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Solannm lyratum extract affected immune response in normal and leukemia murine animal in vivo

Jai-Sing Yang[1], Chia-Chun Wu[2], Chao-Lin Kuo[3], Chin-Chung Yeh[4], Fu-Shin Chueh[5], Cheng-Kuang Hsu[5], Chien-Kuo Wang[6], Ching-Yao Chang[6], Siu-Wan Ip[7], Yuan-Man Hsu[2], Wei-Wen Kuo[2] and Jing-Gung Chung[2,6]

Abstract

Solanum lyratum Thunberg (Solanaceae) has been used as a folk medicine for treating liver, lung and esophagus in the Chinese population. Our previous studies have shown that the crude extract of *S. lyratum* Thunberg (SLE) induced apoptosis in colo 205 human colon adenocarcinoma cells; however, there is no report to show SLE affect immune responses in vivo. In this study, the in vivo effects of SLE on leukemia WEHI-3 cells and immune responses such as phagocytosis and natural killer (NK) cell activity in normal and leukemia mice were investigated. The SLE treatment decreases surface markers of CD3 and Mac-3 in normal and leukemia mice but promoted the cell markers of CD19 and CD11b in normal mice and CD11b in leukemia mice indicating that the precursors of T cells was inhibited and B cells and macrophage were promoted. The SLE treatment promoted the activity of macrophage phagocytosis in the peripheral blood mononuclear cells (PBMC) and peritoneal cells from normal and leukemia mice. The results also showed that NK cells from the normal and leukemia mice after treatment with SLE can kill the YAC-1 target cells. Therefore, the SLE treatment increased macrophage and NK cell activities. These consistent results indicate SLE could be a potent immune responses agent.

Keywords

Solannm lyratum extract, leukemia WEHI-3 cells, immune response, leukemia murine animal model

Introduction

Leukemia is a group of hematological malignancies and they were characterized by clonal expansion of hematopoietic cells with uncontrolled proliferation, decreased apoptosis and blocked differentiation. Leukemia is one of the most notorious enemies of mankind, which accounts for some 300,000 new cases and 222,000 deaths each year worldwide.[1] In Taiwan, 3.8 per 100,000 people die each year of leukemia based on the report from Department of Health, Executive Yuan, ROC (Taiwan). The responses of leukemia to therapeutic agents differ from one type or subtype to another. Current treatments for human leukemia include chemotherapy, interferon therapy and bone marrow transplantation and differentiation therapy. However, the cure for leukemia patients

[1] Department of Pharmacology, China Medical University, Taichung, Taiwan, ROC
[2] Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, ROC
[3] Department of Chinese Medicine Resources, China Medical University, Taichung, Taiwan, ROC
[4] Department of Urology, China Medical University Hospital, Taichung, Taiwan, ROC
[5] Department of Health and Nutrition Biotechnology, Asia University, Wufeng, Taichung, Taiwan, ROC
[6] Department of Biotechnology, Asia University, Wufeng, Taichung, Taiwan, ROC
[7] Department of Nutrition, China Medical University, Taichung, Taiwan, ROC

Corresponding author:
Jing-Gung Chung, Department of Biological Science and Technology, China Medical University, No 91, Hsueh-Shih Road, Taichung 40402, Taiwan, ROC
Email: jgchung@mail.cmu.edu.tw

still is not satisfied. Many evidences showed that increased consumption of plant-based diet can reduce the risk of cancer.[2]

Many leukemia cell lines for in vitro and in vivo assessment of anti-cancer agents are available. The WEHI-3 murine myelomonocytic leukemia cell line was first established in 1969.[3] This cell line was originally derived from the BALB/c mouse and showed characteristics of myelomonocytic leukemia. The orthotopic animal model by intraperitoneal injection of the WEHI-3 cells was established for detecting anti-leukemia activity.[4] It was an ideal system for the studies of potential therapeutic agents that induced maturation and apoptosis.[4] It has been used for induction of leukemia in syngenic BALB/c mice model for evaluating anti-leukemia effects of drugs.[5-9]

Solanum lyratum Thunberg (Solanaceae) have been used for regulating immune function,[10] treating allergic responses[11] and for the treatment of liver, lung and esophagus cancer in Chinese populations since long time ago. It was reported that *S. lyratum* extract (SLE) inhibited cell proliferation of human hepatoma BEL-7402 cells, gastric carcinoma SGC-7901 cells and A375-S2 cells in vitro and in vivo.[12,13] SLE have shown that it promoted the activity of protein kinase A (PKA) in the gastric cancer cells,[14,15] induced apoptosis in human cervical cancer HeLa cells *via* Fas/Fas ligand (FasL) expression.[16] Recently, it was reported that SLE induced apoptosis in human colon cancer colo 205 cells through caspase-3 activation.[17] There are no available information to address that SLE affected the immune responses in normal and leukemia mice in vivo. Therefore, in the present study, we focused on the immune responses action of SLE on the murine leukemia in vivo.

