Phenethyl Isothiocyanate Promotes Immune Responses in Normal BALB/c Mice, Inhibits Murine Leukemia WEHI-3 Cells, and Stimulates Immunomodulations *In Vivo*

Mei-Fen Tsou,[1] Ni Tien,[1] Chi-Cheng Lu,[2] Jo-Hua Chiang,[2] Jai-Sing Yang,[3] Jing-Pin Lin,[4] Ming-Jen Fan,[5] Jang-Jih Lu,[1,6] Su-Peng Yeh,[7,8] Jing-Gung Chung[5,9]

[1]Department of Laboratory Medicine, China Medical University Hospital, Taichung 404, Taiwan

[2]Department of Life Sciences, National Chung Hsing University, Taichung 402, Taiwan

[3]Department of Pharmacology, China Medical University, Taichung 404, Taiwan

[4]School of Chinese Medicine, China Medical University, Taichung 404, Taiwan

[5]Department of Biotechnology, Asia University 413, Taichung, Taiwan

[6]Graduate Institute of Clinical Medical Science, China Medical University, Taichung 404, Taiwan

[7]Division of Hematology and Oncology, China Medical University Hospital, Taichung 404, Taiwan

[8]School of Medicine, China Medical University, Taichung 404, Taiwan

[9]Department of Biological Science and Technology, China Medical University, Taichung 404, Taiwan

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ABSTRACT: Enhanced cruciferous vegetable consumption is associated with the reduction of cancer incidence as shown in epidemiological studies. Phenethyl isothiocyanate (PEITC), one of the important compounds in cruciferous vegetables, has been shown to induce apoptosis in many types of human cancer cell lines, but there is no available information addressing the effects on normal and leukemia mice *in vivo*. The purpose of this study is to focus on the *in vivo* effects of PEITC on immune responses of normal and WEHI-3 leukemia BALB/c mice *in vivo*. Influences of PEITC on BALB/c mice after intraperitoneal (*i.p.*) injection with WEHI-3 cells and normal mice were investigated. In normal BALB/c mice, PEITC did not affect the body weight when compared to the olive oil treated animals. Moreover, PEITC promoted phagocytosis by macrophages from peripheral blood mononuclear cells (PBMC) and peritoneal cavity, increased the levels of CD11b and Mac-3, decreased the level of CD19 and promoted natural killer (NK) cell cytotoxic activity, but it did not alter the level of CD3. Also, PEITC enhanced T cell proliferation after concanavalin A (Con A) stimulation. Otherwise, PEITC increased the body weight, but decreased the weight of liver and spleen as compared to the olive oil-treated WEHI-3 leukemia mice. PEITC also increased the level of CD19, decreased the levels of CD3 and Mac-3 rather than influence in the level of

Correspondence to: J.-G. Chung; e-mail: jgchung@mail.cmu.edu.tw
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CD11b, suggesting that the differentiation of the precursor of macrophages and T cells was inhibited, but the differentiation of the precursor of B cells was promoted in leukemia mice. Furthermore, PEITC enhanced phagocytosis by monocytes and macrophages from PBMC and peritoneal cavity, and also promoted the NK cell cytotoxic activity in comparison with the group of leukemia mice. Based on these observations, the biological properties of PEITC can promote immune responses in normal and WEHI-3 leukemia mice *in vivo*. © 2011 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2011.
Keywords: isothiocyanates; PEITC; immune responses; murine leukemia WEHI-3 cells; leukemia mice

INTRODUCTION

Numerous studies have been shown that the antitumor agents can exhibit the functions for rapid elimination of tumor cells through the induction of cancer cell apoptosis (Guchelaar et al., 1997; Jaffrezou et al., 1998; Debatin, 2000; Woynarowska and Woynarowski, 2002; Chiang et al., 2011). Apoptosis is a programmed cell death process for removal of unwanted cells of the development and homeostasis of multicellular organisms (Arends and Wyllie, 1991). It is well-documented that the characters of apoptosis include membrane blebbing, translocation of phosphatidylserine of the plasma membrane from the inner to the outer leaflet, nuclear and cytoplasmic shrinkage, DNA fragmentation occur, and activation of a family of caspase (Earnshaw et al., 1999; Strasser et al., 2000). The caspase activation is considered to be a key hallmark of apoptosis (Earnshaw et al., 1999; Lu et al., 2010). In addition, apoptotic cell death is also accompanied by a loss of mitochondrial membrane potential ($\Delta\Psi_m$) before induction of cytochrome *c* release and activation of caspase-3 (Kluza et al., 2003; Lim et al., 2003). In Taiwan, leukemia is the twelfth most common cancer and about 4 persons per 100 thousand died annually from leukemia based on the reports from Department of Health, R.O.C. (Taiwan) in 2009 (Lai et al., 2010b). Although many types of treatments, including radiation, chemotherapy, or combination of radiotherapy with chemotherapy have been used in leukemia patients, it is still unsatisfying.

