

# Alpha-phellandrene, a Natural Active Monoterpene, Influences a Murine WEHI-3 Leukemia Model *In Vivo* by Enhancing Macrophage Phagocytosis and Natural Killer Cell Activity

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**Abstract.** *α*-phellandrene (*α*-PA), a cyclic monoterpene, is a natural compound reported to promote immune responses in normal BALB/c mice. The effects of *α*-PA on immune responses in a leukemia mouse model were examined. Mice were injected with mouse leukemia WEHI-3 cells and subsequently treated orally with or without *α*-PA (0, 25 and 50 mg/kg) and olive oil as positive control for two weeks. Leukocytes and splenocytes were isolated and cell markers for CD3, CD19, CD11b and Mac-3, phagocytosis and natural killer cell cytotoxicity effects were analyzed by flow cytometry. *α*-PA increased the percentage of CD3 (T-cell marker), CD19 (B-cell marker) and MAC3 (macrophages) markers but reduced the percentage of CD11b (monocytes) cell surface markers. *α*-PA (25 and 50 mg/kg) increased phagocytosis of macrophages from blood samples and treatment promoted natural killer cell activity at 25 mg/kg from splenocytes. *α*-PA at 25 mg/kg also increased B- and T-cell proliferation.

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**Key Words:** *α*-phellandrene, WEHI-3 leukemia cells, phagocytosis, natural killer cells, leukemia Balb/c mice.

Approximately 50% of childhood cancers are leukemias and lymphomas (1) and leukemia is the second most malignant tumor in children (2). In Taiwan, a 2010 report from the Department of Health, Executive Yuan, R.O.C. (Taiwan) indicated that about 4.2 individuals per 100,000 die from leukemia. Treatment approaches for leukemia patients including immunotherapy, radiotherapy, chemotherapy, or a combination of radiotherapy with chemotherapy are unsatisfactory. Numerous reports have shown that increased consumptions of vegetables and fruits can reduce the formation of human cancers, especially colon cancer (3-5).

The majority of clinical anticancer drugs used come from natural plant products (e.g., docetaxel, vinca alkaloids, topotecan) and identifying new compounds is a high priority in cancer research. *α*-phellandrene (*α*-PA), 5-isopropyl-2-methyl-1, 3-cyclohexadiene, is a component from the essential oil of *Schinus molle* L., *Schinus terebinthifolius* Raddi and in *Zingiber officinale* Roscoe (6, 7). It was reported that *α*-PA (17.5%) from *Solanum eschscholzii* essential oils exhibits antimicrobial activity (8). It has also been shown that *α*-PA is effective against human breast cancer Hs 578T cells and prostate tumor PC-3 cells (8). Recently, we reported that *α*-PA stimulated immune responses in normal Balb/c mice *in vivo* (9). Several reports have shown that *α*-PA has biological activity but there is no report to show an effect of *α*-PA on the immune responses of leukemic mice *in vivo*.

The purpose of the present study was to determine whether *α*-PA stimulated any immune responses on leukemic BALB/c mice injected with leukemia cells.

## Materials and Methods

**Materials and reagents.**  $\alpha$ -phellandrene ( $\alpha$ -PA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and penicillin-streptomycin were obtained from Gibco Life Technologies (Carlsbad, CA, USA). Pharm Lyse™ lysing buffer, PE-labeled anti-mouse Mac-3 antibodies, FITC-labeled anti-mouse CD11b, FITC-labeled anti-mouse CD3 and PE-labeled anti-mouse CD19 were obtained from BD Biosciences Pharmingen Inc. (San Diego, CA, USA).

**Murine leukemia cells (WEHI-3).** The WEHI-3 cell line (murine myelomonocytic leukemia cells) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were cultured in 75-cm<sup>2</sup> plastic culture flasks in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM L-glutamine at 37°C under 5% CO<sub>2</sub> in humidified atmosphere air. Cells were then cultivated for two complete cycles in an incubator (10-12).

