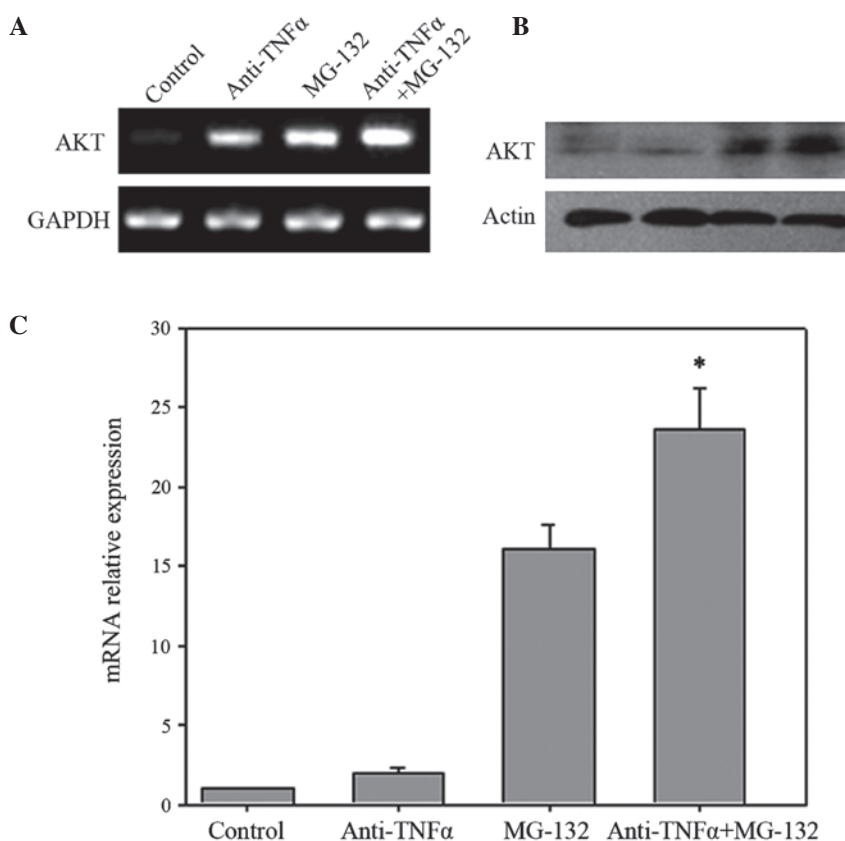


Figure 3. Combined treatment with anti-TNF- $\alpha$  antibody and MG-132 promotes the expression of Akt. K562 cells were treated with anti-TNF- $\alpha$  antibody and MG-132, respectively or in combination. Following treatment, mRNA and protein expression of Akt were detected by (A) RT-PCR, (B) western blotting and (C) RT-qPCR. \*P<0.05 vs. control. mRNA, messenger RNA; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; RT-PCR, reverse transcription-polymerase chain reaction; q, quantitative.

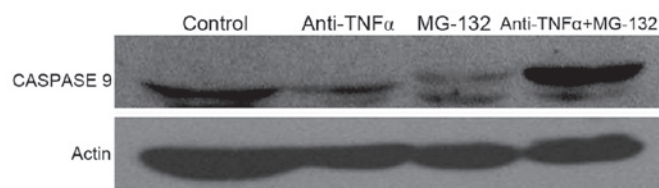


Figure 4. Combined treatment with anti-TNF- $\alpha$  antibody and MG-132 promotes activation of caspase 9. K562 cells were treated with anti-TNF- $\alpha$  antibody and MG-132, respectively or in combination. Following treatment, caspase 9 protein expression was detected via western blotting. TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

*Treatment with a combination of anti-TNF- $\alpha$  antibody and MG-132 promotes Akt expression.* Akt is located downstream of TNF- $\alpha$  and NF- $\kappa$ B signaling (28). The messenger RNA (mRNA) and protein expression levels of Akt in anti-TNF- $\alpha$  antibody and MG-132 co-treated cells, separately treated cells and control cells were therefore detected by RT-PCR (Fig. 3A), western blotting (Fig. 3B) and RT-quantitative (q) PCR (Fig. 3C). The results of the present study revealed that the expression levels of Akt were increased in anti-TNF- $\alpha$  antibody or MG-132 treated cells, while co-treated cells expressed increased levels of Akt compared with those of the separately treated cells or control cells, in terms of mRNA and protein.

*Combined treatment with anti-TNF- $\alpha$  antibody and MG-132 promotes caspase 9 activation.* Caspase 9 is activated in the

intrinsic apoptotic pathway (29), therefore in the present study, expression and activation of caspase 9 in anti-TNF- $\alpha$  antibody and MG-132 co-treated, separately-treated and control cells, was investigated using western blotting. Caspase 9 was identified to be highly expressed and activated in co-treated cells, compared with control cells and separately treated cells (Fig. 4). Caspase 9 activation was promoted following TNF- $\alpha$  and NF- $\kappa$ B inhibitor co-treatment.