Materials and methods

Cell culture

The WEHI-3 murine myelomonocytic leukemia cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 100 Units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine at 75-cm^2 tissue culture flasks at 37°C under a humidified 5% CO_2 atmosphere. SLE was obtained from Dr Chao-Lin Kuo lab (Department of Chinese Medicine Resources, China Medical University) as described previously.[17]

S. lyratum Thunb extract treatment in normal and leukemia animals

Normal BALB/c mice were obtained from animal center of National Taiwan University. Animals were divided into four groups. Group 1 is control; group 2 is dietary 4 mg/kg of SLE; group 3 is dietary 8 mg/kg of SLE and group 4 is dietary 16 mg/kg of SLE for 28 days.

Forty BALB/c mice were divided into four groups. Group I was injected with WEHI-3 cells (1×10^5 cells/100 μL) in RPMI 1640 medium for 2 weeks as control group (10 animals). Groups II, III and IV were injected with WEHI-3 cells and then treated with SLE (4, 8 and 16 mg/kg) in olive oil. All animals were orally given the above dose daily for up to 2 weeks before being weighed.

T and B cell proliferation

Spleen tissues from normal and leukemia mice were isolated and then splenocytes prepared for T and B cells proliferation examinations. About 1×10^5 splenocytes were individually placed in 96-well plates with or without Concanavalin A (Con A, 5 μg/mL) for 72 hours or lipopolysaccharide (LPS, 5 μg/mL) for 120 hours, and then to determine the proliferation by MTS assay (Cell Title96 Kit, Promega, San Luis Obispo, California, USA) as described previously.[6,18]

Phagocytosis by macrophage

Phagocytotic activity was determined by using the PHAGOTEST kit (ORPEGEN Pharma Gesellschaft für biotechnologische, Heidelberg, Germany) as described previously.[6,18,19] Briefly, the cells (1×10^5 cells) from peripheral blood mononuclear cells (PBMC) or peritoneum were incubated for 1 hour at 37°C with opsonized fluorescein isothiocyanate-labelled *Escherichia coli* (20 μL), in compliance with the manufacturer's instruction. The reactions were stopped individually by the addition of ice-cold quenching solution (100 μL). After the completion of phagocytosis, monocytes/macrophages were fixed and DNA was stained by PI according to the manufacturer's instructions. Cell preparations were then analyzed by flow cytometer (FACS Calibur™, Becton Dickinson, New Jersey, USA) and the fluorescence data were collected on 10,000 cells and

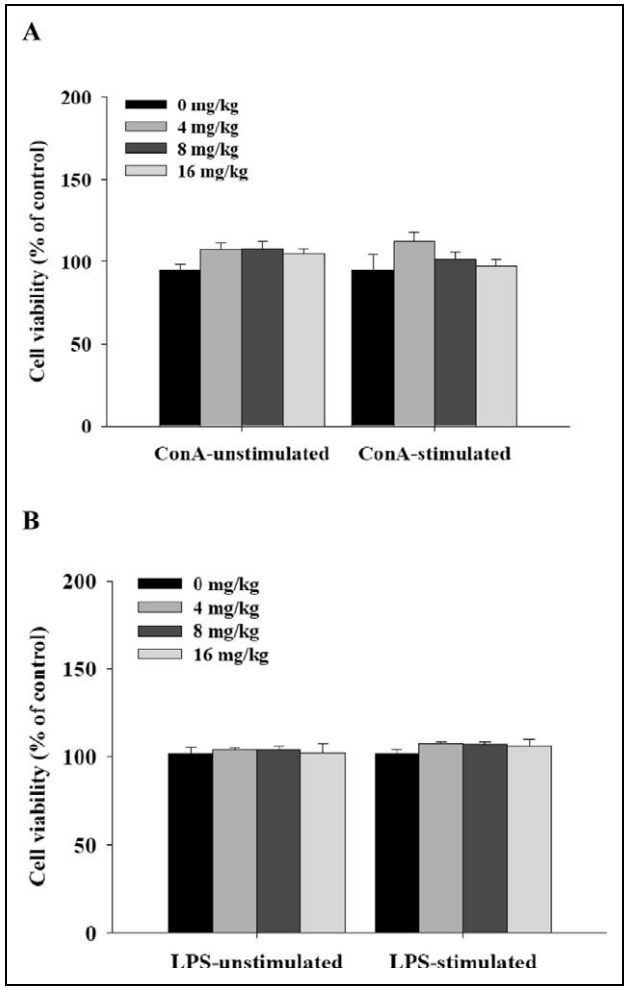

Figure 1. *Solanum lyratum* extract (SLE) affects the cell proliferation of Con A-and lipopolysaccharide (LPS)-stimulated splenocytes from normal BALB/c mice in vitro. Splenocytes were isolated from normal BALB/c mice then splenocytes were stimulated with Con A and LPS for 72 and 120 hours, respectively, and proliferation examinations by CellTiter 96 assay kit as described in 'Materials and methods.' A: Con-A stimulated; B: LPS-stimulated.