Conaway et al. demonstrated that dietary intake of cruciferous vegetables may decrease the risk of various types of malignancies (Conaway et al., 2002). The organic isothiocyanates (ITCs) one of the components in these vegetables (Conaway et al., 2002), including phenethyl ITC (PEITC), allyl ITC (AITC), and benzyl ITC (BITC) (Kumar and Sabbioni, 2010). PEITC exhibits cancer chemopreventive activity in rat (Stoner et al., 1991). PEITC induces apoptosis in HT-29 cells through the inhibition of cytochrome P450 (CYP) enzymes and the induction of phase II detoxification enzymes (12). Also, PEITC suppresses 4-(methylnitrosamino)-1-(3-pyridyl)-1-butone-induced pulmonary neoplasia in A/J mouse lung (Morse et al., 1989) and reduces azoxymethane-induced colonic aberrant crypt foci formation (Zhang et al., 1994).

Many reports have shown that PEITC induced apoptosis in many types of tumor cell lines (Chen et al., 2002; Kuang and Chen, 2004; Rose et al., 2005; Wu et al., 2005; Hwang and Lee, 2010). Our previous studies also showed that PEITC inhibited migration and invasion of human gastric cancer AGS cells (Yang et al., 2010b) and colon cancer HT29 cells (Lai et al., 2010a), but there are no reports to show that PEITC promoted immune responses in normal and WEHI-3 leukemia mice *in vivo*. Therefore, the purpose of the present study was to investigate the hypothesis of PEITC-affecting the immune responses in normal and leukemia mice *in vivo*.

MATERIALS AND METHODS

Materials and Reagents

PEITC and olive oil were obtained from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and L-glutamine were obtained from Invitrogen Life Technologies (Carlsbad, CA).

BALB/c Mice

Ninety male BALB/c mice 8 weeks of age (~22–28 g) were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan).

WEHI-3 Cells

The murine myelomonocytic leukemia cell line (WEHI-3) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained onto 75 cm^2 cell culture flasks with RPMI 1640 medium containing 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 m*M* L-glutamine and 10% FBS at 37°C under a humidified 5% CO_2 and one atmosphere (Yu et al., 2010).

Normal Mice Studies

PEITC Treatment

Forty BALB/c mice were divided into four groups (10 animals per group). Group I was control and group II was only treated with olive oil orally. Group III was orally treated with PEITC (40 mg kg^{-1}) in olive oil. Group IV was treated with PEITC (80 mg kg^{-1}) in olive oil by oral administration (Xiao and Singh, 2010). Mice were treated

daily for 30 days before being weighed and sacrificed (Yang et al., 2010a).

WEHI-3 Leukemia Mice Studies

Establishment of Leukemia Mice and PEITC Treatment

Fifty BALB/c mice were divided into five groups (10 animals per group). Group I was control and group II was only orally treated with olive oil. Group III was intraperitoneally (i.p.) injected with WEHI-3 cells (1×10^5 cells/mice) in phosphate buffered saline (PBS) only. Group IV and V was intraperitoneally injected with WEHI-3 cells (1×10^5 cells/mice) and then treated with PEITC (80 and 160 mg kg^{-1}, respectively) in olive oil. After WEHI-3 cells intraperitoneal injection for 2-week incubation, animals were orally treated daily for 2 weeks before being weighed and sacrificed (Yang et al., 2010a; Yu et al., 2010).

Blood Collections and Immunofluorescence Staining

All mice were treated as described above, and 1 mL of whole blood was collected from all animals (normal and WEHI-3 cells-injected mice). The blood sample was immediately exposed to 1× Pharm Lyse™ lysing buffer (BD Biosciences, San Jose, CA) for lysing of the red blood cells and then was centrifuged at 1500 rpm at 4°C for 15 min as previously described (Lin et al., 2009) for isolation of white blood cells (WBC). Isolated WBC were measured for cell surface markers of T-cell (CD3), B-cell (CD19), monocyte, and macrophage (CD11b and Mac-3) (WEHI-3 is a myelomonocytic leukemia cell line) by using anti-CD3-FITC, CD11b-FITC, CD19-PE, and Mac-3-PE antibodies (BD Pharmingen, San Diego, CA) for determining the levels of cell marker by flow cytometry (FACS Calibur™, Becton Dickinson, Franklin Lakes, NJ) as described elsewhere (Hsu et al., 2011; Yang et al., 2010a).

Body Weight, Liver, and Spleen Tissues

All mice were weighed before blood was drawn for the other tests. Liver and spleen were isolated and also weighed for individual mice from both study animals (Su et al., 2008).

Phagocytic Activity of Macrophages

The level of phagocytosis by macrophages was measured by using the PHAGOTEST kit (Glycotope Biotechnology, Heidelberg, Germany). Cells were isolated from PBMC and peritoneum in experimental animals of both studies. Isolated cells from each mice were individually incubated for 1 h at 37°C with opsonised fluorescein isothiocyanate (FITC)-labeled *E. coli* (20 μL) according to the manufacturer's protocols. The quenching solution (100 μL) was added to stop the reaction. Phagocytosis by monocytes and macrophages was determined, and the analysis of DNA content was measured for viable cells as previously described (Chang et al., 2009). Fluorescent data were collected and analyzed by flow cytometery. All data were calculated by using BD CellQuest™ Pro Software (Becton Dickinson) (Su et al., 2008; Tsou et al., 2009).