**Animals.** Fifty male Balb/c mice at 8-weeks-old and weight 22-25 g were obtained from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan, ROC). Mice were housed in a filtered laminar air flow room in the Animal Center of China Medical University. Cages, bedding and food were autoclaved before use. Water and food were provided *ad libitum*. Treatment of animals was according to Institutional guidelines (Affidavit of Approval of Animal Use Protocol), which was approved by the Institutional Animal Care and Use Committee (IACUC), China Medical University (Taichung, Taiwan).

**Injection of WEHI-3 cells into mice and  $\alpha$ -PA treatment.** Fifty normal Balb/c mice were used and ten mice were not intraperitoneally (I.P.) injected with WEHI-3 cells, setting it as the normal group (Group I). Forty Balb/c mice were intraperitoneally (*i.p.*) injected with  $1 \times 10^5$  WEHI-3 cells. After 2 weeks, animals were randomly separated into four groups of 10 mice per group. Group III was the control group not injected with WEHI-3 cells. Group II was a vehicle (olive oil) control group. Group IV,  $\alpha$ -PA (25 mg/kg). Group V,  $\alpha$ -PA (50 mg/kg).  $\alpha$ -PA was administered by oral gavage daily for 2 weeks after which time, mice were weighed and sacrificed using CO<sub>2</sub> (9-11).

**Immunofluorescence staining for surface markers.** Blood (1 ml) was collected from mice before they were euthanized. Blood samples were lysed with 1 $\times$ Pharm Lyse™ lysing buffer (BD Biosciences), were then centrifuged for 15 min at 1,500 rpm at 4°C to isolate white blood cells. Cells were stained using FITC-labeled anti-mouse CD3 and FITC-labeled anti-mouse CD11b, PE-labeled anti-mouse Mac-3 antibodies and PE-labeled anti-mouse CD19 (BD Biosciences Pharmingen Inc., San Diego, CA, USA) for 30 min. Samples were washed twice with PBS, and then analyzed for cell markers using flow cytometry as previously described (9-11).

**Macrophage phagocytosis.** Macrophages were isolated from PBMC and the peritoneum of each mouse. Macrophages were maintained in a 96-well plate, then 50  $\mu$ l of *E. coli*-FITC was added and phagocytosis was determined using the PHAGOTEST® kit according to