analyzed using the CELLQUEST software as described previously.[7,8]

Natural killer (NK) cells activity

YAC-1 cells were prepared following the guideline (Sigma-Aldrich Corp. St. Louis, Missouri, USA). Briefly, YAC-1 cells in 15-mL tubes were washed twice with serum-free RPMI-1640 medium and centrifuged for discarding the supernatant, then PKH-67/Dil.C buffer (Sigma-Aldrich Corp.) was added to the cells in each well and thoroughly mixed for 2 min at 25°C; then 2 mL phosphate buffered saline (PBS) was added. After 1 min, 4 mL RPMI-1640 medium was added and cells were incubated for 10 min at 1200 rpm at 25°C. YAC-1 cells (2×10^5) were plated on 96-well plates and splenocytes (2×10^7; 1×10^7 and 5×10^6) were added to each well and incubated at 37°C for 12 hours. NK cell activation was determined by flow cytometry as described previously.[19,20]

Surface markers of PBMC

Approximately 1 ml of blood was collected from each animal (normal and leukemia mice) and the red blood cells were lysed by ammonium chloride, and then were centrifuged at 1500 rpm (1000*g*) at 4°C for 15 min for isolating white blood cells as described previously.[6,18,21] Each isolated cells were further examined for cell surface markers (T cell: CD3; B cell: CD19; monocyte and macrophage: CD11b and Mac-3) by using staining with anti-CD3-FITC, -CD11b-FITC, -CD19-PE and -Mac-3-PE antibodies (BD Pharmingen, San Diego, California, USA). Stained cells from each sample were washed with PBS for determining the cell marker levels by flow cytometry as described.[19,21]

Statistics analyses

The experimental results were expressed as mean $\pm$ SD and the differences among control and experimental groups were analyzed by Student's t test. $^*p < .05$, $^{**}p < .01$ and $^{***}p < .001$ were considered as significant.

Results

SLE affects the cell proliferation of Con A-and LPS-stimulated splenocytes from normal BALB/c mice in vitro

The spleens from normal BALB/c mice after treatment with or without various doses of SLE were isolated and then splenocytes were stimulated with Con A and LPS for 72 and 120 hours, respectively. They were collected and analyzed for cell proliferation. The results are presented in Figure 1A and B, which indicated that after Con A (Figure 1A) and LPS (Figure 1B) stimulation, all doses of SLE extract did not promote the significant proliferation of splenocytes.

SLE affects PBMC and peritoneum phagocytosis and NK activity in BALB/c mice in vivo

PBMC and peritoneum macrophage were isolated from 0, 4, 8 and 12 mg/kg/day of SLE-treated normal BALB/c mice for 28 days, then phagocytes were used for phagocytosis determination. The results are presented in Figure 2A. The percentage of phagocytic activity with FITC green fluorescent particles in SLE treatment from PBMC increased in 4, 8 and 16 mg/kg/day of SLE treatment (Figure 2A); however, cells from peritoneum increased the percentage of phagocytosis only at 4 mg/kg/day treatment group (Figure 2B).

Splenocytes were isolated from 4 and 8 mg/kg/day of SLE-treated BALB/c mice for 28 days, and then NK cells were used for NK activity determination. The results are presented in Figure 2C. The YAC-1 target cells were killed by NK cells from the normal mice after treatment with SLE extract at 4 and 8 mg/kg/day treatment in target cells ratio of 100: 1 and 50: 1.

SLE affects the cell markers of white blood cells from normal BALB/c mice

The data for cell markers of white blood cells from normal BALB/c mice after exposure to 4, 8 and 16 mg/kg of SLE in olive oil are presented in Figure 3A–D. The results indicated that SLE decreased the levels of CD3 (Figure 3A) at 8 and 16 mg/kg treatment, increased CD19 (Figure 3B) at 4 mg/kg treatment, increased CD11b (Figure 3C) at 16 mg/kg treatment, respectively, but it did not affect levels of Mac-3 (Figure 3D).

SLE affects the cell proliferation of Con A-and LPS-stimulated splenocytes from leukemia BALB/c mice in vitro

The spleens from leukemia BALB/c mice after treatment with or without various doses of SLE was isolated and then splenocytes were stimulated with Con A and LPS for 72 and 120 hours, respectively. Splenocytes were collected and analyzed for proliferation. The results presented in Figure 4A and B indicated that with or without Con A stimulation, 4 and 8 mg/kg of SLE promoted the T cells proliferation (Figure 4A), however, with or without LPS stimulation, 4 and 8 mg/kg of SLE inhibited the B cell proliferation (Figure 4B).