Natural Killer (NK) Cell Cytotoxic Activity

Tissues of spleen were isolated from each mouse of both studies and then were prepared for the splenocytes as previously described (Chang et al., 2009; Lin et al., 2010). The $\sim 1 \times 10^5$ splenocytes in 1 mL of RPMI 1640 medium were cultured in each well of 96-well plates. YAC-1 (2.5×10^7 cells) target cells in 15 mL tubes with serum-free RPMI 1640 medium and the PKH-67/Dil. C buffer (Sigma-Aldrich Corp.) was added to the cells then mixed thoroughly for 2 min at 25°C and then 2 mL PBS was added for 1 min. Four milliliter medium was added for a 10-min incubation and then centrifuged at 1200 rpm and 25°C. YAC-1 cells (2.5×10^6) were placed into 96-well plates for 100 μL before the addition of the leukocytes to the well for 6 h and determination of the NK cell cytotoxic activity by flow cytometry as described elsewhere (Chang et al., 2009; Lin et al., 2010).

Determinations for T and B Cell Proliferation

Spleen tissues from mice in each group were isolated and the splenocytes (1×10^5 cells/well) were isolated from the spleens of each mouse. One hundred microliters RPMI 1640 medium was added, and splenocytes were placed in 96-well plates and stimulated with concanavalin A (Con A, 5 μg mL^{-1}) for 3 days and lipopolysaccharide (LPS, 5 μg mL^{-1}) for 5 days. Splenocytes were collected and the cells were also determined for the cell proliferation by using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) as previously described (Chang et al., 2009; Yang et al., 2010a).

Statistical Analysis

All data were expressed as mean ± S.D. and differences between control and PEITC-experimental groups were analyzed by one-way ANOVA followed by Dunnett's test or Student's *t* test. **P* less than 0.05 was used as the level of significance.

RESULTS

PEITC Affected the Body Weight, Spleen, and Liver from Normal or WEHI-3 Cells-Injected Mice

The effects of PEITC on the body weights of normal and WEHI-3 cells-injected leukemia mice are presented in

