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# Bufalin Enhances Immune Responses in Leukemic Mice Through Enhancing Phagocytosis of Macrophage *In Vivo*

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**Abstract.** *Background/Aim: Bufalin, bufadienolide present in Chan Su, has been shown to induce cancer cell apoptosis in many human cancer cells, including human leukemia cells, but its effects on immune responses are unknown. Materials and Methods: This study investigated whether bufalin affected immune responses of mice with WEHI-3 cell-generated leukemia in vivo. BALB/c mice were intraperitoneally injected with WEHI-3 cells to develop leukemia and then were treated with oral treatment with bufalin at different doses (0, 0.1, 0.2 and 0.4 mg/kg) for 2 weeks. At the end of treatment, all mice were weighted and blood was collected; liver and spleen tissues were collected for cell marker, phagocytosis, natural killer (NK) cell activity and T- and B-cell proliferation measurements by using flow cytometric assays. Results: When compared with the leukemia control group, bufalin increased the body weight, but reduced liver and spleen weights, and reduced CD3, CD16 and Mac-3 cell markers at 0.4 mg/kg treatment and increased CD11b marker at 0.1 and 0.2 mg/kg treatment. Furthermore, bufalin at 0.4 mg/kg increased phagocytosis by macrophages isolated from peripheral blood mononuclear cells and at 0.1 mg/kg by those from the peritoneal cavity. Bufalin (0.2 and 0.4 mg/kg) increased NK cell cytotoxic activity at effector:target ratio of 50:1. Bufalin increased B-cell proliferation at 0.1 and 0.2 mg/kg treatment but only increased T-cell proliferation at 0.1 mg/kg. Bufalin increased glutamate oxaloacetate transaminase level at all dose treatments, increased glutamic pyruvic transaminase level only at 0.1 mg/kg treatment, but reduced the level of lactate dehydrogenase at all dose levels in mice with WEHI-3 cell-induced leukemia in vivo. Conclusion: Bufalin increased immune responses by enhancing phagocytosis in mice with leukemia mice.*

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Leukemia is a kind of malignant disease arising from unregulated proliferation of immature blood cells (1). It includes chronic and acute myelomonocytic leukemia (MML) (2). In Western countries, chronic MML remains the most common type of leukemia in adults (3) and is a clonal hematopoietic malignancy which includes two features, myelodysplastic syndrome and myeloproliferative neoplasm (4) which are recognized by the World Health Organization classification of 2008 (5). In about 15%-30% of patients, chronic MML evolves into acute myeloid leukemia (AML) (5, 6). AML is also the most common pediatric malignancy

(7, 8). Currently, chemotherapy is still the main curative treatment in patients with leukemia but it has relatively low efficacy and high toxicity (9). Therefore, numerous studies are focused on the identification and development of novel agents to treat this disease.

Bufalin, a digoxin-like immunoreactive component, is a bufadienolide present in *Chan Su* and is used as Chinese medicine (10). Bufalin has biological activities including inducing cancer cell apoptosis (11-17) and DNA damage in NCI-H460 human lung cancer cells (18). Recently, we also found that bufalin significantly inhibits cell adhesion, migration and invasion of gefitinib-resistant NCI-H460 cells (19) and induced apoptosis of U-2 OS human osteosarcoma cells (20) and SCC 4 human tongue cancer cells (21).

Although several studies have demonstrated bufalin-induced cytotoxic effects *in vitro* through the induction of apoptosis in many human cancer cells and also have antitumor activity *in vivo*, there is no report to show how it affects immune response. Therefore, we investigated the immune response-modulating potential of bufalin on a *in vivo* model of leukemia.

## Materials and Methods

*Materials and reagents*. Bufalin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). IMDM and RPMI-1640 culture medium, fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin were obtained from Gibco Life Technologies (Carlsbad, CA, USA). Antibodies against CD3 (T-cell marker), CD19 (B-cell marker), CD11b (monocyte marker) and Mac-3 (macrophage marker) were obtained from BD Biosciences Pharmingen Inc. (San Diego, CA, USA). Bufalin was dissolved in DMSO at 1% stock solution and kept at −20˚C in a 50 ml shielded from light before use.