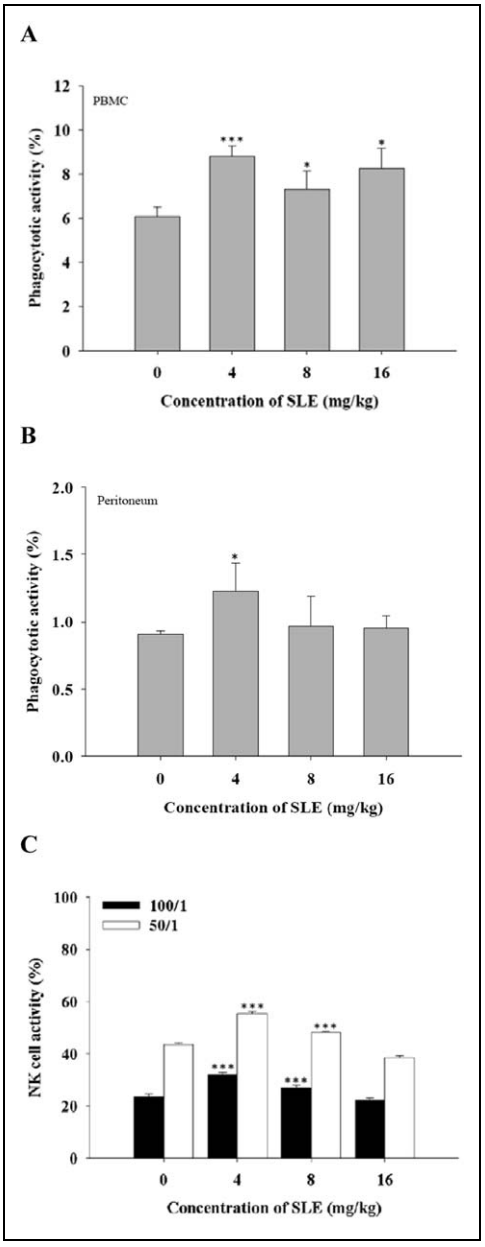

Figure 2. *Solanum lyratum* extract (SLE) affects phagocytosis of peripheral blood mononuclear cells (PBMC; 2A) and peritoneum (2B) and NK activity (2C) in normal BALB/c mice in vivo. Phagocytes were isolated from PBMC and/or peritoneum of normal BALB/c mice after being exposed to 4, 8 and 16 mg/kg/day of SLE for 28 days. The percentages of phagocytes with phagocyted green fluorescent particles (FITC – *Escherichia coli*) at SLE treatment were determined by flow cytometric analysis as described in 'Materials and methods.' Each point is mean ± SD of three experiments. ***$p < .001$. SLE also affects activity of natural killer (NK) cells in normal BALB/c mice. The YAC-1 target cells were killed by NK cells from the mice after being treated with SLE extract at 4, 8 and 16 mg/kg/day in target cells ratio of 25:1 and 50:1. Each point is mean ± SD of three experiments. *$p < .05$, ***$p < .001$.