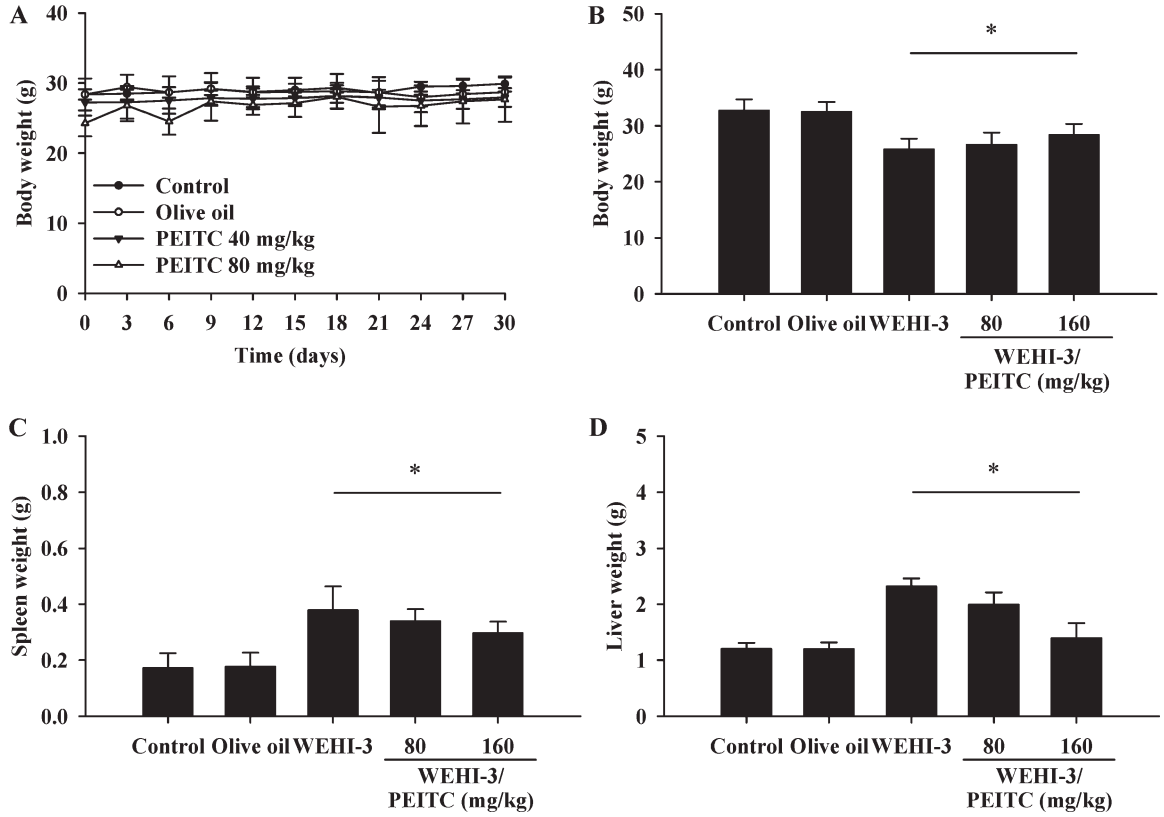

Fig. 1. PEITC affected the body weight, spleen, and liver from normal and WEHI-3 cells-injected leukemia mice. Animals including normal and leukemia BALB/c mice were treated with or without PEITC for 30 days or 2 weeks, respectively. Blood samples were isolated from each mouse; body weight from normal mice (A) and WEHI-3 cells leukemia mice (B) was measured and then were sacrificed for examinations of tissues of spleen (C) and liver (D) and were individually weighed. Each point is mean ± S.D. ($n = 10$) and the difference is considered statistically significant at P values <0.05.

Figure 1(A,B), which indicated that PEITC did not influence the body weight in normal mice [Fig. 1(A)], but it can increase the body weight of leukemia mice [Fig. 1(B)] as compared with WEHI-3 only injected-mice. Tissues of spleen and liver were isolated from leukemia mice and weighed individually. The effects of PEITC on the weights of spleen and liver from WEHI-3 injected mice are shown in Figure 1(C,D), indicating that PEITC decreased the weight of spleen [Fig. 1(C)] and liver [Fig. 1(D)]. These effects are dose-dependent manners.

PEITC Affected the Phagocytotic Activity of Normal BALB/c and WEHI-3 Leukemia Mice

Changes of macrophages with phagocytosed green fluorescent (fluorescein isothiocyanate, FITC) particles from PBMC and peritoneal cavity of control and PEITC-treated groups from both studies are presented in Figure 2(A–D). In normal mice, PEITC promoted the phagocytosis by macrophages from PBMC [Fig. 2(A)] and peritoneal cavity [Fig. 2(B)]. In WEHI-3 leukemia mice groups, PEITC also enhanced the phagocytotic activity in macrophages from PBMC [Fig. 2(C)] and peritoneal cavity [Fig. 2(D)]. Base on these results, PEITC induced significant differences in phagocytotic activity by macrophages isolated from PBMC and peritoneal cavity as compared with the control or WEHI-3 leukemia groups.

PEITC Affected the Whole Blood Cell Surface Markers of Normal BALB/c Mice and Leukemia Mice

Alterations of cell markers in white blood cells from normal BALB/c mice and mice were injected with WEHI-3 cells after treatment with PEITC or olive oil treatment only are shown in Figures 3 and 4, respectively. In normal mice, PEITC increased the levels of CD11b and Mac-3 [Fig. 3(C,D)] and decreased the level of CD19 [Fig. 3(B)], but it did not affect the level of CD3 [Fig. 3(A)]. In WEHI-3 leukemia mice, PEITC promoted the level of CD19 [Fig.