## Discussion

NF- $\kappa$ B is a nuclear transcription factor, which may mediate survival pathways in a number of types of cancer, including leukemia (30). NF- $\kappa$ B induces the expression of genes involved in cell proliferation, angiogenesis and metastasis, and possesses significant roles in carcinogenesis and chemoresistance (31). NF- $\kappa$ B activity is detectable in the majority of leukemic cells, and its expression has been observed to be significantly increased in leukemic cells compared with that of healthy bone marrow cells (32,33). NF- $\kappa$ B inhibition exerts antitumor activity in leukemia cells (27). Cosimo *et al* (34) reported that inhibition of NF- $\kappa$ B was able to induce apoptosis in chronic lymphocytic leukemia cells. Therefore, inhibition of the activity of NF- $\kappa$ B may be a potential novel therapeutic strategy for the treatment of leukemia. However, a significant limitation of NF- $\kappa$ B inhibition in the treatment of leukemia is the low efficiency. NF- $\kappa$ B inhibition induces apoptosis in leukemia cells, however additionally causes inflammatory molecules (particularly TNF- $\alpha$ )

to increase the sensitivity of normal hematopoietic cells to cell death signals (18), which limit its clinical application.

TNF- $\alpha$  is a central regulator of inflammation, which exerts various functions in leukemia and normal hematopoietic cells (35). TNF- $\alpha$  is continually expressed in a number of types of leukemia cell (36), and has been demonstrated to upregulate certain molecules involved in cell growth and proliferation via the NF- $\kappa$ B-dependent or -independent pathway in leukemia (37). TNF- $\alpha$  is critical for the growth and survival of leukemic cells. However, in healthy hematopoietic cells, TNF- $\alpha$  induces death signaling (20). TNF- $\alpha$ -induced death signaling has been observed to be enhanced by NF- $\kappa$ B inhibition in healthy hematopoietic cells. The toxicity induced by NF- $\kappa$ B inhibition was observed to be significantly suppressed in TNF- $\alpha$  or TnfR knockout mice (25). In the present study, it was suggested that inhibition of TNF- $\alpha$  may be capable of enhancing NF- $\kappa$ B inhibitor-induced apoptosis in leukemia cells. Combined treatment with NF- $\kappa$ B and TNF- $\alpha$  inhibitors may be a specific and effective novel therapeutic strategy for the treatment of leukemia. Leukemia cells were treated with anti-TNF- $\alpha$  antibody and MG-132, respectively or in combination, and the apoptotic rates of these various treatment groups were compared. It was identified that combination treatment with anti-TNF- $\alpha$  antibody and MG-132 enhanced apoptosis in a number of leukemia cell types, including K562 and HL-60 cells. K562-ADM is a doxorubicin-resistant leukemia cell line. In K562-ADM cells, which are chemoresistant, a significant increase in apoptosis was also observed following co-treatment with MG-132 and anti-TNF- $\alpha$  antibody.

The molecular mechanism by which inhibition of TNF- $\alpha$  and NF- $\kappa$ B promotes apoptosis in leukemia cells was additionally elucidated. Previously, it was demonstrated that Akt is a downstream molecule influenced by NF- $\kappa$ B signaling (38). In addition, TNF- $\alpha$  induced activation of the phosphoinositide 3-kinase/Akt pathway, and therefore the transcriptional activation of NF- $\kappa$ B, in C4HD murine mammary tumor cells (28). In the present study, the expression of Akt in the presence of anti-TNF- $\alpha$  antibody and MG-132, respectively or in combination, was detected. The present study identified that the expression of Akt was significantly increased, at the mRNA and protein levels, when leukemia cells were treated with anti-TNF- $\alpha$  antibody and MG-132 in combination. This result further confirmed the involvement of Akt in the regulation of TNF- $\alpha$  and NF- $\kappa$ B inhibition-induced apoptosis in K562 cells.

The results of the present study also revealed that caspase 9 was activated following anti-TNF- $\alpha$  antibody and MG-132 co-treatment. However, low levels of cleavage of caspase 9 were identified in cells treated with anti-TNF- $\alpha$  antibody or MG-132 alone. Caspase 9 is activated during the intrinsic apoptotic pathway (29). In K562 cells, inhibition of NF- $\kappa$ B and TNF- $\alpha$  signaling induced activation of the intrinsic apoptotic pathway, during which caspase 9 was activated.

In conclusion, the present study revealed that inhibition of TNF- $\alpha$  enhanced NF- $\kappa$ B inhibition-induced apoptosis in leukemia cells. Akt was significant in the regulation of TNF- $\alpha$  and NF- $\kappa$ B inhibition-induced apoptosis. During this process, the intrinsic apoptotic pathway was activated. A combination of treatment with NF- $\kappa$ B inhibitor and TNF- $\alpha$  inhibitor may therefore present a specific and effective novel therapeutic strategy for the treatment of leukemia.

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