*Cell culture*. Murine myelomonocytic leukemia cells (WEHI-3 cells) and Yac-1 were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C.). About $1\times10^6$ cells/ml were maintained in 75 $cm^2$ tissue culture flasks with IMDM or RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37˚C in 100% humidity, with 5% $CO_2$ as previously described (15).

*Male BALB/c mice and bufalin treatment*. Forty male BALB/c mice, aged 5 weeks, weighing approximately 22-25 g each, were purchased from the National Laboratory Animal Center (Taipei, Taiwan, R.O.C.). All mice were kept at 25˚C on a 12 h light/dark cycle at the Animal Center of China Medical University (Taichung, Taiwan, R.O.C.). The animal study projects were reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University (approval ID: 104-10-B) and all animals were cared for following the institutional animal ethical guidelines of the China Medical University as described previously (22). The mice were randomly divided into five groups (n=8). Group I mice received a normal diet (Prolab RMH 2500; LabDiet, PMI Nutrition, Brentwood, MO, USA) as control. For the other four groups, the mice were intraperitoneally injected with $1\times10^6$ WEHI-3 leukemia cells to generate leukemic mice as described previously (22). Group II mice received a normal diet and treatment with olive oil as leukemia control (positive control). Groups III-V mice received a normal diet and treatment with bufalin (0.1, 0.2 or 0.4 mg/kg, respectively) in olive oil. Bufalin in olive oil was given to mice by oral gavage every 2 days for 14 days. All mice were weighed during the oral treatment every 2 days. At the end of treatment, all mice were weighed and blood was collected before mice were then sacrificed. After sacrifice, liver and spleen were isolated and weighed (22).

*Immunofluorescence staining for surface markers in isolated white blood cells*. After treatment, blood was collected individually from all mice from each group as described previously (22). Cell markers from isolated leukocytes were measured. A sample of 200 μl blood from each animal was lysed to destroy the red blood cells with 1×Pharm Lyse™ lysing buffer (BD Biosciences) as per guidelines from BD Biosciences and leukocytes were collected. Isolated leukocytes were individually stained by phycoerythrin (PE)-labeled anti-mouse CD3, PE-labeled anti-mouse CD19, fluorescein isothiocyanate (FITC)-labeled anti-mouse CD11b and FITC-labeled anti-mouse Mac-3 for 1 h and washed with phosphate-buffered saline (PBS) three times, and then analyzed by flow cytometry to determine the percentage of cell markers, as previously described (22).

*Measurement of macrophage phagocytosis*. Macrophages were isolated from peripheral blood mononuclear cells (PBMCs) and the peritoneum of each animal as described previously (22). FITC-conjugated *Escherichia coli* as target cells (50 μl) were added to the macrophages from mice under each treatment according to PHAGOTEST® kit manufacturer's instructions (ORPEGEN Pharma Gesellschaft für biotechnologische, Heidelberg, Germany) and mixed well. All samples were analyzed for phagocytosis using flow cytometry and quantified by the CellQuest software (Becton Dickinson), as described previously (22).

*Measurement of NK cell cytotoxic activity*. Splenocytes isolated from mice were placed in 96-well plate ($2.5\text{-}5\times10^5$ cells/well) with 100 μl of RPMI 1640 medium. Yac-1 cells ($1\times10^4$ cells) and PKH-67/Dil.C buffer (Sigma-Aldrich Corp.) were added to the cells and mixed thoroughly for 2 min at 25˚C then 2 ml PBS was added to well for 1 min. Next, 4 ml medium was added and cells were incubated for 10 min. After incubation, all cells were centrifuged at 290 × *g* for 2 min and then NK cell cytotoxic activity was measured by flow cytometry as described elsewhere (22).