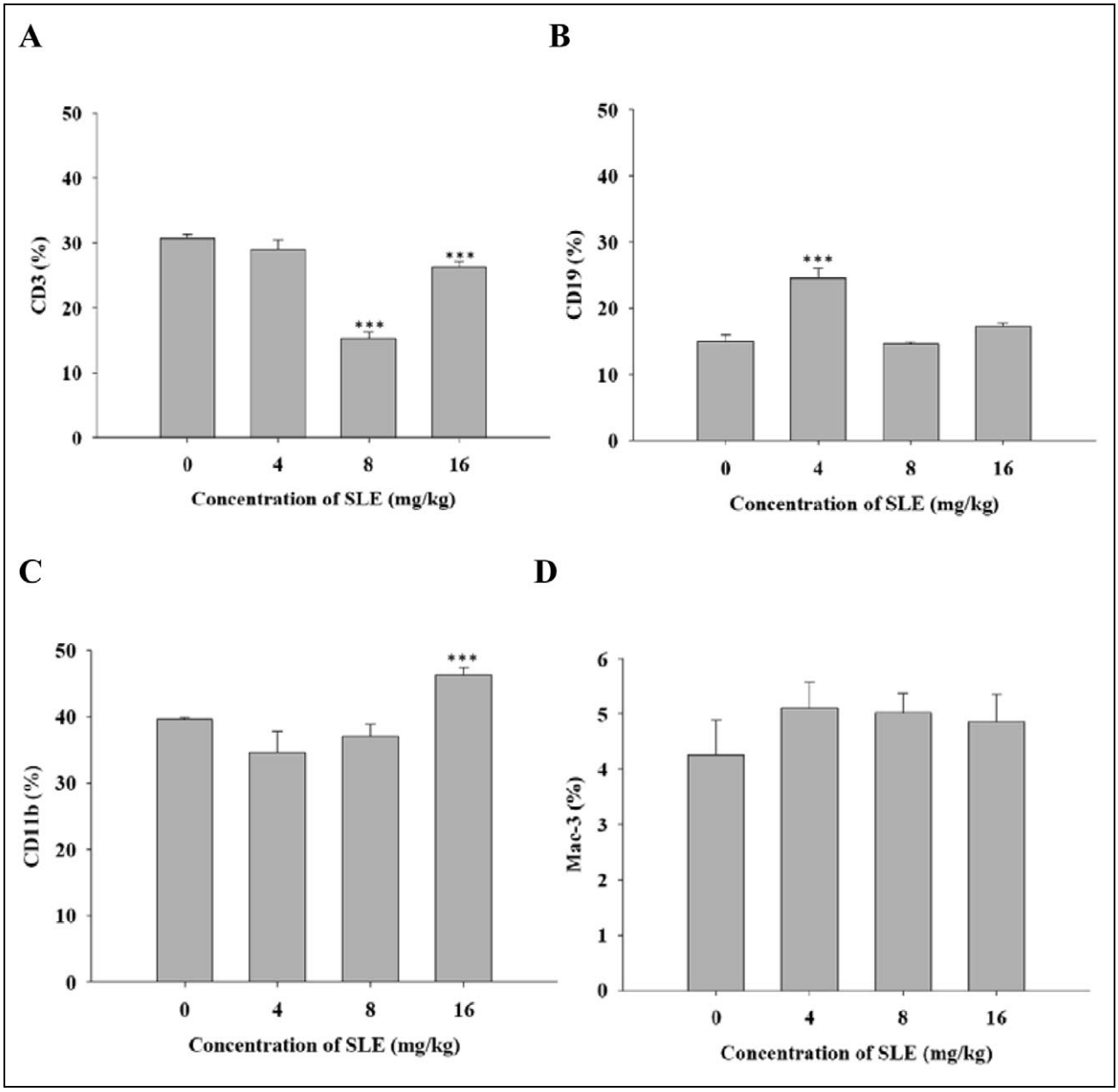

Figure 3. *Solanum lyratum* extract (SLE) affects the cell markers in (A) CD3 (B) CD19 (C) CD11b and (D) Mac-3 of white blood cells from normal BALB/c mice. The animals were oral treated with or without SLE (4, 8 and 16 mg/kg) for 28 days. Blood was collected from individual animal and analyzed for cell marker by flow cytometry as described in 'Materials and methods.' Each point is mean $\pm$ SD of three experiments. ***$p < .001$.

SLE affects phagocytosis and NK activity of PBMC and /or peritoneum in leukemia BALB/c mice in vivo

PBMC and peritoneum phagocytes were isolated from 0, 4, 8 and 12 mg/kg/day of SLE-treated leukemia BALB/c mice for 14 days, then macrophage were used for phagocytosis determination. The results are presented in Figure 5A, the percentage of phagocytes with phagocyted green fluorescent particles at SLE treatment from PBMC increased in 4, 8 and 12 mg/kg/day of SLE treatment (Figure 5A), however, cells from peritoneum increased the percentage of phagocytosis only at 4 and 16 mg/kg/day treatment (Figure 5B). Splenocytes were isolated from 4, 8 and 16 mg/kg/day of SLE-treated leukemia BALB/c mice for 14 days, then NK cells were used for activity determination. The results are presented in Figure 5C,