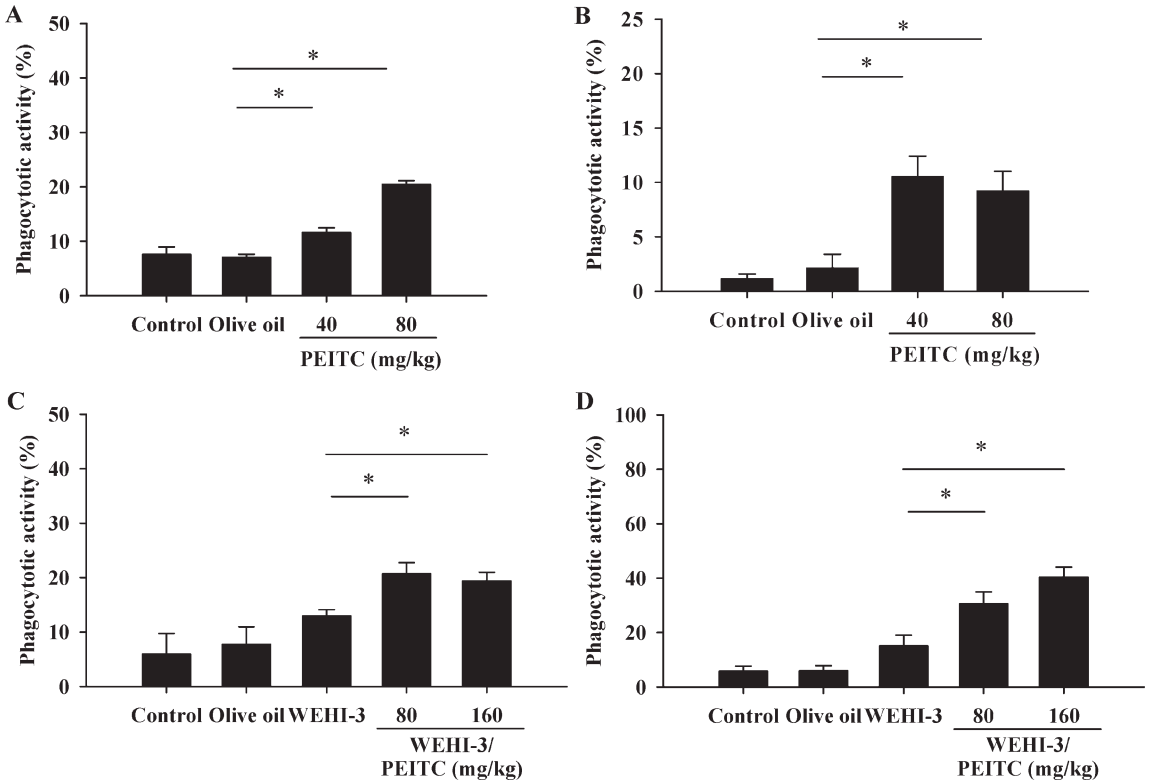

Fig. 2. PEITC affected the phagocytostic activity by macrophages from normal BALB/c mice and WEHI-3 cells-injected leukemia mice. Mice were intraperitoneally (i.p.) injected without or with WEHI-3 cells (1×10^5 cells/mice) in PBS for 2 weeks and then orally treated with present or absent PEITC for 2 weeks. Cells were collected from animals of PBMC and peritoneal cavity, and they were analyzed for phagocytotic activity and macrophages by flow cytometry as described in "Materials and Methods." Each point is mean ± S.D. ($n = 10$) and P values less than 0.05 are considered significant. A: PBMC from normal mice; B: macrophages from peritoneal cavity of normal mice; C: PBMC from WEHI-3 cells-injected mice; D: macrophages from peritoneal cavity of leukemia mice.

4(B)], reduced the levels of CD3 and Mac-3 [Fig. 4(A,D)], but it did not alter the level of CD11b [Fig. 4(C)], suggesting that the differentiation of the precursor of macrophage and T cells was inhibited, but the differentiation of the B cells was promoted.

PEITC Affected the Cytotoxic Activity of Natural Killer Cells from BALB/c Mice and Mice were Injected with WEHI-3 Cells

The YAC-1 target cells killed by NK cells were isolated from spleens in mice from both studies after exposure to PEITC in target cells ratio of 25:1, 50:1, and 100:1. Results can be seen in Figure 5(A,B). In normal mice, PEITC (80 mg kg^{-1}) treatment stimulated significant differences between control and the tested agent treatment in target cells ratio of 25:1 and 50:1 [Fig. 5(A)]. In mice after WEHI-3 cells injection, PEITC (80 mg kg^{-1}) treatment promoted significant differences between the WEHI-3 control and PEITC treatment in target cells ratio of 50:1 and 100:1 [Fig. 5(B)]. Based on the above observations, PEITC is likely to promote NK cell cytotoxic activity in BALB/c normal and leukemia mice *in vivo*.

PEITC Affected the Proliferation of T Cells from Normal BALB/c Mice

We investigated whether PEITC is able to increase T cell proliferation on normal BALB/c mice and results are revealed in Figure 6. PEITC (80 mg kg^{-1}) enhanced T cell proliferation [Fig. 6(A)], but it did not show significant differences in B cell proliferation in BALB/c mice [Fig. 5(B)].

DISCUSSION

PEITC, a type of isothiocyanates from cruciferous vegetables, has been reported to have anticancer activity such as:

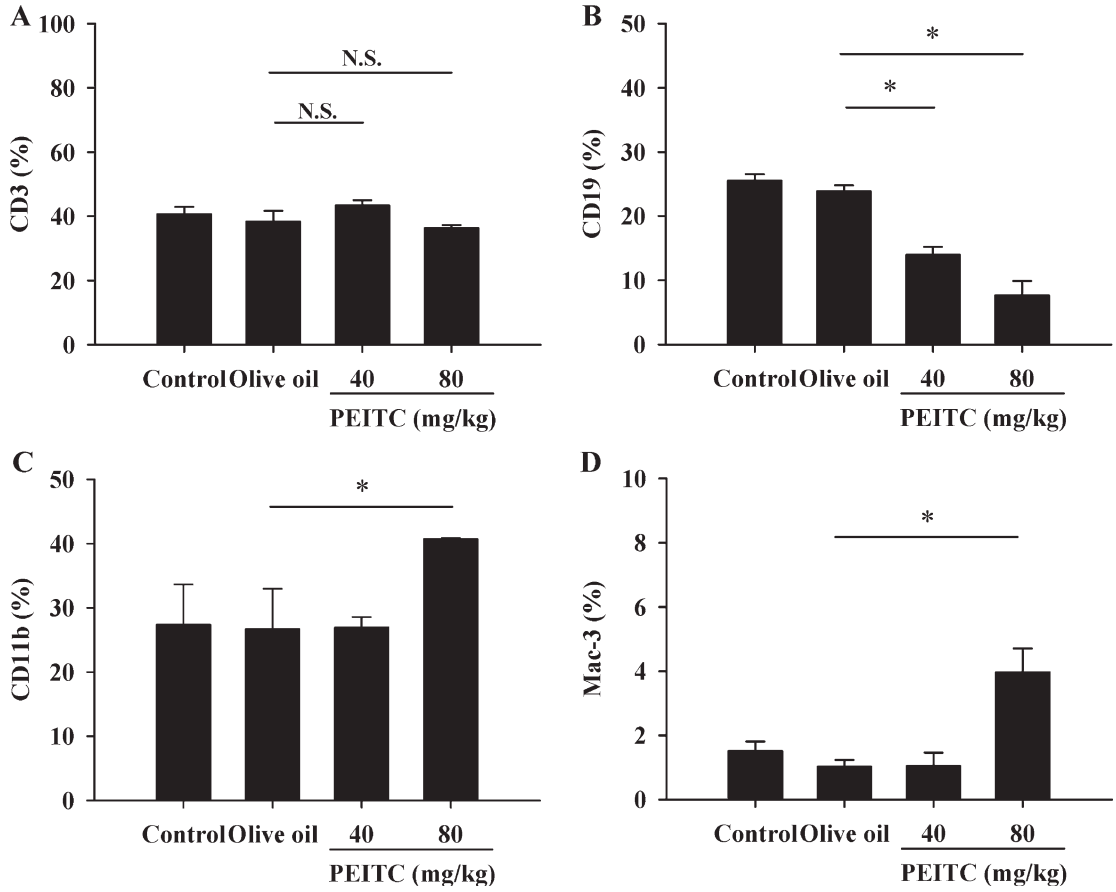

Fig. 3. PEITC affected the whole blood cell surface markers of normal BALB/c mice. The animals were orally treated with or without PEITC (40 and 80 mg kg^{-1}) for 30 days. Blood was collected from individually animals and analyzed for cell markers including CD3 (A), CD19 (B), CD11b (C), and Mac-3 (D) by flow cytometry as described in "Materials and Methods." Each point is mean ± S.D. of three experiments ($n = 10$). A P value less than 0.05 was considered significant and N.S. = not significant.

(1) induction of apoptotic cell death in different human cancer cell lines (Zhang et al., 2000; Nguyen et al., 2004; Granado-Serrano et al., 2006; Lee et al., 2006; VijayabaBu et al., 2006); (2) effects on cytokine and growth factor-secretion (Huang et al., 1999; Lee et al., 2004) and; (3) inhibition of migration and invasion of human hepatoma SK-HEP-1 cells and gastric cancer AGS cells (Hwang and Lee, 2006; Yang et al., 2010b). However, the roles of PEITC on normal and leukemia mice *in vivo* are not being fully investigated. Our primary experiments also demonstrated that PEITC-induced cytotoxicity and triggered apoptosis in human gastric cancer cells (Yang et al., 2010b). Therefore, this raises the possibility that PEITC could affect normal and leukemia mice *in vivo*. The present study indicated that PEITC effectively suppressed leukemia BALB/c mice injection with WEHI-3 cells. Furthermore, PEITC promoted the phagocytosis by macrophages and NK cell cytotoxic activity in normal and leukemia WEHI-3 cells-injected mice *in vivo*. In this study, we investigated WEHI-3 cells that were intraperitoneally injected to the BALB/c mice and set up leukemia animal model. The major reason is that the WEHI-3 was a mouse leukemia cell line (a murine myelomonocytic leukemia) (Abe et al., 1986; Lai et al., 2010b) and it also has a low cost and needs short periods of time to easily develop leukemia mice *in vivo*. This leukemia model has been used for potential anticancer agents (Yang et al., 2006, 2010a; Wen et al., 2010; Yu et al., 2010).

Our data indicated that PEITC did not affect the body weight of normal mice [Fig. 1(A)]. However, PEITC significantly decreased the averaged body weight [Fig. 1(B)] and weights of liver [Fig. 1(C)] and spleen [Fig. 1(D)] of BALB/c mice intraperitoneally injected with leukemia WEHI-3 cells when compared with the olive oil-treated only WEHI-3 group [Fig. 1(B–D)]. Also, PEITC increased phagocytosis by macrophages from isolations of PBMC