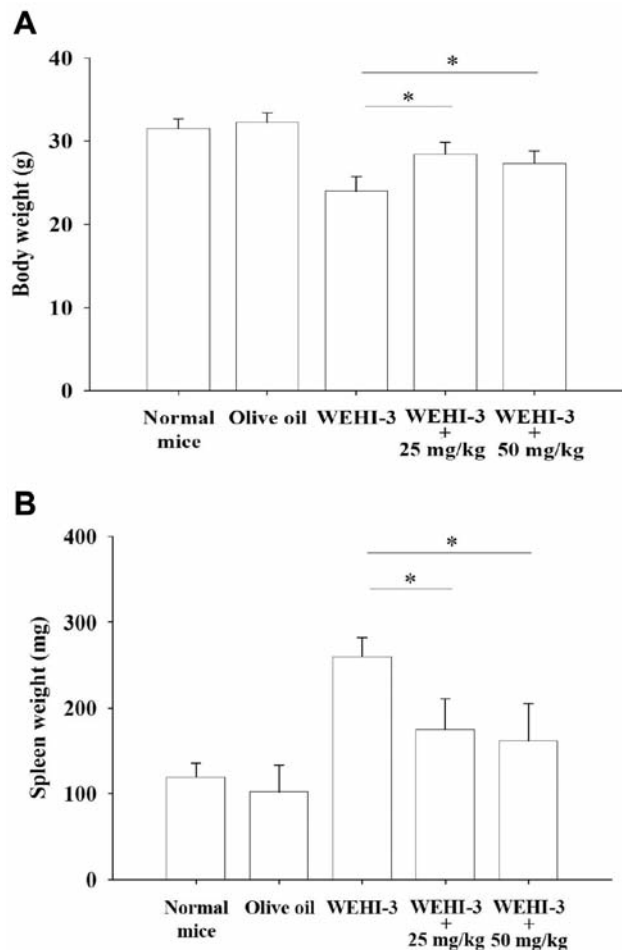


Figure 1.  $\alpha$ -PA effects on the body and spleen weights of leukemic mice. Fifty normal mice were used in this experiment, ten mice were not intraperitoneally (I.P.) injected with WEHI-3 cells as normal group (Group I) and other forty mice were I.P. injected with WEHI-3 cells for 2 weeks before being randomly separated into four groups (each group contains 10 mice). Group II mice were treated with olive oil alone. Group III, IV and V mice were treated with  $\alpha$ -PA at 0, 25 and 50 mg/kg, respectively in olive oil. During the treatment, all animals were monitored and measured for the weights of body (A) and spleen (B). \* $p < 0.05$  Significant difference between control and  $\alpha$ -PA-treated groups.

manufacturer's instructions (ORPEGEN Pharma Gesellschaft für biotechnologische, Heidelberg, Germany). Phagocytosis of each sample was analyzed by flow cytometry and quantified by the CellQuest software (Becton Dickinson), as described previously (9-11).

**Natural killer (NK) cell cytotoxic activity.** At the end of treatment, the spleens of all mice were collected and prepared for splenocyte collection, as previously described (9-11). Splenocytes (at a density of  $1 \times 10^5$ ) from each mouse were maintained in each well of a 96-well plates in 1 ml of RPMI 1640 medium. The target YAC-1 cells ( $2.5 \times 10^7$  cells) in serum-free RPMI 1640 medium and the PKH-67/Dil.C buffer (Sigma-Aldrich Corp.) was added to each well then mixed thoroughly for 2 min at 25°C and 2 ml PBS was added for