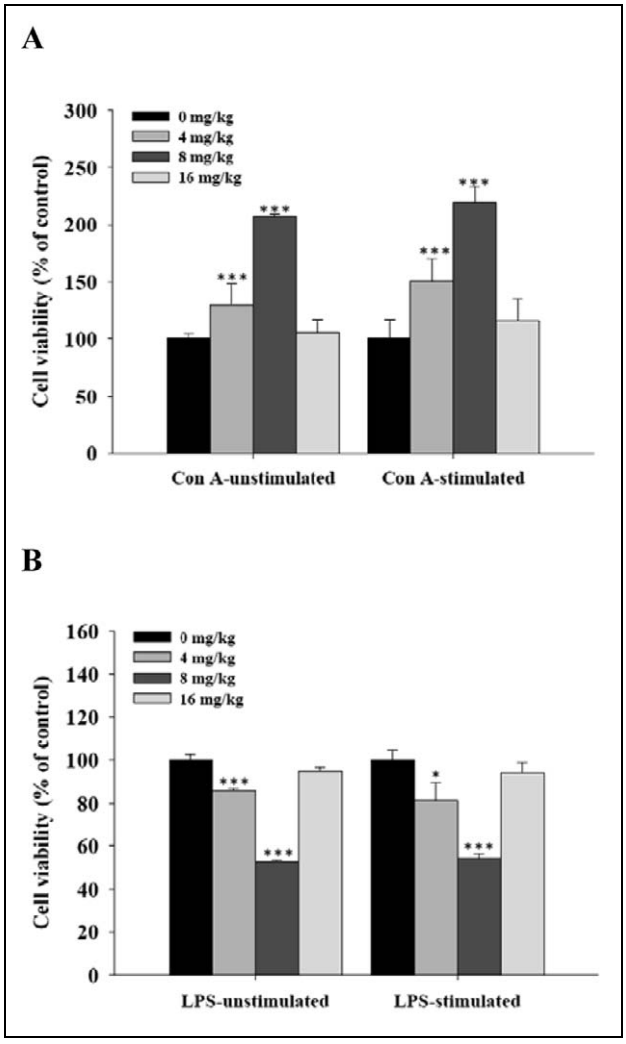

Figure 4. *Solanum lyratum* extract (SLE) affects the cell proliferation of Con A-and lipopolysaccharide (LPS)-stimulated splenocytes from leukemia BALB/c mice in vitro. Splenocytes were isolated from leukemia BALB/c mice, then splenocytes were isolated and were treated with various doses of SLE extract for 72 hours for proliferation examinations by CellTiter 96-assay kit as described in 'Materials and methods.' A: Con-A stimulated; B: LPS-stimulated, *$p < .05$, ***$p < .001$.

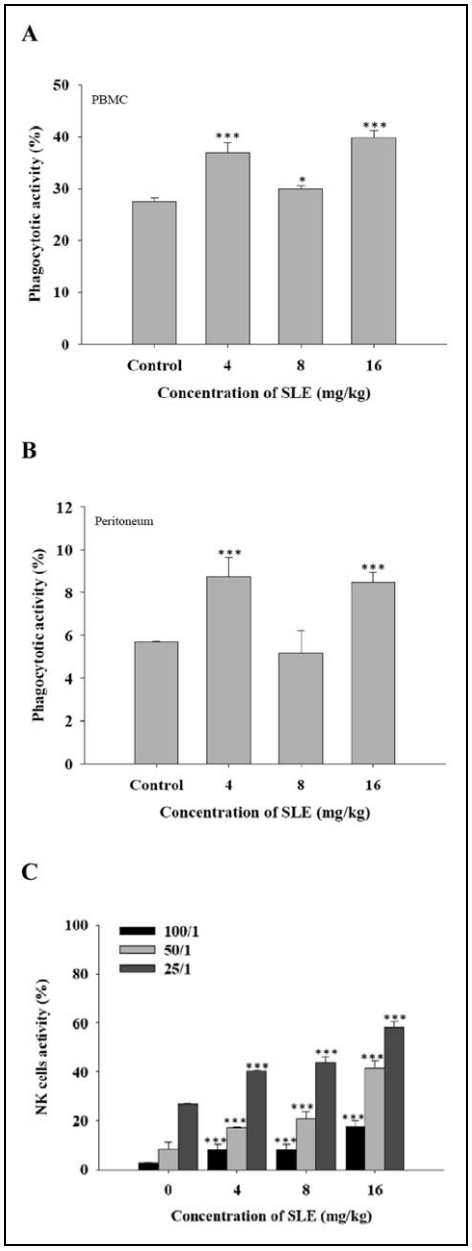

Figure 5. *Solanum lyratum* extract (SLE) affects phagocytosis of PBMC (A) and/or peritoneum (B) and NK activity (C) in leukemia BALB/c mice in vivo. Phagocytes were isolated from peripheral blood mononuclear cells (PBMC) and/or peritoneum of leukemia BALB/c mice after being exposed to 4, 8 and 16 mg/kg/day of SLE for 2 weeks. The percentages of phagocytes with phagocyted green fluorescent particles (FITC – *Escherichia coli*) at SLE treatment were determined by flow cytometric analysis as described in 'Materials and methods.' Each point is mean ± SD of three experiments. SLE also affects activity of natural killer (NK) cells in leukemia BALB/c mice. The YAC-1 target cells were killed by NK cells from the mice after being treated with SLE extract at 4, 8 and 16 mg/kg/day in target cells ratio of 25:1 and 50:1. Each point is mean ± SD of three experiments. *$p < .05$, ***$p < .001$.

the YAC-1 target cells were killed by NK cells from the mice after treatment with SLE extract at 4, 8 and 16 mg/kg/day treatment in target cells ratio of 25:1, 50:1 and 100:1.