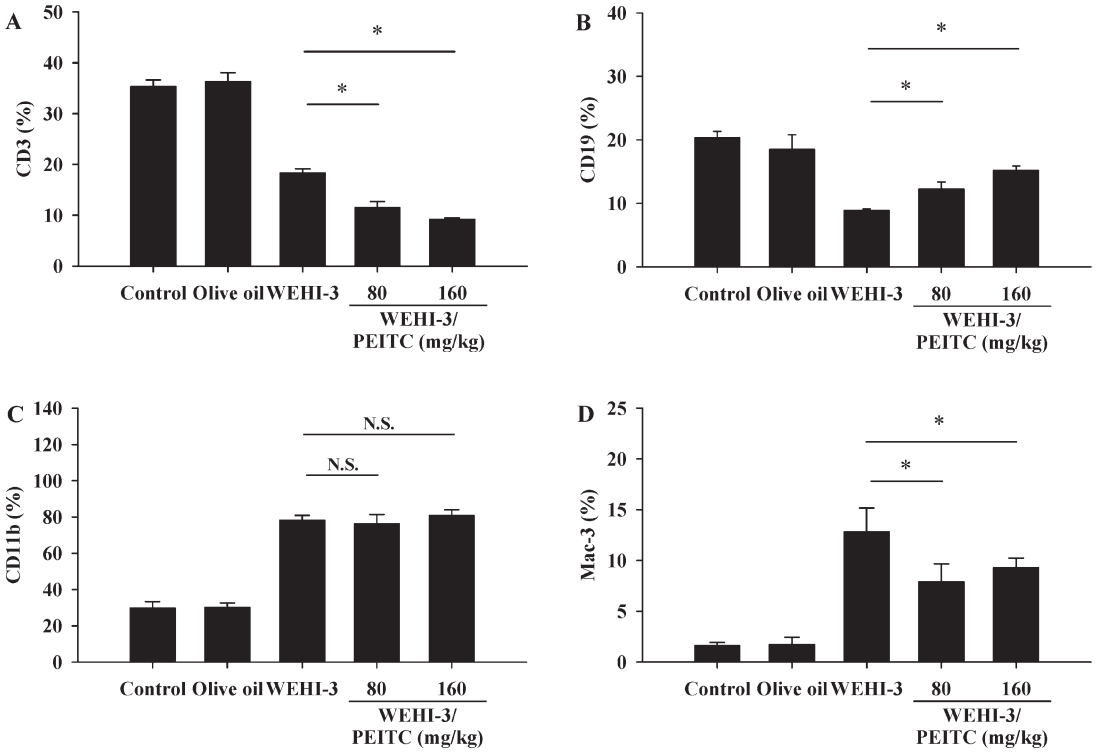

Fig. 4. PEITC affected cells' markers of white blood cells from leukemia mice. The animals were intraperitoneally injected with WEHI-3 cells (1×10^5 cells/mice) for 2 weeks and treated without or with PEITC by dietary oral gavage administration for 2 weeks. Blood was collected from individual animals and analyzed for cell markers such as CD3 (A), CD19 (B), Mac-3 (C), and CD11b (D) by flow cytometry as described in "Materials and Methods." Each point is mean ± S.D. ($n = 10$). *$P < 0.05$ was considered statistically significant and N.S. = not significant.

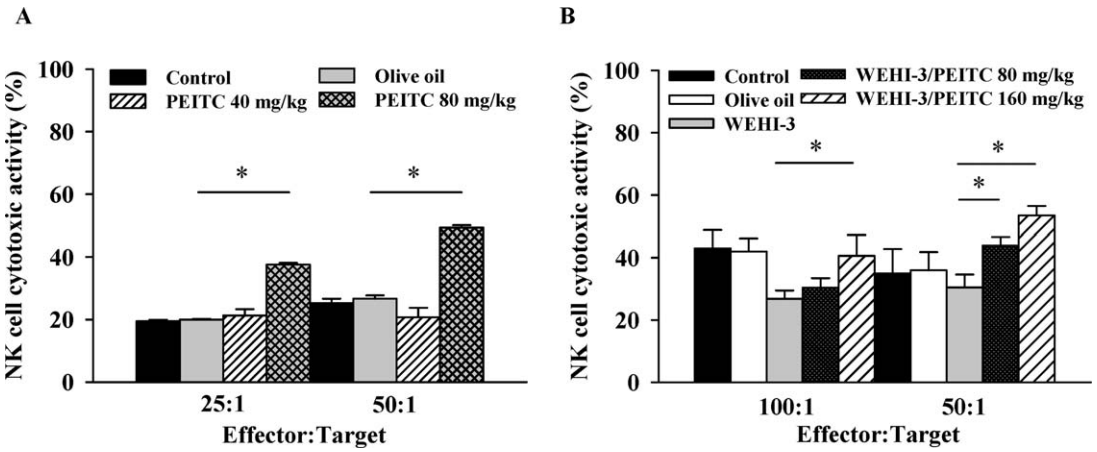

Fig. 5. PEITC affected the natural killer (NK) cell cytotoxic activity from BALB/c normal mice and leukemia mice. The effects of PEITC on the YAC-1 target cells which were killed by NK cells from the mice after treatment with or without PEITC at 40, 80, or 160 mg kg^{-1} in target cells ratio of 25:1, 50:1, or 100:1. Groups of normal (A) and leukemia (B) mice were analyzed for NK cell cytotoxic activity by flow cytometry as described in "Materials and Methods." Each point is mean ± S.D. of three experiments ($n = 10$). There was a significant difference when P value less than 0.05.