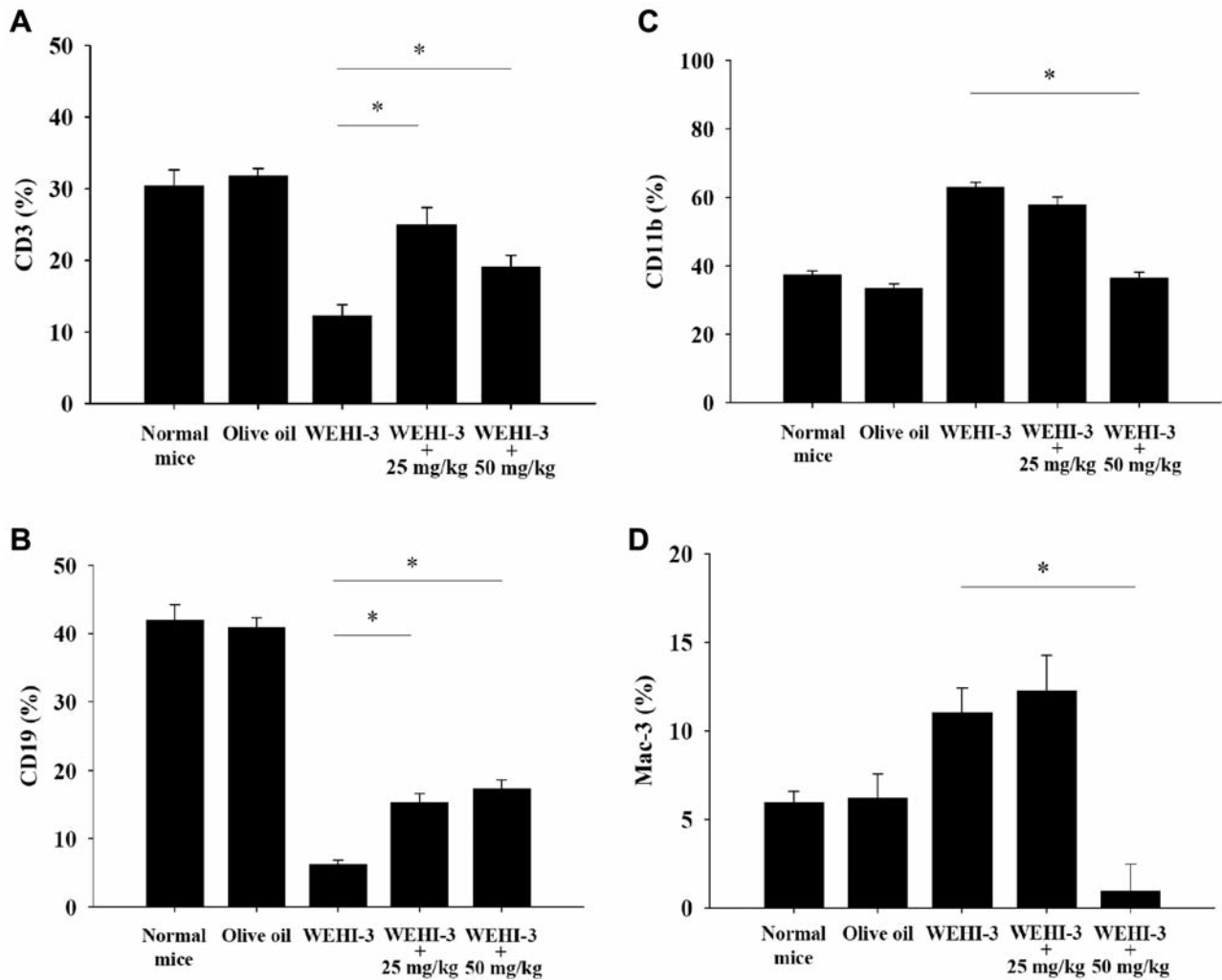


Figure 2.  $\alpha$ -PA affected surface markers on whole blood cells from WEHI-3-leukemic BALB/c mice. Blood samples were collected from each animal of each group and then leukocytes were collected, to measure cell surface markers from  $\alpha$ -PA-treated and -untreated (control) groups. Levels of CD3 (A), CD19 (B), CD11b (C) and Mac-3 (D) were measured for all samples by flow cytometry, as described in Materials and Methods. \* $p < 0.05$  Significant difference between control and  $\alpha$ -PA-treated groups.

1 min. Finally 4 ml of medium were added to each well. Cells were then incubated for 10 min and then centrifuged at 1,200 rpm at 25°C. NK cell cytotoxic activity was analyzed by flow cytometry, as described elsewhere (9-11).

**Measurements for T- and B-cell proliferation.** Isolated splenocytes ( $1 \times 10^5$  cells/well) were placed in a 96-well plate containing 100  $\mu$ l of RPMI-1640 medium. For the T-cell proliferation test, samples were stimulated with concanavalin A (Con A, 5  $\mu$ g/ml) for 3 days. For the B-cell proliferation test, samples were stimulated with lipopolysaccharide (LPS, 5  $\mu$ g/ml) for 5 days. Cell proliferation was measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA), as previously described (9-11).

**Statistical analysis.** All assays were performed in triplicates, and the results are presented as means  $\pm$  SD. Differences between control and

$\alpha$ -PA groups were analyzed by Student's *t*-test. Differences were considered significant at  $p < 0.05$ .

## Results

**$\alpha$ -PA effects on body and spleen weights of leukemic mice.** Results indicated that  $\alpha$ -PA in a two-dose treatment did not significantly affect body weight (Figure 1A), but reduced spleen weight (Figure 1B) when compared to the olive oil-treated and normal groups.