*Measurement of B-and T-cell proliferation*. Splenocytes ($1\times10^5$ cells/well) in 100 μl of RPMI-1640 medium were placed in 96-well plate. Lipopolysaccharide (LPS, 5 μg/ml) (Sigma Chemical Co., St. Louis, MO, USA) was added to the splenocytes to stimulate for 3 days for measuring B-cell proliferation. Concanavalin A (Con A, 5 μg/ml) (Sigma Chemical Co.) was added to the splenocytes to stimulate them for 5 days for measuring T-cell proliferation. After stimulation, all samples were measured by using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) as previously described (22).

*Biochemical measurement of blood glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactate dehydrogenase (LDH) of BALB/c mice after exposure to bufalin*. Isolated blood samples from each animal were assayed for levels of GOT, GPT and LDH using quantification kit as described previously (22). The

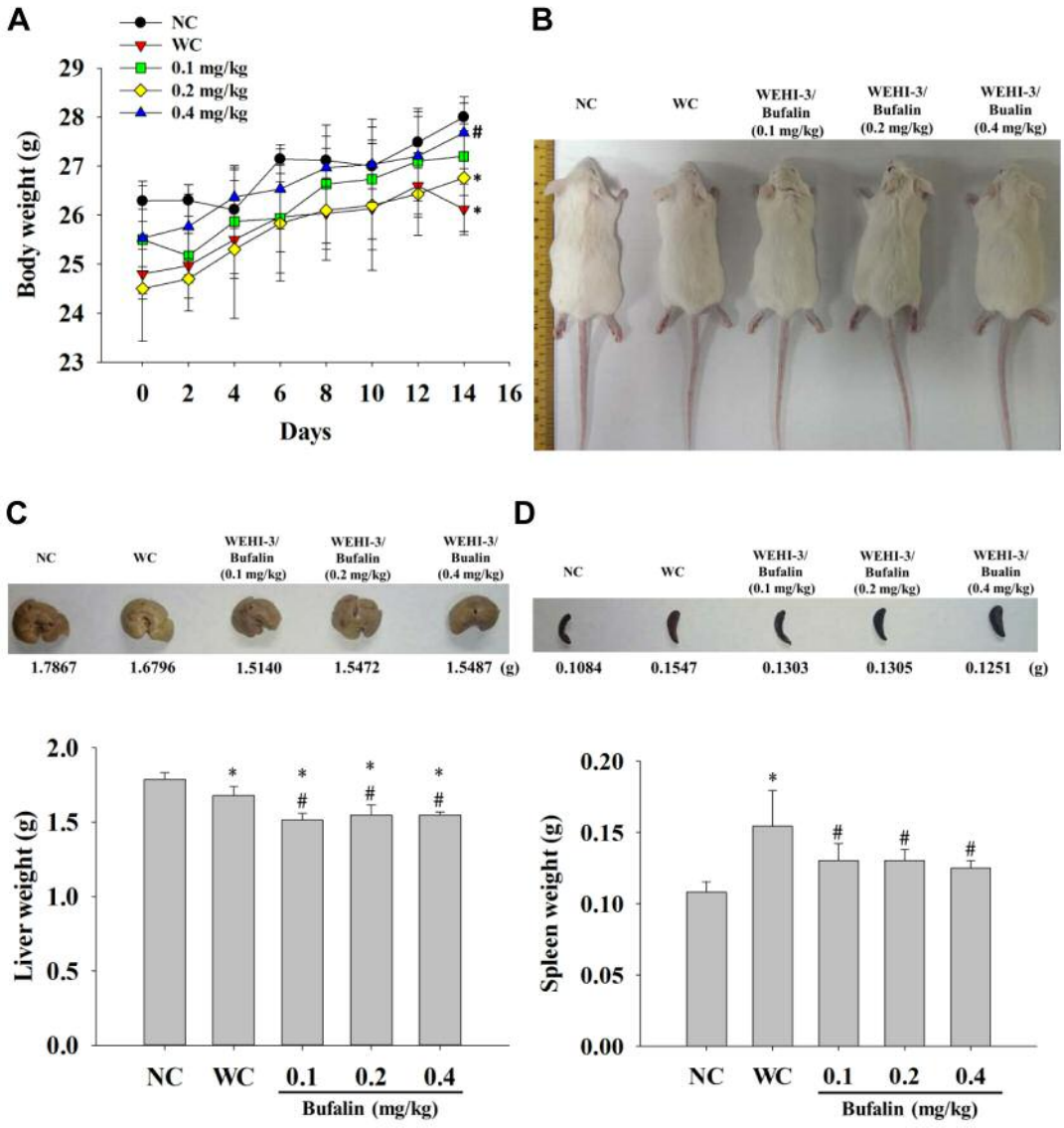


Figure 1. *Bufalin affect the body, liver and spleen weights of leukemia BALB/c mice. Group I was untreated as normal control (NC). Remaining mice were intraperitoneally injected with WEHI-3 cells then were divided into four groups: Group II was treated with normal diet (WC); group III was treated with 0.1 mg/kg of bufalin; group IV was treated with 0.2 mg/kg of bufalin; group V was treated with 0.4 mg/kg of bufalin. Body weights (A), animal appearance (B), liver (C), and spleen (D) weights are presented. The body weights were measured every 2 days. Significantly different at $p<0.05$ vs. *normal control group, vs. #leukemia control group.*

liquiUV Test (Aspartate Aminotransferase) for GOT, liquiUV Test (Alanine Aminotransferase) for GPT and liquiUV Test (Lactate Dehydrogenase) for LDH were obtained from Human Gesellschaft fur Biochemica und Diagnosica mbH (Wesbaden, Germany).

*Statistical analysis*. All values are expressed as mean±standard error (S.D.). Comparisons between groups were analyzed by one-way analysis of variance and Tukey test for multiple comparisons (SigmaPlot for Windows version 12.0; Systat Software, Inc., San Jose, CA, USA). A value of $p<0.05$ was considered to indicate a statistically significant difference.

## Results

*Bufalin affected the weights of body, liver and spleen from leukemic BALB/c mice*. After treatment, blood, liver and spleen were collected from mice; results are presented in Figure 1. The leukemia control group had significantly lower body weight compared with normal control group, while bufalin at 0.4 mg/kg significantly increased body weight compared with the leukemic control group. Representative images of liver and spleen tissues and their weights are

