in vivo *27*: 627-634 (2013)

Epigallocatechin Gallate (EGCG), Influences a Murine WEHI-3 Leukemia Model *In Vivo* Through Enhancing Phagocytosis of Macrophages and Populations of T- and B-Cells

AN-CHENG HUANG[1], HSIU-YUEH CHENG[1], TSU-SHUN LIN[1,2], WEN-HSEIN CHEN[1,2], JU-HWA LIN[3], JEN-JYH LIN[4,5], CHI-CHENG LU[6], JO-HUA CHIANG[6], SHU-CHUN HSU[3], PING-PING WU[7], YI-PING HUANG[8] and JING-GUNG CHUNG[3,9]

[1]Department of Nursing, St. Mary's Junior College of Medicine, Nursing and Management, Yilan, Taiwan, R.O.C.;
[2]Pharmacy Department of St. Mary's Hospital Luodong, Luodong Township, Yilan County, Taiwan, R.O.C.;
Departments of [3]Biological Science and Technology and [8]Physiology, and
Schools of [4]Chinese Medicine and [7]Pharmacy, China Medical University, Taichung, Taiwan, R.O.C.;
[5]Division of Cardiology, China Medical University Hospital, Taichung, Taiwan, R.O.C.;
[6]Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan, R.O.C.;
[9]Department of Biotechnology, Asia University, Taichung, Taiwan, R.O.C.

Abstract. *Epigallocatechin gallate (EGCG) is the major polyphenol in green tea, and has been reported to have anticancer effects on many types of cancer cells. However, there is no report to show its effects on the immune response in a murine leukemia mouse model. Thus, in the present study, we investigated the effects of EGCG on the immune responses of murine WEHI-3 leukemia cells in vivo. WEHI-3 cells were intraperitoneally injected into normal BALB/c mice to establish leukemic BALB/c mice, which were then oral-treated with or without EGCG at 5, 20 and 40 mg/kg for two weeks. The results indicated that EGCG did not change the weight of the animals, nor the liver or spleen when compared to vehicle (olive oil) -treated groups. Furthermore, EGCG increased the percentage of cluster of differentiation 3 (CD3) (T-cell), cluster of differentiation 19 (CD19) (B-cell) and Macrophage-3 antigen (Mac-3) (macrophage) but reduced the percentage of CD11b (monocyte) cell surface markers in EGCG-treated groups as compared with the untreated leukemia group. EGCG promoted the phagocytosis of macrophages from 5 mg/kg treatment and promoted natural killer cell activity at 40 mg/kg, increased T-cell proliferation at 40 mg/kg but promoted B-cell proliferation at all three doses. Based on these observations, it appears that EGCG might exhibit an immune response in the murine WEHI-3 cell line-induced leukemia in vivo.*

Correspondence to: Jing-Gung Chung, Department of Biological Science and Technology, China Medical University, No 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C. Tel: +886 422053366 ext. 2161, Fax: +886 422053764, e-mail: jgchung@mail.cmu.edu.tw and Yi-Ping Huang Department of Physiology, China Medical University, Taichung 404, Taiwan, R.O.C. Tel: +886 422053366 ext. 2195, Fax: +886 422053764, e-mail: yphuang@mail.cmu.edu.tw

Key Words: Epigallocatechin gallate (EGCG), WEHI-3 leukemia cells, leukemia model, phagocytosis, macrophage, B-cell.

Numerous studies have demonstrated that daily consumption of green tea can reduce the risk of oxidative stress and damage, atherosclerosis, cancer, and cardiovascular diseases (1-3). Green tea contains abundant polyphenolic compounds, such as catechins. Epigallocatechin gallate (EGCG) is the major polyphenol component of green tea (4). EGCG has been shown to present many biological activities, including antioxidant and immunomodulatory activities and is also effective against some pathogens (5-7). In addition, it has an anticancer activity towards many cancer cell lines (8-12). EGCG has been reported to inhibit cytokine-induced interleukin-8 (IL-8) production in both nasal fibroblasts and bronchial epithelial cells (13). It has been reported that intraperitoneal administration of EGCG can protect mice against lethal endotoxemia, and rescue mice from lethal sepsis (14).