**$\alpha$ -PA effects on the production of surface markers of blood cells from mice injected with WEHI-3-leukemia cells.** In order to evaluate whether  $\alpha$ -PA affects the cell population of

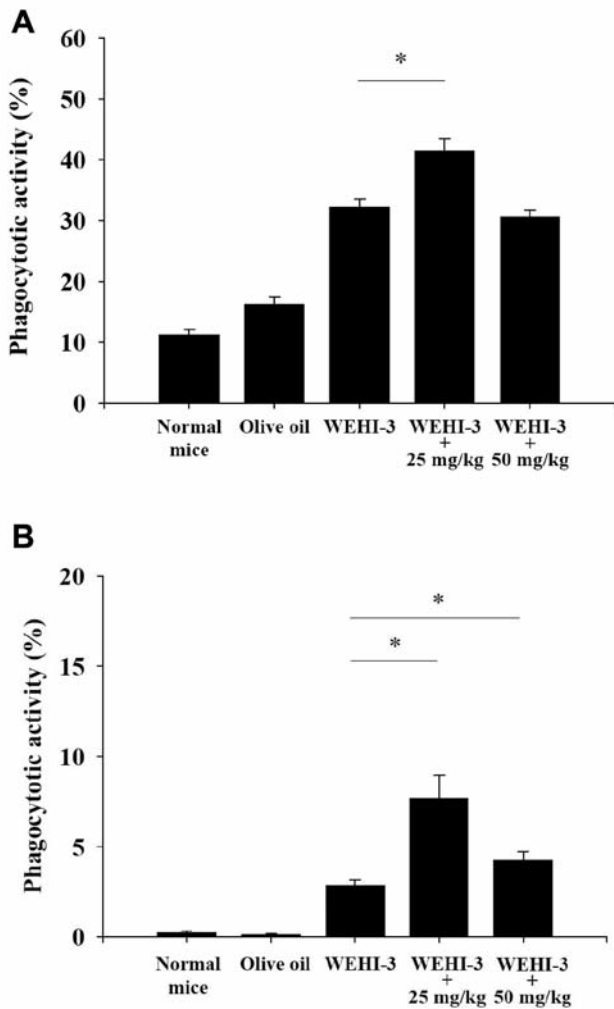


Figure 3.  $\alpha$ -PA affected macrophage phagocytosis from WEHI-3-leukemic BALB/c mice. Isolated macrophages from each animal of group were measured for phagocytosis by flow cytometry and quantified by CellQuest, as described in Materials and Methods. PBMCs (A); peritoneal (B) cavity. \* $p < 0.05$  Significant difference between control and  $\alpha$ -PA-treated groups.

leukocytes, we isolated blood samples from each animal and collected leukocytes in order to measure cell surface markers from  $\alpha$ -PA-treated and -untreated (control) groups. Levels of CD3, CD19, CD11b and Mac-3 were measured by flow cytometry and results are shown in Figure 2A, B, C and D. Data in Figure 2A show that  $\alpha$ -PA at a low dose (25 mg/kg) increased CD3 (T-cells) but this was not observed for the two higher doses. However, both 5 and 25 mg/kg reduced the CD19 (B-cell) (Figure 2B) population. It was noted that  $\alpha$ -PA at 25 and 50 mg/kg significantly increased the CD11b (monocytes) population (Figure 2C). The Mac-3 cell population (macrophage) was increased by  $\alpha$ -PA but only at 25 mg/kg and not at a lower dose (Figure 2D).

*Effects of  $\alpha$ -PA on macrophage phagocytosis in mice injected with WEHI-3 leukemia cells.* Blood samples and spleens were collected from  $\alpha$ -PA-treated and -untreated (control) mice and phagocytosis was determined. Figure 3A shows that macrophage phagocytosis in blood was increased in mice injected with WEHI-3 cells.  $\alpha$ -PA treatment of mice injected with WEHI-3 cells has a significant effect on phagocytosis of macrophages.