SLE affects the cell markers of white blood cells from leukemia BALB/c mice

The data for cell markers of white blood cells from leukemia BALB/c mice after exposed to 4, 8 and 16 mg/kg/mice of SLE in olive oil are presented

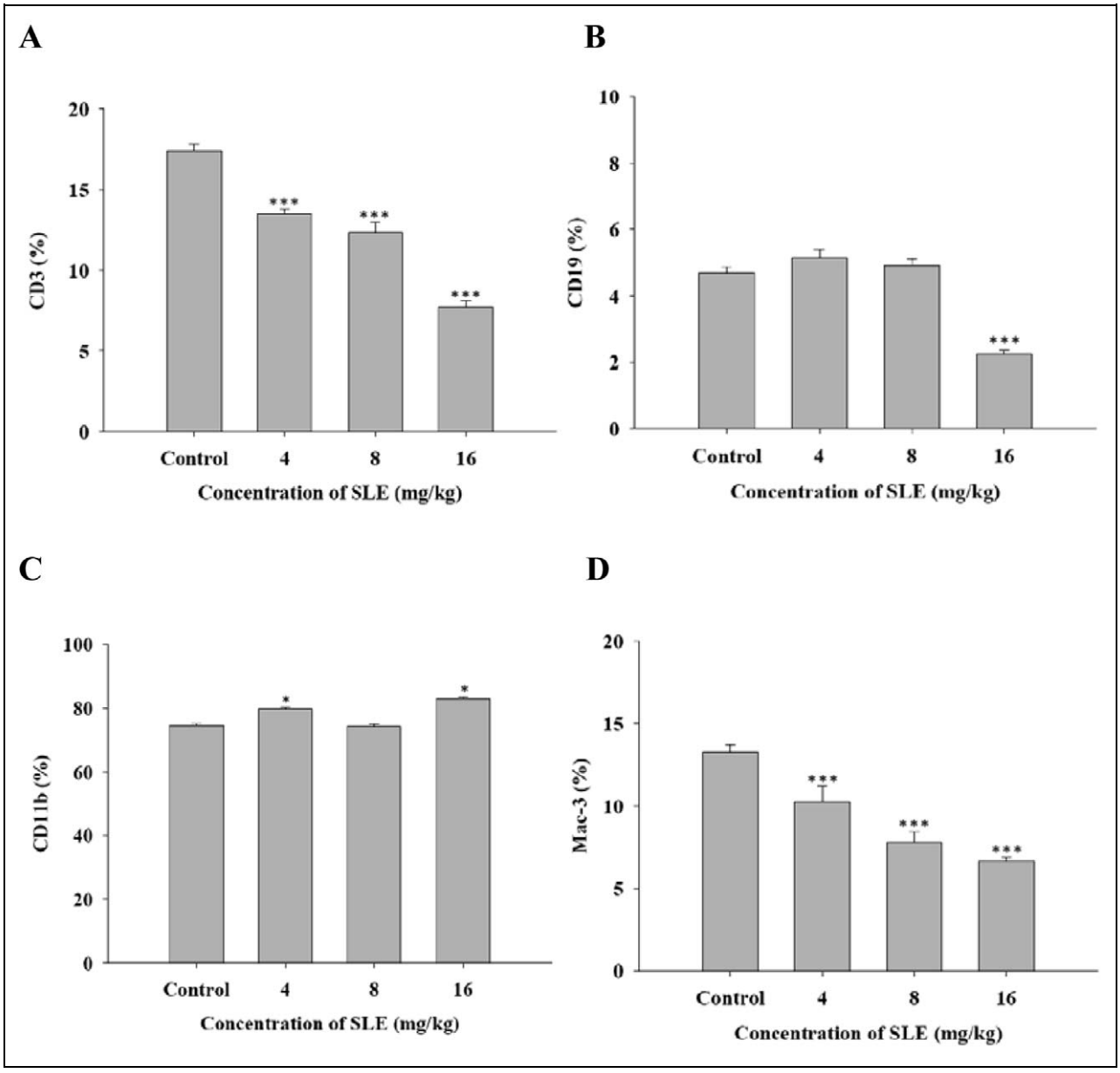

Figure 6. *Solanum lyratum* extract (SLE) affects the cell markers in (A) CD3 (B) CD19 (C) CD11b and (D) Mac-3 of white blood cells from leukemia BALB/c mice. The animals were injected with WEHI-3 cells (1×10^5 cells/100 μL) for 2 weeks and treated with or without SLE (4, 8 and 16 mg/kg) for 28 days. Blood was collected from individual animal and analyzed for cell surface marker by flow cytometry as described in 'Materials and methods.' Each point is mean ± SD of three experiments. *$p < .05$, ***$p < .001$.

in Figure 6A–D. The results indicated that SLE decreased the levels of CD3 (Figure 6A) at 4, 8 and 16 mg/kg/mice treatment, decreased the levels of CD19 (Figure 6B) at 16 mg/kg/mice treatment, decreased the levels of CD11b (Figure 6C) at 4 and 16 mg/kg/mice treatment and decreased the levels of Mac-3 (Figure 6D) at 4, 8 and 16 mg/kg/mice treatment.