Leukemia and lymphoma have been reported to account for almost 50% of all childhood cancers (15), with leukemia frequently occurring in children under 14 years of age (16). It was also reported that leukemia is the second most malignant tumor in children (17). The treatments of leukemia patients have included immune modulatory, radiotherapy, chemotherapy, or a combination of radiotherapy with chemotherapy, however, the results are still unsatisfactory. Many investigators have focused on finding novel

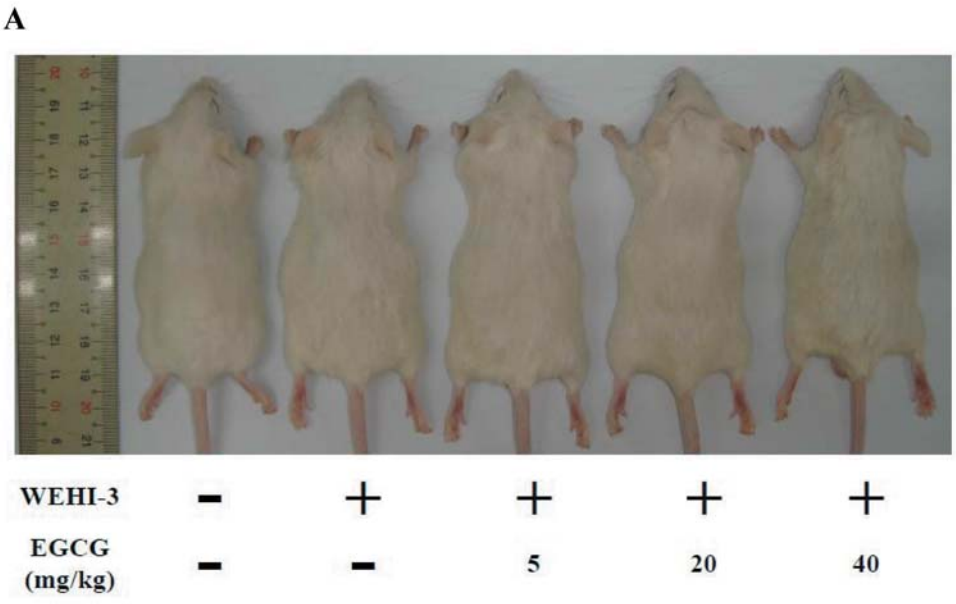

Figure 1. *Continued*

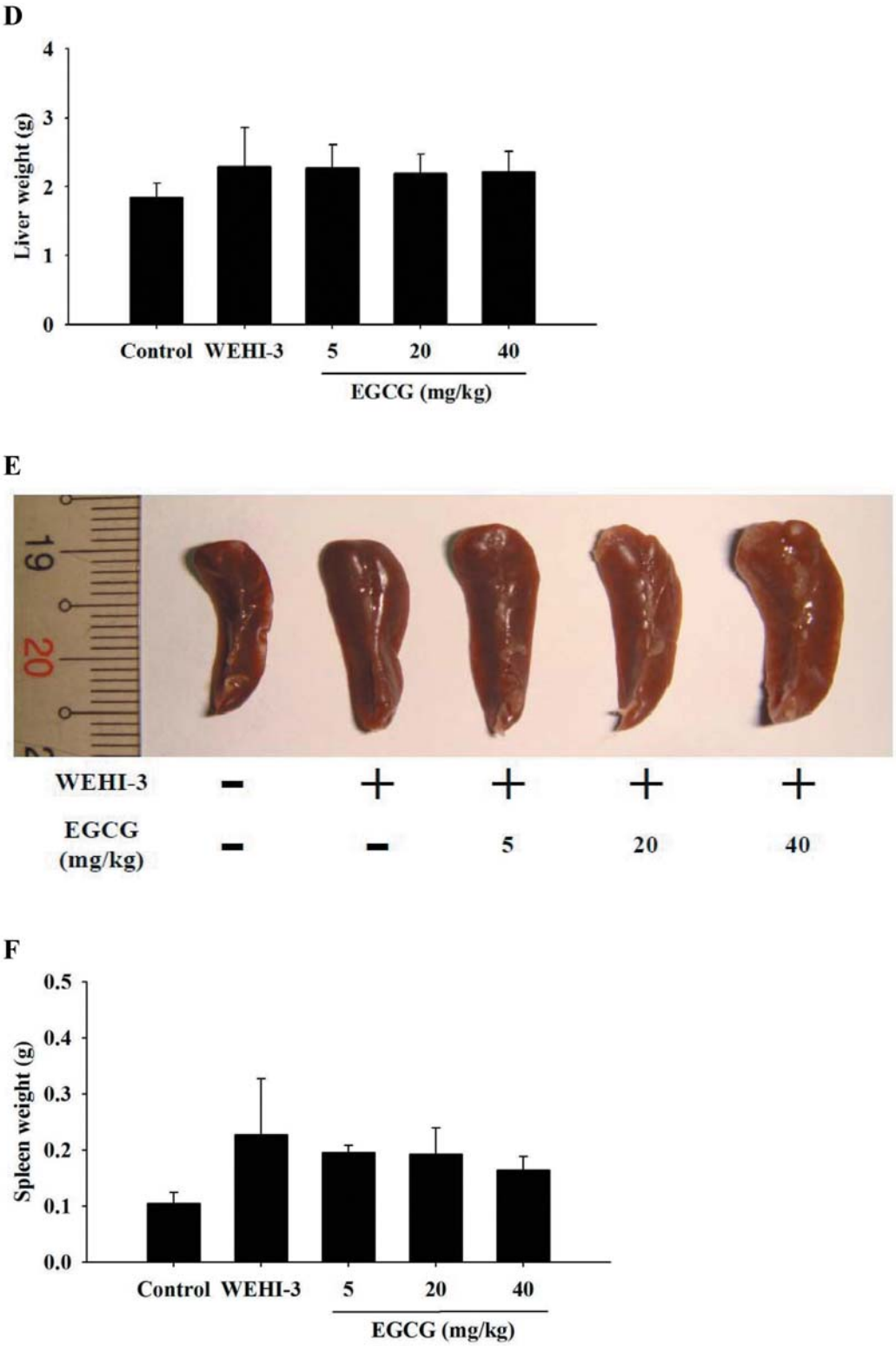

Figure 1. *Epigallocatechin gallate (EGCG) affects WEHI-3 cell-generated in BALB/c mice. All mice except the normal groups were intraperitoneally injected with WEHI-3 cells, and after two weeks they were divided into four groups, Group I included normal mice treated with normal diet. Group II was injected with WEHI-3 cells and treated with olive oil. Groups III-IV were injected with WEHI-3 cells and oraly treated with EGCG at 5 mg/kg, 20 mg/kg and 40 mg/kg, respectively. Representative animals (A), liver (C) and spleen (E), body weights (B), liver weights (D) and spleen weights (F) are shown. *p<0.05 represents, significant difference between control and EGCG-treated groups.*