*$\alpha$ -PA stimulated NK cell activity in mice injected with WEHI-3 cells.* Spleens were collected from  $\alpha$ -PA-treated and -untreated (control) mice injected with WEHI-3 cells and NK cell activity was examined. Figure 4 shows that  $\alpha$ -PA (50 mg/kg) promoted NK cell activity. A lower dose however (25 mg/kg) did not affect NK cell activity.

*Effects of  $\alpha$ -PA on T- and B-cell proliferation in mice injected with WEHI-3 leukemia cells.* Spleens were collected from mice injected with WEHI-3 leukemia cells and treated with or without  $\alpha$ -PA. Figures 5A and 5B show that  $\alpha$ -PA (25 mg/kg) increased B- and T-cell proliferation, respectively.

## Discussion

Essien *et al.* (2012) demonstrated that *Solanumerianthum* essential oils contain  $\alpha$ -PA (17.5%), which has been shown to have anti cancer properties in human breast cancer Hs 578T cells and human prostate tumor PC-3 cells (8). We have shown that  $\alpha$ -PA promoted immune responses in normal Balb/c mice but there is no available information to show that  $\alpha$ -PA affects the growth of leukemia mice *in vivo*. In the present study, we investigated the effect of  $\alpha$ -PA on immune-associated cell markers, phagocytosis and NK cell cytotoxic effects in mice injected with WEHI-3 cells.

$\alpha$ -PA at a low dose (25 mg/kg) increased the CD3 marker (T-cells) population and at the higher dose (50 mg/kg) decreased the CD19 (B-cell) marker (Figure 2) levels. It is well-known that infiltrating T-cells at the site of disease play an important role in tumor control (13-15) and reversal of *in situ* T-cell exhaustion during effective human anti-leukemia responses to donor lymphocyte infusion (16). However, both doses of  $\alpha$ -PA did not significantly affect the CD3 marker population. CD19 is an activated B-cell surface marker (17) and B-cell differentiation also requires the interaction of various cytokines that come from macrophages or T-cell secretion (3).  $\alpha$ -PA at 25 and 50 mg/kg increased the CD11b population and at 25 mg/kg also increased Mac-3 levels. A high dose of  $\alpha$ -PA stimulated B- (Figure 5A) and T- (Figure 5B) cell proliferation. Those findings suggest that  $\alpha$ -PA may activate immune responses through the proliferation of B- and T-cells. Figure 3A indicated that  $\alpha$ -PA at 25 and 50 mg/kg increased macrophage phagocytosis.  $\alpha$ -PA (25 mg/kg) also increased NK cell cytotoxic activity. These findings are