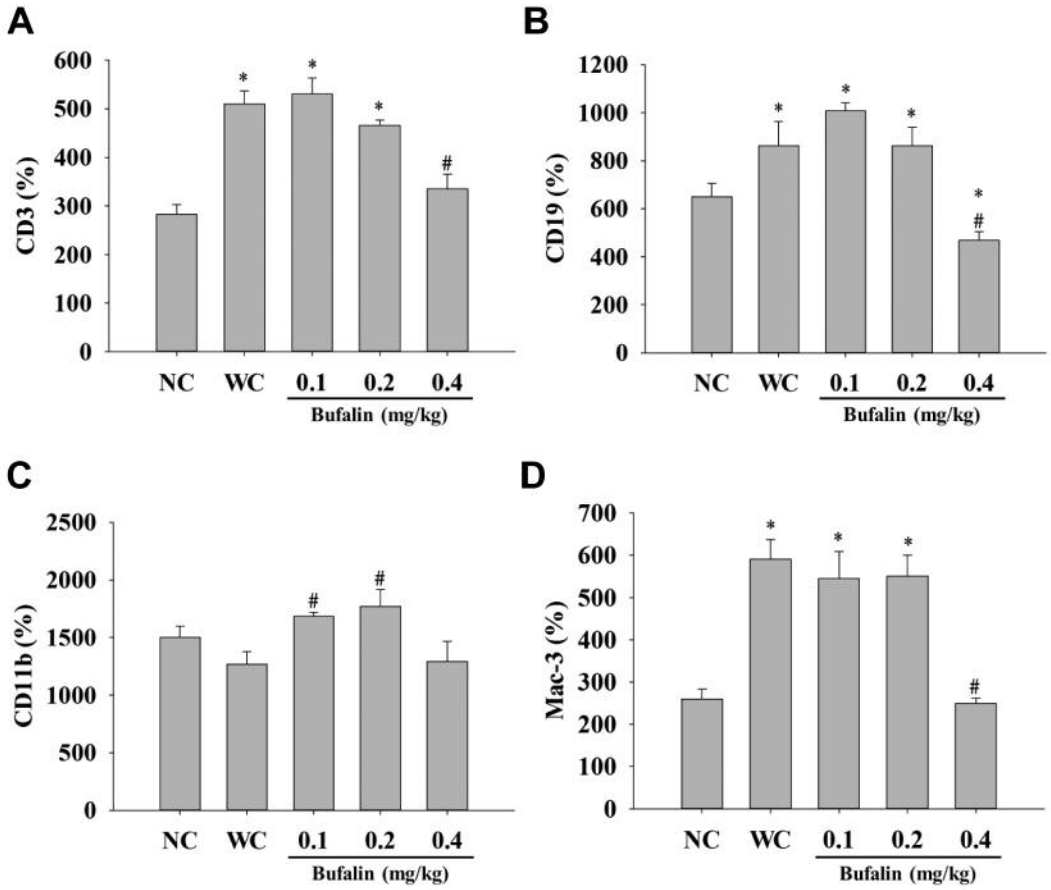


Figure 2. *Bufalin affected the levels of cell markers in white blood cells from BALB/c mice with leukemia. Blood was collected from all mice and analyzed for cell markers (A: CD3; B: CD19; C: CD11b and D: Mac-3) by flow cytometry, as described in the Materials and Methods. Significantly different at p<0.05 vs. *normal control group (NC), vs. #leukemia control group (WC).*

shown in Figure 1C and D. These results indicated that bufalin significantly reduced liver (Figure 1C) and spleen (Figure 1D) weights at all doses of bufalin treatment when compared with the leukemic control group.

*Bufalin affected cells markers of white blood cells from leukemic BALB/c mice*. Blood samples were collected for measuring the levels of cell markers CD3, CD19, CD11b and Mac-3. Results indicated that bufalin reduced CD3 (T-cells) (Figure 2A), CD19 (B-cells) (Figure 2B) and Mac-3 (macrophages) (Figure 2D) at 0.4 mg/kg treatment, however, bufalin promoted CD11b (monocytes) at 0.1 and 0.2 mg/kg treatment (Figure 2C) when compared with the leukemic control group.

*Bufalin affected phagocytosis by macrophages from PBMCs and peritoneal cavity of leukemic BALB/c mice*. After treatment, macrophages were isolated from PBMCs and peritoneal cavity in order to measure the level of phagocytosis by flow cytometry. Bufalin treatment at 0.4 mg/kg significantly increased phagocytosis by macrophages isolated from PBMCs (Figure 3A), and at 0.1 mg/kg treatment by those from the peritoneal cavity but it was reduced at 0.2 and 0.4 mg/kg treatment (Figure 3B) when compared with the leukemic control group.

*Bufalin affected the cytotoxic activity of NK cells from leukemic BALB/c mice*. Yac-1 cells were used as targets for isolated splenocytes and then were assayed by flow cytometry. Results indicated that bufalin did not affect NK cell cytotoxic activity at 25:1 (splenocytes:target cells), but 0.2 and 0.4 mg/kg treatment reduced NK cell cytotoxic activity at 50:1 when compared to the leukemic control group.