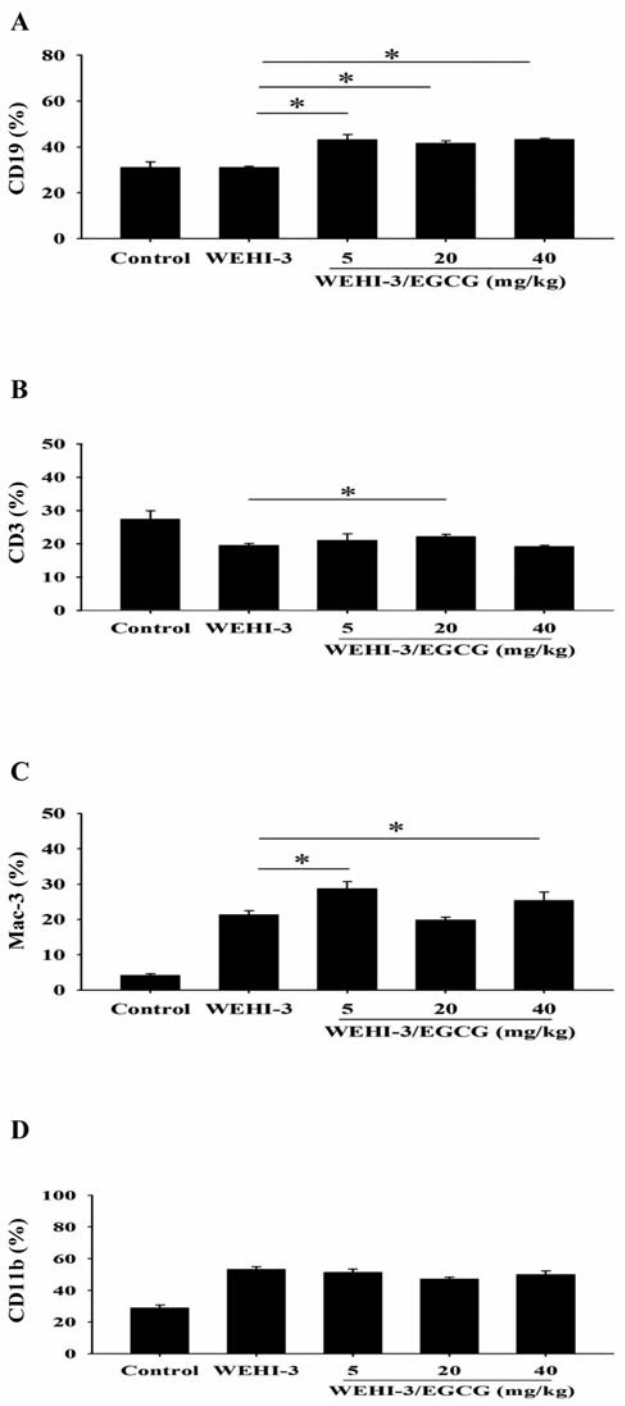

Figure 2. *Epigallocatechin gallate (EGCG) affected the levels of cell markers in white blood cells from leukemic BALB/c mice. All mice except the normal groups were intraperitoneally injected with WEHI-3 cells, after two weeks, followed by oral treatment with or without EGCG for four weeks. Blood was collected from each animal and was analyzed for cell markers (A: CD19; B: CD3; C: Mac-3 and D: CD11b) by flow cytometry as described in Materials and Methods. The data are expressed as the mean±S.D. of three experiments (n=10). *p<0.05 represent significant difference between leukemic control and EGCG-treated groups.*

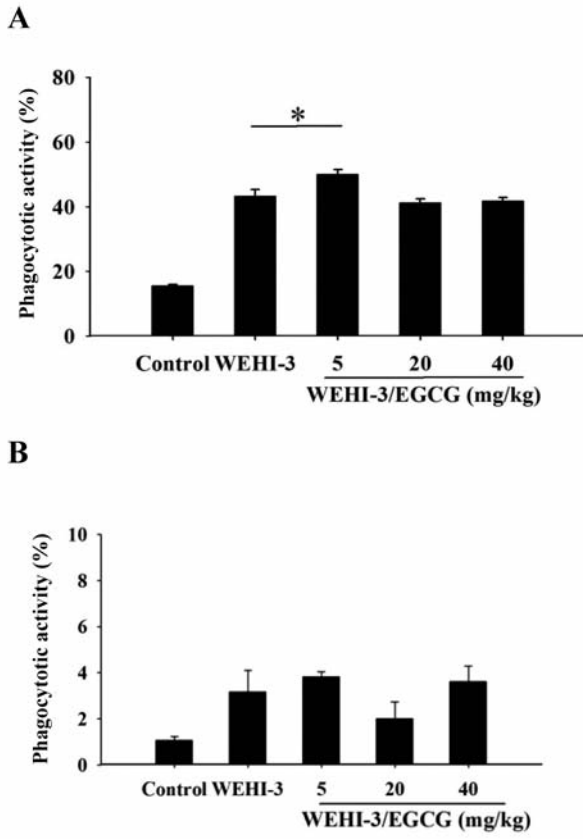

Figure 3. *Epigallocatechin gallate (EGCG) promoted phagocytosis by macrophages from peripheral blood mononuclear cells (PBMCs) and the peritoneal cavity of leukemic BALB/c mice. After blood samples were collected from control and experimental groups, macrophages were isolated from PBMCs and the peritoneum of each mouse. Isolated macrophages were placed and 50 μl of E. coli-FITC were added according to PHAGOTEST® kit. Each sample was analyzed by flow cytometry and quantified by CellQuest as described in Materials and Methods. A: PBMCs; B: peritoneal cavity. *p<0.05, Significant difference between leukemic control and EGCG-treated groups.*

compounds from natural products. An interesting point is that numerous experiments have shown that increased consumption of a plant-based diet can reduce the risk of cancer development (4-6).

A literature review shows that EGCG has certain biological activity, and anticancer functions. However, there are no reports to show the effects of EGCG on the immune responses of leukemic mice *in vivo*. Thus, in the present study, we investigated whether EGCG affects the immune response of leukemic BALB/c mice *in vivo*.

Materials and Methods

Materials and reagents. EGCG was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin were obtained from