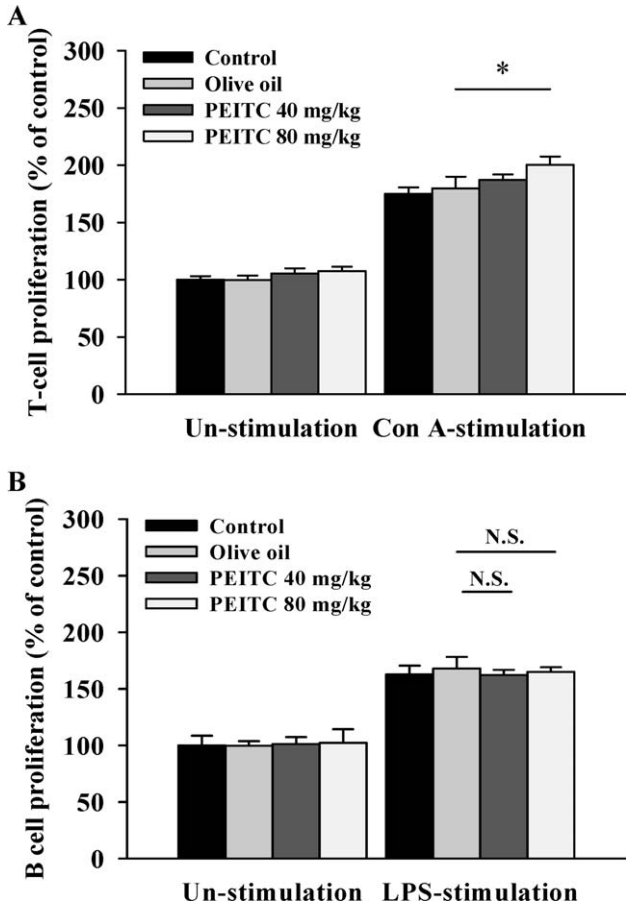

Fig. 6. PEITC affected the proliferation of T cells (Con A-stimulated splenocytes) from normal BALB/c mice. Splenocytes were isolated from each group of BALB/c mice after oral treatment with PEITC and then Con A (A) stimulation for 3 days or LPS (B) stimulation for 5 days for examinations of T and B cell proliferation respectively as described in "Materials and Methods." Each point is mean ± S.D ($n = 10$). The analysis reveals a significant difference when $P < 0.05$ and N.S. = not significant.

[Fig. 2(A,C)] and peritoneal cavity [Fig. 2(B,D)] of both examined mice. Moreover, PEITC promoted NK cell cytotoxic activity from both groups of study mice (Fig. 5).

Our results also indicated that PEITC increased the levels of CD11b and Mac-3 [Fig. 3(C,D)] and decreased the level of CD19 [Fig. 3(A)], but it did not alter the levels of CD3 [Fig. 3(A)] in normal mice. In leukemia animal groups, however, PEITC stimulated the level of CD19 [Fig. 3(B)] and attenuated the levels of CD3 and Mac-3 [Fig. 3(A,D)], but PEITC has no effect on the level of CD11b [Fig. 4(C)], suggesting that the differentiation of the precursor of macrophage and T cells was inhibited, but the differentiation of B cells was promoted. The B cell population in normal mice compared with PEITC treatment was slightly reduced based on the level of CD19 (B-cell surface marker) as can be seen in Figure 3(B). CD19 is a 95-kDa transmembrane protein expressed from the early stage of B cell development up to the stage of plasma cell differentiation. It is well-known as an essential downstream element of B cell receptor signaling required for B cell maturation and activation, T cell dependent, antigen-specific antibody responses, and germinal center formation (Otero et al., 2003; Otero and Rickert, 2003). In normal mice, our results also revealed that PEITC promoted the proliferation of T cells after Con A stimulation [Fig. 5(A)], but it had no significant effects in B cell proliferation even LPS exposure.

The proportions of NK cell cytotoxic activity between with or without PEITC treatment from normal and leukemia WEHI-3 cells-injected mice were much lower in absent PEITC-treated group than present exposure mice in YAC-1 target cells ratio of 50:1 (Fig. 5) (NK cell cytotoxicity, 21.6% vs. 47.7% from the normal mice group; NK cell activities, 27.3% vs. 56.4% in the leukemia mice group). It is known that NK cells are an early source of IFN-γ and, in general, to promote a type 2 response (Godfrey et al., 2000; Stein-Streilein et al., 2000). Therefore, the increase in NK cell cytotoxic activity could be responsible in part for the increased immune response of infections (Moretta, 2007).

In addition to the lymphocyte population, PEITC also regulated the generation and differentiation of CD11b positive cells (monocytes) and promoted the function of phagocytosis by macrophages and monocytes (Fig. 2). The proportion of CD11b positive in neonatal mice was 25% of the adult level [Fig. 3(C)]. The proportions of phagocytosis by macrophages between present or absent PEITC treatment from normal and leukemia mice were significantly different (Fig. 2). It is well-documented that macrophages are major contributors to innate immunity (Bu et al., 2006; Mantovani and Sica, 2010). Although the percentage of CD11b positive cells in leukemia mice was not significantly affected by PEITC; however, the functions of phagocytosis by macrophages are promoted by PEITC in groups of animals.

Overall, PEITC can promote the phagocytosis by macrophages and NK cell cytotoxic activity from normal and leukemia mice, indicating that PEITC treatment significantly increased a wide spectrum of immune cells in mice *in vivo*. The regulation of immune cell populations and function by PEITC might directly affect functional outcomes in BALB/c mice *in vivo*.

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