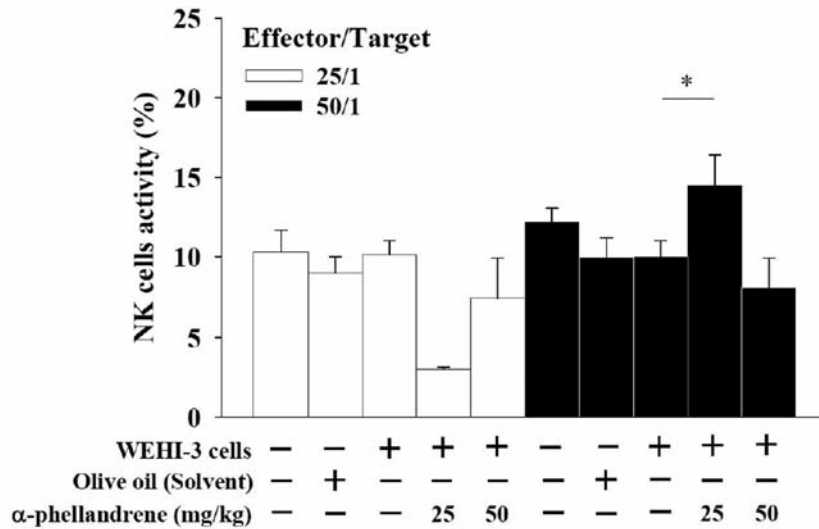


Figure 4.  $\alpha$ -PA affected natural killer cell activity from WEHI-3-leukemic BALB/c mice. Isolated splenocytes ( $1 \times 10^5$ ) were added to YAC-1 cells ( $2.5 \times 10^7$  cells) for the determination of NK cell cytotoxic activity by flow cytometry and quantified by CellQuest, as described in Materials and Methods. \* $p < 0.05$  Significant difference between control and  $\alpha$ -PA-treated groups.

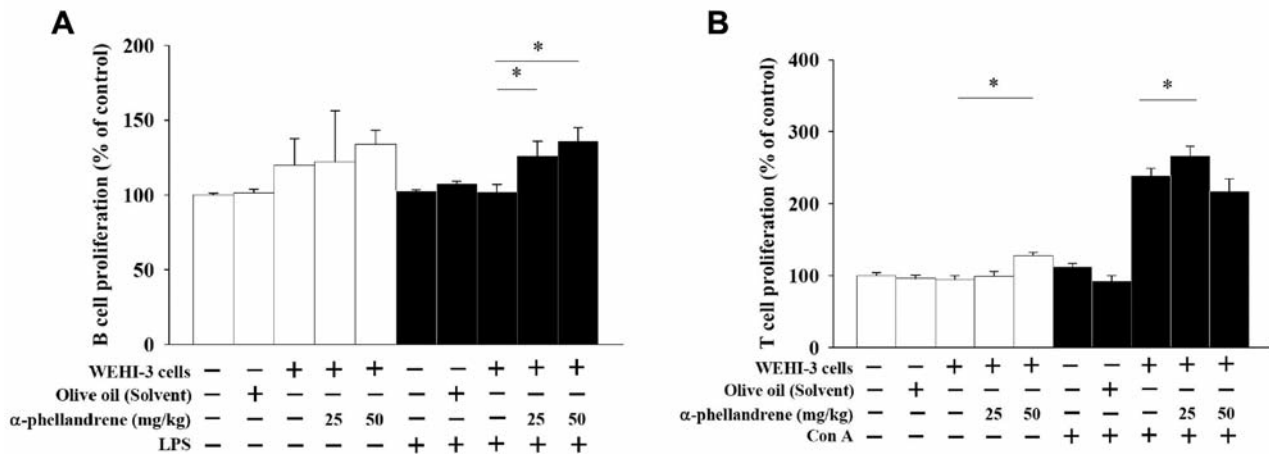


Figure 5.  $\alpha$ -PA affected T- and B-cell proliferation from WEHI-3-leukemic BALB/c mice. Spleens were collected from  $\alpha$ -PA-treated and -untreated (control) mice, then were individually measured for proliferation of B cells (A) and T cells (B) by flow cytometry and quantified by CellQuest, as described in Materials and Methods. \* $p < 0.05$  Significant difference between control and  $\alpha$ -PA-treated groups.

in agreement with our earlier studies in normal mice (9). Macrophages play an important role in generating inflammatory reaction responses upon tissue damage or invading pathogens and the modulation of macrophage responses is critical for maintaining tissue homeostasis (18). NK cells have been shown to detect and clear the transformed or virus-infected cells and were reported to recognize and respond to bacteria-infected cells (19).

Taken together, our results indicate that  $\alpha$ -PA promoted immune response through stimulation of macrophage phagocytosis and NK cell activation in mice injected with mice WEHI-3 leukemia cells. Our findings are the first to show that oral administration of  $\alpha$ -PA can activate immune responses in mice injected with leukemia cells. Our results provide the rationale for further studies on the potential use of  $\alpha$ -PA as a treatment for leukemia.

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