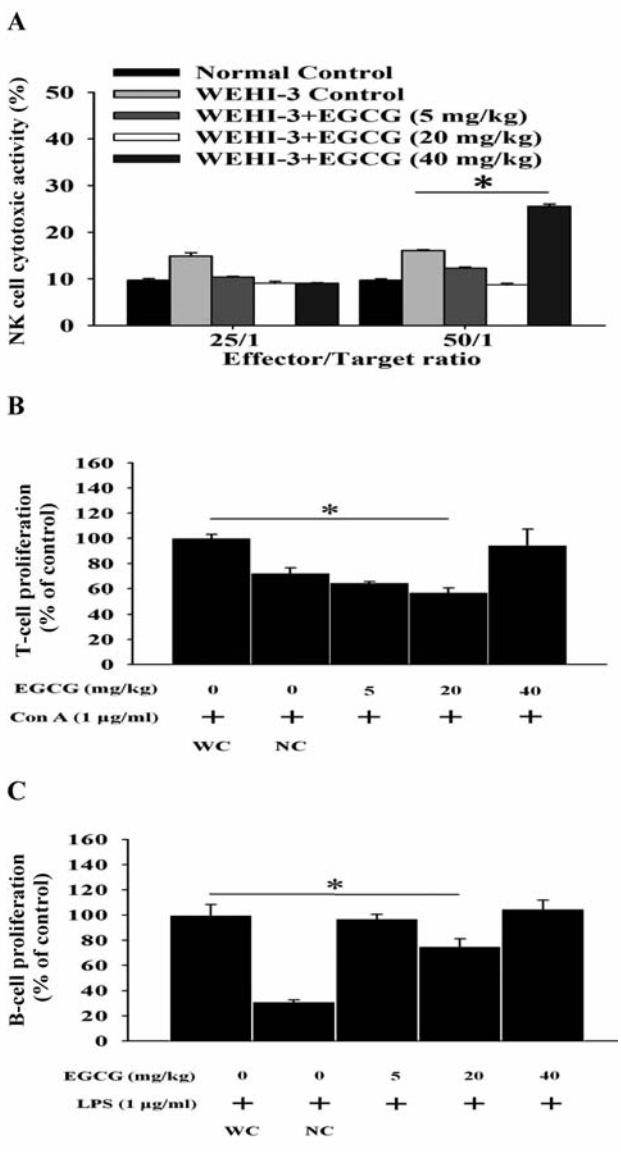

Figure 4. *Epigallocatechin gallate (EGCG) affected the cytotoxic activity of natural killer (NK) cells and T- and B-cell proliferation from leukemic BALB/c mice. Isolated spleen tissues were prepared for the splenocytes. Approximately 1×10^5 splenocytes were placed in 1 ml of RPMI-1640 medium in 96-well plates. Target YAC-1 cells (2.5×10^7 cells) with serum-free RPMI-1640 medium and PKH-67/Dil.C buffer were added to the cells for the determination of the NK cell cytotoxic activity by flow cytometry as described in Materials and Methods. A: NK cell activity; B: T-cell proliferation; C: B-cell proliferation.*

Gibco Life Technologies (Carlsbad, CA, USA). EGCG was dissolved in pyrogen-free water at a concentration of 5 mg/ml and kept at –20˚C in a tube covered with black paper to protect it from light.

WEHI-3 murine leukemia cells. The WEHI-3 murine myelomonocytic leukemia cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). WEHI-3 cells were maintained in plastic culture flasks (75 cm^2) in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37˚C (100% humidity, 5% CO_2, 95% air). All cells were cultured for two complete cycles in an incubator.

Male BALB/c mice. Fifty male BALB/c mice 22-25 g in weight and aged about eight weeks were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan, ROC) and kept in the animal center of China Medical University. We followed the institutional guidelines (Affidavit of Approval of Animal Use Protocol) which was approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan, ROC).

Establishment of leukemic mice and EGCG treatment. A total of fifty BALB/c mice were used for the whole experiment. Ten BALB/c mice were used as control without WEHI-3 cell injection (normal treatment, Group I). Forty BALB/c mice were individually intraperitoneally (*i.p.*) injected with 1×10^5 WEHI-3 cells. After two weeks, animals were randomly separated into four groups as a model of leukemia. Group II mice were treated with olive oil (vehicle) as positive control. Group III, IV and V animals were treated with EGCG at 5, 20 and 40 mg/kg in olive oil, respectively. EGCG was administered by oral gavage to the treatment groups at the above doses daily for two weeks and at the end of treatment all mice were weighed and sacrificed by euthanasia with CO_2 (18).

Immunofluorescence staining for surface markers. All animals from each group were treated with EGCG for 2 weeks except Groups I and II before being sacrificed for further investigations. Each animal was individually weighed before blood was sampled. The liver and spleen were removed and were weighed individually. For surface marker measurements, blood samples of 1 ml from all experimental mice were collected and then were lysed to destroy red blood cells with 1×Pharm Lyse™ lysing buffer (BD Biosciences Pharmingen Inc., San Diego, CA, USA). All samples were centrifuged at 1500 ×*g* at 4˚C for 15 min to isolate white blood cells. All isolated cells from each groups were stained by the R-Phycoerythrin (PE)-labeled anti-mouse Mac-3, Fluorescein isothiocyanate (FITC)-labeled anti-mouse cluster of differentiation molecule 11b (CD11b), FITC-labeled anti-mouse CD3 and PE-labeled anti-mouse CD19 (BD Biosciences Pharmingen Inc., San Diego, CA, USA) for 30 min before being analyzed for cell markers by flow cytometry, as previously described (18).