*Bufalin affected B- and T-cell proliferation from leukemic BALB/c mice*. Isolated splenocytes were assayed for cell proliferation. Results indicated that bufalin at 0.1 and 0.2 mg/kg

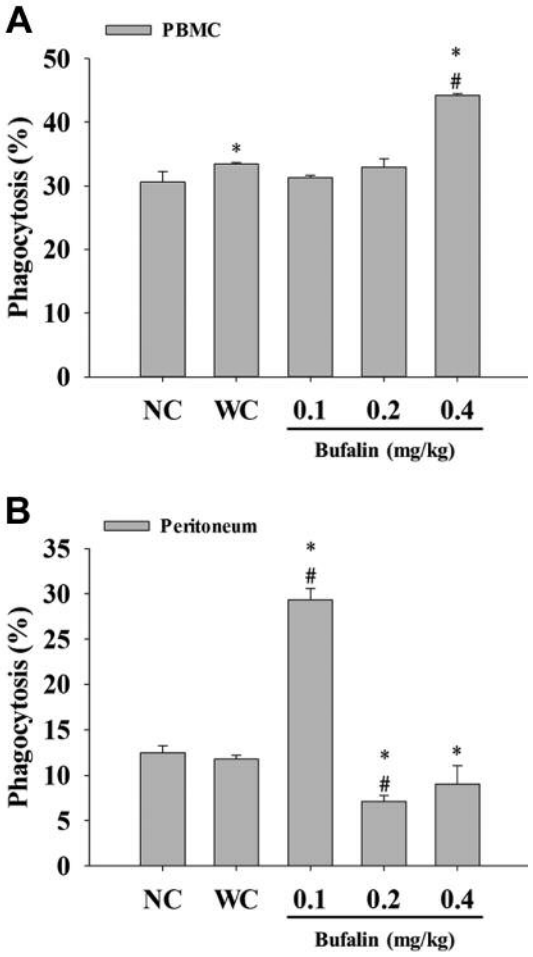


Figure 3. *Bufalin promoted macrophage phagocytosis from peripheral blood mononuclear cells (PBMCs) and peritoneal cavity of BALB/c mice with leukemia. Blood samples were collected from mice then macrophages were isolated from PBMCs (A) and peritoneum (B) of each mouse. Macrophage phagocytosis was measured by flow cytometry, as described in the Materials and Methods. Significantly different at p<0.05 vs. *normal control group (NC), vs. #leukemia control group (WC).*

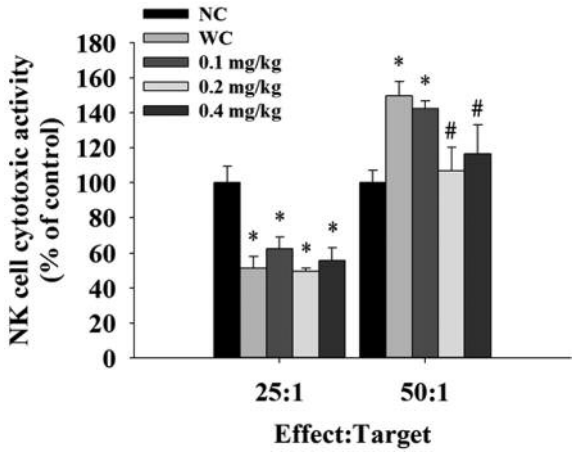


Figure 4. *Bufalin affected the cytotoxic activity of natural killer (NK) cells in BALB/c mice with leukemia. Splenocytes were cultured in 96-well plates and target YAC-1 cells were added. NK cell cytotoxic activity was measured using flow cytometry, as described in the Materials and Methods. Significantly different at p<0.05 vs. *normal control group (NC), vs. #leukemia control group (WC).*

significantly increased B-cell proliferation (Figure 5A) when compared with the leukemic control group, however, only 0.1 mg/kg treatment of bufalin significantly increased T-cell proliferation (Figure 5B) when compared with the leukemic control group.

*Bufalin affected the blood levels of GOT, GPT, and LDH of BALB/c mice*. Blood samples were collected for measurement of GOT, GPT and LDH levels. Results indicated that bufalin significantly increased GOT level at all doses (Figure 6A), increased GPT level at 0.1 mg/kg treatment (Figure 6B), but reduced the level of LDH (Figure 6C) at all doses when compared with the leukemic control group.