Discussion

The immune system may recognize and eliminate tumors that have been considered since long ago. The ability or inability of the immune system to respond to human cancers was one of the most controversial areas in medical research.[22] The immune tolerance to self is not simply mediated via the deletion of self-reactive T cells but also by active mechanisms

to suppress T cell activity in the periphery.[23] The antigen presenting cells (dendritic cells) trough the 'danger' signals has been recognized to presenting antigens in a stimulatory way to T cells.[24] Tumors can evade the immune system including reduced immunogenicity of host, resistance to killing by immune effector cells or subversion of the immune response.[25]

The relationship between cancers and the immune system will guide the design of future therapeutic strategies. Although numerous studies have shown that SLE exhibits anti-cancer activity, there is no report to show SLE affect immune responses. Therefore, in the present study, we investigated SLE-affected immune responses in normal and leukemia mice in vivo.

Our results showed that SLE did not affect the proliferation of T and B cells in normal mice (Figure 1A and B), but it promoted the T cell proliferation and inhibited B cell proliferation in leukemia mice (Figure 4A and B). T cells play an important role in anti-tumor immune systems, therefore, the promotion of T cell proliferation may lead to the function of anti-leukemia activity based on the information regarding T cells are key effectors cells in anti-tumor immunity.[26] However, our results from CD marker analysis from normal animal after being exposed to 8 and 16 mg/kg/mice was decreased in CD3 marker, that is mean T cells is decreased (Figure 3A), but B cells marker is increased (CD19; Figure 3B) after exposed to 4 mg/kg/mice of SLE. SLE also decreased the CD3 cell marker in leukemia mice after exposure to 4, 8 and 16 mg/kg/mice of SLE (Figure 6A) and decreased CD19 in 16 mg/kg/mice of SLE treatment (Figure 6B) of leukemia mice.

Macrophages are essential for innate immune response and they also play a role in the activation of the adaptive immune system by acting as professional antigen-presenting cells.[27] Phagocytosis by macrophages has also been implicated in the mechanism of action of other therapeutic monoclonal antibodies.[28] Our results showed that SLE promoted phagocytosis from PBMC (Figure 2A) in normal mice after dietary 4, 8 and 16 mg/kg of SLE treatment and from peritoneum of mice of 4 mg/kg SLE (Figure 2B) treatment. SLE also promoted the phagocytosis from PBMC (Figure 5A) in leukemia mice after dietary 4, 8 and 16 mg/kg of SLE treatment and from peritoneum of mice of 4 and 16 mg/kg SLE (Figure 5B) treatment. However, our results from CD marker analysis from normal animal after being exposed to 4, 8 and 16 mg/kg/mice indicated that SLE in all treated doses did not induce significant changes in Mac-3 marker (Figure 3D) but it increased the marker of CD11b (Figure 3C). SLE in three doses of treatment in leukemia also decreased the Mac-3 marker (Figure 6D), however, 4 and 16 mg/kg/mice of SLE increased CD11b (Figure 6C).

The inactivation of NK and T cells has shown to increase susceptibility to tumor development from different models.[28,29] Other reports also showed that NK cell-deficient mice have an increased incidence of spontaneous cancers[30] and of metastatic disease.[31] Furthermore, NK cells were found to mediate tumor rejection of MHC class I-deficient tumors.[32] MCA (methylcholanthrene)-induced tumors were more common than in wild-type mice in gene-targeted mice lacking the NK T lymphocyte subset.[32] Our results showed that SLE promoted NK cells activity from splenoctes (Figure 2C) in normal mice after dietary 4 and 8 mg/kg of SLE treatment and from leukemia mice after dietary 4, 8 and 16 mg/kg of SLE treatment (Figure 5C).

In summary, this study shows that SLE suppresses surface markers of CD3 and Mac-3 in normal and leukemia mice but promoted the cell markers of CD19 and CD11b in normal mice and CD11b in leukemia mice. SLE promoted did not affect T and B cell proliferation in normal mice, however, SLE promoted T cell proliferation but inhibited B cells proliferation in leukemia mice. SLE promoted phagocytosis in normal and leukemia mice. Furthermore, SLE also increased the NK cells activity in normal and leukemia mice. These consistent results indicate that SLE is a potent immune responses agent.

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