Assay for phagocytosis by macrophages. After blood samples were collected from control and experiment groups, macrophages were isolated from peripheral blood mononuclear cell (PBMC) and peritoneum of each mouse. Isolated macrophages were placed in 15 ml centrifugal tube and added 50 μl of *Escherichia coli*-FITC according to PHAGOTEST® kit manufacturer's instructions (ORPEGEN Pharma Gesellschaft für biotechnologische, Heidelberg, Germany). All samples were shaken in a shaker bath for 30 min at 37˚C, centrifuged at 1,000 ×*g* for 5 min then the supernatant was discarded and the pellets were mixed with DNA and stained as described previously (18). Each sample was analyzed by flow cytometry and quantified by CellQuest software (Becton Dickinson).

Assay for natural killer (NK) cell cytotoxic activity. Spleen tissues were processed for the isolation of splenocytes, as previously described (18, 19). Approximately 1×10^5 splenocytes were placed in 1 ml of RPMI-1640 medium and then were cultured in each well of 96-well plates. Target YAC-1 cells (2.5×10^7 cells, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC) with serum-free RPMI-1640 medium and PKH-67/Dil.C buffer (Sigma-Aldrich Corp.) was added to the cells, then mixed thoroughly for 2 min at 25˚C, and 2 ml PBS was added for 1 min, then 4 ml medium was added for a 10-min incubation before centrifuging at 1200 × *g* (25˚C). The determination of the NK cell cytotoxic activity by flow cytometry is described elsewhere (18, 19).

Determinations of T- and B-cell proliferation. Splenocytes (1×10^5 cells/well) were placed in 96-well plate, then 100 μl of RPMI-1640 medium was added, and cells were stimulated with concanavalin A (Con A, 5 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) for three days and lipopolysaccharide (LPS, 5 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) for five days. Cell proliferation was determined by using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) as previously described (18, 19).

Statistical analysis. All data are expressed as the mean±S.D. and differences between control and EGCG experimental groups were analyzed by Student's *t*-test. *value of *p* less than 0.05 was used as the level of significance.

Results

EGCG did not affect the body, spleen and liver weights of leukemic BALB/c mice. After the animals from control and experimental groups were sacrificed, they were individually weighed, and their spleen and liver were isolated and weighed. The results are shown in Figure 1. EGCG did not significantly affect their body weight (Figure 1A and B), liver weight (Figure 1C and D) or spleen weight (Figure 1E and F) when compared with the control untreated leukemic mice.

EGCG affected markers of white blood cells from leukemic BALB/c mice. After the experiment, whole blood from each animal was collected and the levels of cell markers CD19, CD3, Mac-3 and CD11b were analyzed by flow cytometry. The results indicate that EGCG increased the level of CD19 (Figure 2A) at all three doses, increased CD-3 level (Figure 2B) at 20 mg/kg treatment, increased Mac-3 level (Figure 2C) at 5 and 40 mg/kg treatment but did not significantly affect the CD11b levels (Figure 2D), when compared with the leukemia group.

EGCG promoted phagocytosis by macrophages from PBMCs and the peritoneal cavity of leukemic BALB/c mice. Cells collected from PBMCs and the peritoneal cavity from each group were analyzed for phagocytosis by macrophages. EGCG at 5 mg/kg promoted and stimulated phagocytotic activity by macrophages which were isolated from PBMCs (Figure 3A). However, it had no significant effects on the phagocytotic activity of macrophages which were isolated from the peritoneal cavity (Figure 3B).

EGCG affected the cytotoxic activity of NK cells from leukemic BALB/c mice. YAC-1 target cells were killed by NK cells which were isolated from normal, WEHI-3-injected and WEHI-3-injected EGCG-treated mice. In WEHI-3-injected mice, EGCG at 40 mg/kg showed a significant cytotoxic activity of NK cells compared to the control at a target cell ratio of 50:1 (Figure 4A) but not at 25:1. It also showed that EGCG (5 and 20 mg/kg) reduced Con A-treated T-cell proliferation (Figure 4B). However, EGCG at 20 mg/kg treatment led to a significantly decreased B-cell proliferation (Figure 4C).

Discussion

Numerous studies have demonstrated that EGCG induces cytotoxic effects on various cancer cells through cell-cycle arrest and apoptosis but no information is available regarding the effect of EGCG on leukemic mice *in vivo*. Thus, herein, we investigated the effect of WEHI-3-induced leukemia in mice *in vivo*. Based on the lack of effect on body, liver and spleen weights, EGCG does produce a significant toxic effect on animals. However, EGCG does affect cellular populations of immune-associated leukocytes.