## Discussion

In the present study, we injected murine WEHI-3 cells into normal BALB/c mice to generate an animal model of leukemia and these mice were randomly divided into different groups for oral treatment with bufalin at different doses to examine the effects of bufalin on immune responses *in vivo*. It is well known that several types of white blood cell interact with each other to produce immune responses in humans in order to act against foreign antigens (23). Many plant-derived bioactive compounds have been used to treat patients with cancer such as paclitaxel from *Taxus brevifolia* and camptothectin from *Camptotheca acuminata* (24). Although numerous reports have shown that bufalin induced cytotoxic effects *via* induction of cell-cycle arrest and apoptosis in many human cancer cell lines *in vitro* and has antitumor activity *in vivo*, however, there is no available information or data in literature regarding the effects of bufalin on immune responses in mice with leukemia *in vivo*. Our results are the first to demonstrate bufalin increases immune responses in leukemia BALB/c mice *in vivo*.

It is well documented that normal BALB/c mice develop leukemia after injection with WEHI-3 cells (25), which can be seen from their increased spleen weight when compared to normal mice (26). In the present study, we successfully generated leukemia in BALB/c mice using WEHI-3 cells and showed that bufalin significantly increased markers of immune-associated leukocytes *in vivo*. In particular, bufalin enhanced macrophage phagocytosis in these leukemic mice *in vivo*. It is well known that macrophages play a key role in

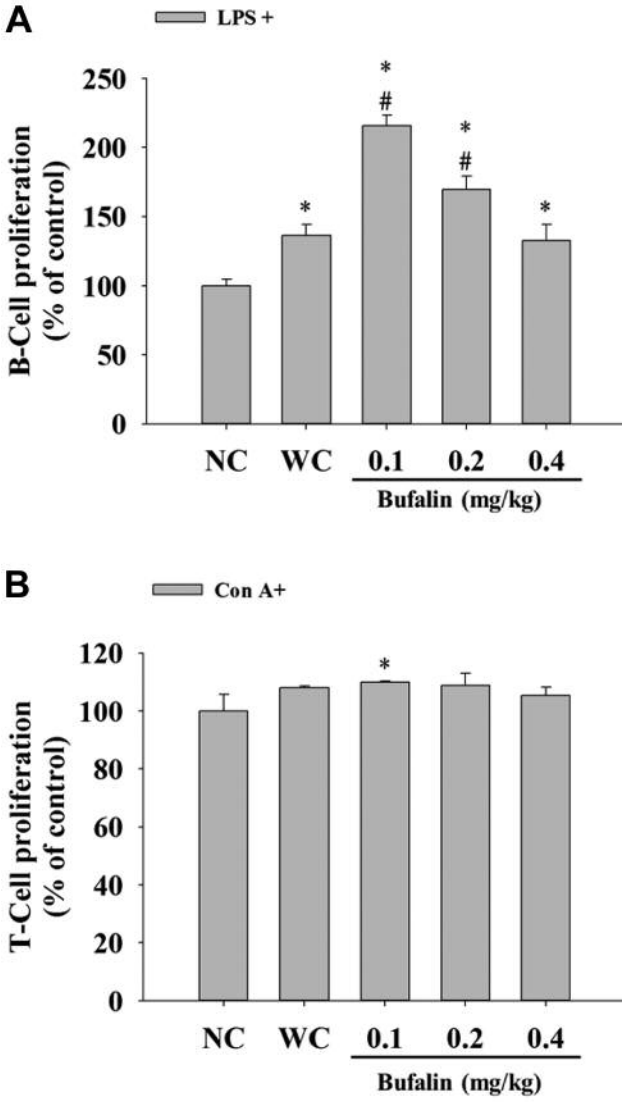


Figure 5. *Bufalin affected B- and T-cell proliferation in BALB/c mice with leukemia. Isolated B- and T-cells were pretreated with lipopolysaccharide (LPS) and concanavalin A (Con A) for determination of B-cell (A) and T-cell (B) proliferation, respectively, and then were analyzed by flow cytometry as described in the Materials and Methods. Significantly different at p<0.05 vs. *normal control group (NC), vs. #leukemia control group (WC).*

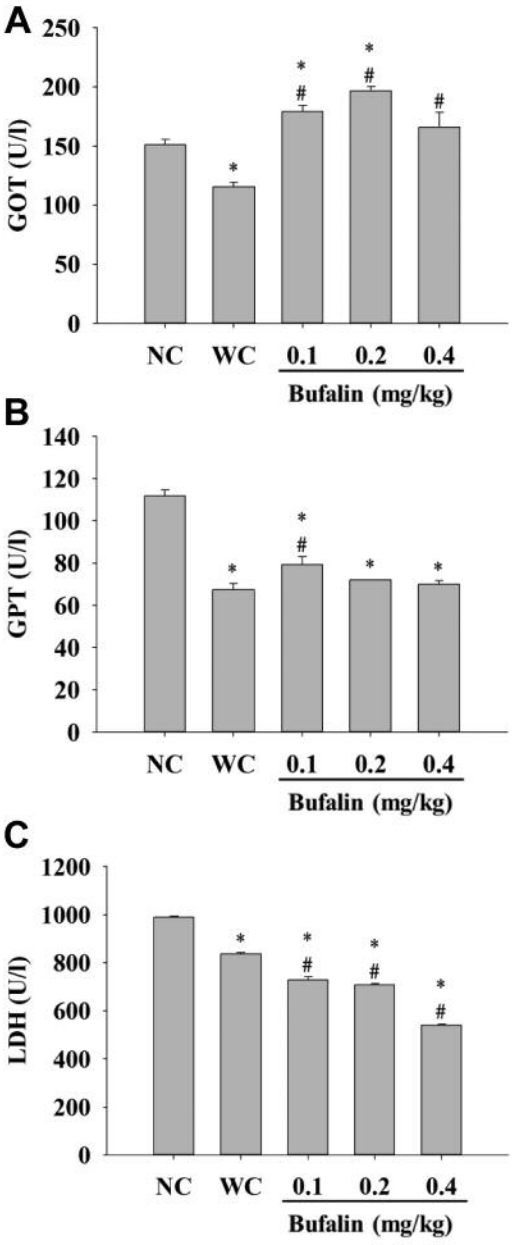


Figure 6. *Measurement of blood glutamic oxaloacetic transaminase (GOT) (A), glutamic pyruvic transaminase (GPT) (B), lactate dehydrogenase (LDH) (C) activity of BALB/c mice after exposed to bufalin. After treatment, blood samples were collected from each mouse and then the activity of GOT, GTP and LDH was measured, as described in the Materials and Methods. Significantly different at p<0.05 vs. *normal control group (NC), vs. #leukemia control group (WC).*

innate immune responses to entry of antigens that is necessary to control or eliminate infection (27, 28). The exact function of bufalin associated with Mac-3 marker and macrophage and NK cell activities needs further study as to whether they have any effect on survival of such mice with leukemia. Macrophages have great plasticity and can differentiate into several functional states in response to microenvironmental signals (29). Thus, some studies have focused on the roles of immune cell subtypes and their capacity to function in the tumor microenvironment (30). We examined macrophage phagocytosis using FITC-labelled *E. coli* as a target and assay by flow cytometry, which is a reliable method for this investigation (31). Yac-1 cells were used as targets for NK cell

activity and were also assay by flow cytometry (32). The stimulation of NK cell cytotoxicity may increase immune response (33), thus the role of NK associated with bufalin-increased immune responses needs further investigation.

In conclusion, based on these observations, we suggest that bufalin may modulate immune responses not only through increasing monocyte (CD11b) population and T- and B-cell proliferation, but also by increasing macrophage phagocytosis in leukemic mice *in vivo*. Whether this translates into increased survival needs further investigation.

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