It is well-documented that the development of cellular immunity is essential in the host defense to infection agents such as *Legionella pneumophila* (20). Agents to activate macrophages can lead to suppression of intracellular bacterial growth which is an essential effector mechanism for the resolution of infection (21). Our results also show that in leukemic mice, oral treatment with EGCG at 5 and 40 mg/kg promoted the Mac-3-expression in the cell population (Figure 2C), indicating that EGCG may stimulate macrophage proliferation (Mac-3) *in vivo*.

It was reported that the *L. pneumophila* growth can be inhibited by macrophages and monocytes which were activated by T-helper 1 cell (Th1) cytokine and gamma interferon (IFN-γ) (22, 23). Results shown on Figure 2 indicate that EGCG increase the levels of CD3 (Figure 2B) and Mac-3 (Figure 2C) thus, it may *via* T-cells (CD3) affect macrophage (Mac-3) and lead to increased macrophage phagocytosis. It was reported that Th1 cells play an essential role in the development of cell-mediated immunity to pathogens (24). Cell development and humoral immune responses are controlled and regulated by the CD22 and CD19 cell surface receptors *in vivo* (25) and CD19 is an activated B-cell surface marker (26, 27). Furthermore, it was reported that B-cell differentiation requires the interaction of various cytokines which come from macrophages or T-cells (28). In the present study, Figure 2A indicated that

EGCG promoted the population of CD19 marker levels and that EGCG may also promote the B-cell population at all three doses of treatment. However, treatment of EGCG did not significantly induce cell proliferation of T-cells and B-cells after Con A and LPS stimulation, respectively (Figure 4B and C).

In conclusion, based on these observations, we suggest that EGCG promotes the immune response through increasing the levels of T-cell and macrophage cell surface markers in WEHI-3-generated leukemic BALB/c mice *in vivo*.

Acknowledgements

This work was supported by grant 1 CMU100-ASIA-04 from China Medical University, Taichung, Taiwan and grant 2 from St. Mary's Hospital Luodong, Luodong Township, Yilan County, Taiwan.

References

1 Frei B and Higdon JV: Antioxidant activity of tea polyphenols *in vivo*: evidence from animal studies. J Nutr *133*: 3275S-3284S, 2003.

2 Vita JA: Tea consumption and cardiovascular disease: Effects on endothelial function. J Nutr *133*: 3293S-3297S, 2003.

3 Crespy V and Williamson G: A review of the health effects of green tea catechins in *in vivo* animal models. J Nutr *134*: 3431S-3440S, 2004.

4 Hirao K, Yumoto H, Nakanishi T, Mukai K, Takahashi K, Takegawa D and Matsuo T: Tea catechins reduce inflammatory reactions *via* mitogen-activated protein kinase pathways in toll-like receptor 2 ligand-stimulated dental pulp cells. Life Sci *86*: 654-660, 2010.

5 Katiyar SK, Challa A, McCormick TS, Cooper KD and Mukhtar H: Prevention of UVB-induced immunosuppression in mice by the green tea polyphenol (–)-epigallocatechin-3-gallate may be associated with alterations in IL-10 and IL-12 production. Carcinogenesis *20*: 2117-2124, 1999.

6 Sakagami H, Takeda M, Sugaya K, Omata T, Takahashi H, Yamamura M, Hara Y and Shimamura T: Stimulation by epigallocatechin gallate of interleukin-1 production by human peripheral blood mononuclear cells. Anticancer Res *15*: 971-974, 1995.

7 Yam TS, Hamilton-Miller JM and Shah S: The effect of a component of tea (*Camellia sinensis*) on methicillin resistance, PBP2' synthesis, and beta-lactamase production in *Staphylococcus aureus*. J Antimicrob Chemother *42*: 211-216, 1998.

8 Paschka AG, Butler R and Young CY: Induction of apoptosis in prostate cancer cell lines by the green tea component, (–)-epigallocatechin-3-gallate. Cancer Lett *130*: 1-7, 1998.

9 Du GJ, Zhang Z, Wen XD, Yu C, Calway T, Yuan CS and Wang CZ: Epigallocatechin gallate (EGCG) is the most effective cancer chemopreventive polyphenol in green tea. Nutrients *4*: 1679-1691, 2012.

10 Liu X, Zhang DY, Zhang W, Zhao X, Yuan C and Ye F: The effect of green tea extract and EGCG on the signaling network in squamous cell carcinoma. Nutr Cancer *63*: 466-475, 2011.

11 Lambert JD, Sang S, Hong J and Yang CS: Anticancer and anti-inflammatory effects of cysteine metabolites of the green tea polyphenol, (–)-epigallocatechin-3-gallate. J Agric Food Chem *58*: 10016-10019, 2010.

12 Chen D, Wan SB, Yang H, Yuan J, Chan TH and Dou QP: EGCG, green tea polyphenols and their synthetic analogs and prodrugs for human cancer prevention and treatment. Adv Clin Chem *53*: 155-177, 2011.

13 Kim IB, Kim DY, Lee SJ, Sun MJ, Lee MS, Li H, Cho JJ and Park CS: Inhibition of IL-8 production by green tea polyphenols in human nasal fibroblasts and A549 epithelial cells. Biol Pharm Bull *29*: 1120-1125, 2006.

14 Li W, Ashok M, Li J, Yang H, Sama AE and Wang H: A major ingredient of green tea rescues mice from lethal sepsis partly by inhibiting HMGB1. PLoS One *2*: e1153, 2007.

15 O'Neill KA, Bunch KJ and Murphy MF: Intrauterine growth and childhood leukemia and lymphoma risk. Expert Rev Hematol *5*: 559-576, 2012.

16 Diamantaras AA, Dessypris N, Sergentanis TN, Ntouvelis E, Athanasiadou-Piperopoulou F, Baka M, Fragandrea I, Moschovi M, Polychronopoulou S, Stiakaki E, Panagiotakos D and Petridou E: Nutrition in early life and risk of childhood leukemia: A case–control study in Greece. Cancer Causes Control *24*: 117-124, 2013.

17 Chen X, Zhou M, Ning B, Song H, Yang S and Tang Y: Transfusion-Associated HIV Infection in Pediatric Leukemia Patients (Two Case Reports). Iran J Pediatr *22*: 417-420, 2012.

18 Lin CC, Yu CS, Yang JS, Lu CC, Chiang JH, Lin JP, Kuo CL and Chung JG: Chrysin, a natural and biologically active flavonoid, influences a murine leukemia model *in vivo* through enhancing populations of T- and B-cells, and promoting macrophage phagocytosis and NK cell cytotoxicity. In Vivo *26*: 665-670, 2012.

19 Tsou MF, Tien N, Lu CC, Chiang JH, Yang JS, Lin JP, Fan MJ, Lu JJ, Yeh SP and Chung JG: Phenethyl isothiocyanate promotes immune responses in normal BALB/c mice, inhibits murine leukemia WEHI-3 cells, and stimulates immunomodulations *in vivo*. Environ Toxicol *28*: 127-136, 2013.

20 Matsunaga K, Klein TW, Friedman H and Yamamoto Y: Epigallocatechin gallate, a potential immunomodulatory agent of tea components, diminishes cigarette smoke condensate-induced suppression of anti-*Legionella pneumophila* activity and cytokine responses of alveolar macrophages. Clin Diagn Lab Immunol *9*: 864-871, 2002.

21 Horwitz MA: Cell-mediated immunity in Legionnaires' disease. J Clin Invest *71*: 1686-1697, 1983.

22 Bhardwaj N, Nash TW and Horwitz MA: Interferon-gamma-activated human monocytes inhibit the intracellular multiplication of *Legionella pneumophila*. J Immunol *137*: 2662-2669, 1986.

23 Nash TW, Libby DM and Horwitz MA: IFN-gamma-activated human alveolar macrophages inhibit the intracellular multiplication of *Legionella pneumophila*. J Immunol *140*: 3978-3981, 1988.

24 Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A and Murphy KM: Development of TH1 CD4+ T-cells through IL-12 produced by Listeria-induced macrophages. Science *260*: 547-549, 1993.

25 Kwon SH, Nam JI, Kim SH, Kim JH, Yoon JH and Kim KS: Kaempferol and quercetin, essential ingredients in Ginkgo bilboa extract, inhibit interleukin-1beta-induced MUC5AC gene expression in human airway epithelial cells. Phytother Res *23*: 1708-1712, 2009.

26 Asano N, Fujimoto M, Yazawa N, Shirasawa S, Hasegawa M, Okochi H, Tamaki K, Tedder TF and Sato S: B Lymphocyte signaling established by the CD19/CD22 loop regulates autoimmunity in the tight-skin mouse. Am J Pathol *165*: 641-650, 2004.

27 Jarasch-Althof N, Wiesener N, Schmidtke M, Wutzler P and Henke A: Antibody-dependent enhancement of coxsackievirus B3 infection of primary CD19+ B lymphocytes. Viral Immunol *23*: 369-376, 2010.

28 Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL and Bertagnolli MM: Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. Carcinogenesis *21*: 921-927, 2000.

Received April 28, 2013
Revised June 19, 2013
Accepted June 20, 2013