

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

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Abstract. Chrysin (5,7-dihydroxyflavone), a natural and biologically active flavonoid found in plants, possesses many biological activities and anticancer effects. However, there is no available evidence regarding the antileukemia responses to chrysin in a mouse model. We hypothesized that chrysin affects murine WEHI-3 leukemia cells *in vitro* and *in vivo*. The present study showed that chrysin at concentrations of 5-50 μ M reduced the cell viability in concentration- and time-dependent manners. In an *in vivo* study, WEHI-3 leukemic BALB/c mice were established in order to determine antileukemia activity of chrysin. Our results revealed that chrysin increased the percentage of CD3 (T-cell maker), CD19 (B-cell maker) and Mac-3 (macrophages) cell surface markers in treated mice as compared with the untreated leukemia group. However, chrysin did not significantly influence the level of CD11b (a monocyte maker) in treated mice. Moreover, there was a significant increase in phagocytosis by macrophages from peripheral

blood mononuclear cells, but no effect in those from the peritoneal cavity in leukemic mice after chrysin treatment. Isolated splenocytes from chrysin-treated leukemic mice demonstrated an increase of natural killer (NK) cell cytotoxicity. Based on these observations, chrysin might exhibit antileukemia effects on a murine WEHI-3 cell line-induced leukemia *in vivo*.

In Taiwan, based on a 2010 report from the Department of Health, Executive Yuan, R.O.C. (Taiwan) 4.2 individuals per 100,000 thousand die from leukemia. Currently, the treatments for leukemia are radiotherapy, chemotherapy, or a combination of radiotherapy with chemotherapy; however, these treatments have proven unsatisfactory. Thus, many investigators have focused on new compounds for the treatment of leukemia patients. Numerous studies have been demonstrated on the track that an increased consumption of a plant-based diet can reduce the risk of cancer development (1-3). Therefore, new compounds from natural plant are the major focus for investigators because diet can play a vital role in cancer prevention (4).

Chrysin (5,7-dihydroxyflavone) is a naturally active compound of the flavone group and can be obtained from honey, propolis and plants (4-6). Chrysin has been reported to exert multiple biological activities such as anti-inflammatory (7) and anti-oxidation effects (8), and cancer chemopreventive activity through inducing cell cycle arrest (5, 9) and apoptosis in melanoma (5) and leukemia cells (9). Chrysin reduced melanoma cell proliferation and induced cell differentiation in both A375 human and B16-F1 murine

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melanoma cells (10). Interestingly, chrysin has been shown to induce apoptosis in U937 (9, 11), HL-60 and L1210 leukemia cells (12). Furthermore, chrysin has been shown to inhibit tumor angiogenesis *in vivo* and angiogenesis is a critical step in cancer cell metastasis (13).

Currently there are no reports to address the effects of chrysin on the immune system of leukemic mice *in vivo*. Thus, in the present study, we investigated whether chrysin can promote phagocytosis of macrophages and increase activity of natural killer NK cells from leukemic BALB/c mice *in vivo*.

Materials and Methods

Materials and reagents. Chrysin, dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase A and Triton X-100 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin were purchased from Gibco Life Technologies (Carlsbad, CA, USA).

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). Cells were immediately maintained in plastic culture flasks (75 cm²) in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C under a humidified atmosphere with 5% CO₂. The cells were cultivated for two complete cycles in an incubator.

Viability determination. About 2×10⁵ WEHI-3 cells/well were placed into 24-well plates for 24 h. Chrysin was dissolved in DMSO then was individually added to the wells at final concentrations of 0, 5, 10, 20, 30, 40 and 50 µM, and 0.1% of DMSO in culture medium was added to the well as the control group. After treatments for 24 and 48 h, cells from each well were harvested for the determination of viability by using a flow cytometric method as described previously (14, 15).

Male BALB/c mice. Thirty male BALB/c mice of 8 weeks of age and around 22-25 g in weight were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan, ROC). This study following the institutional guidelines (Affidavit of Approval of Animal Use Protocol) was approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan).

Establishment of leukemic mice and chrysin treatment. Thirty BALB/c mice were intraperitoneally (*i.p.*) individually injected with 1×10⁵ WEHI-3 cells for 2 weeks and then were randomly separated into three groups as a model of leukemia. Group I mice were treated with olive oil (vehicle) as control (10 animals). Group II mice were treated with chrysin (10 mg/kg) in olive oil (10 animals). Group III animals were treated with chrysin (50 mg/kg) in olive oil (10 animals). Chrysin was administered by oral gavage to the treatment groups at the above doses daily for 2 weeks before mice were weighed and sacrificed by euthanasia with CO₂ (16).

Quantification of phagocytic activity of macrophages. The measurement of phagocytosis in each animal was performed by the

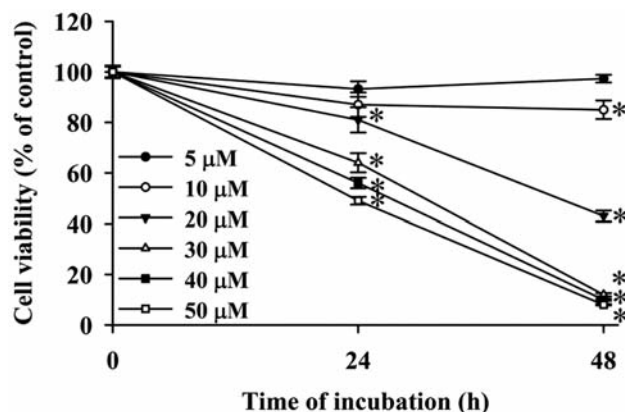


Figure 1. Chrysin reduced the percentage of viable WEHI-3 cells. Cells were treated with 0, 5, 10, 20, 30, 40 and 50 µM chrysin for 24 and 48 h. The cells were harvested and the percentages of viable WEHI-3 cells were determined as described in Materials and Methods. Each point is the mean±S.D. of three experiments. **p*<0.05, Significantly different compared with DMSO-treated control.

PHAGOTEST kit (GlycoTep Biotechnology GmbH, Heidelberg, Germany) as previously described (16). Peritoneal macrophages from each mouse in chrysin-treated or untreated groups were isolated as previously described (17). Briefly, a total of 1×10⁵ leukocytes in 100 µl from each animal were incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-labelled *Escherichia coli* (20 µl). Quenching solution (100 µl) was then added to the reaction according to the manufacturer's instruction. After the completion of phagocytosis by monocytes/macrophages, DNA was then stained according to the manufacturer's protocol and cells from each treatment were analyzed by flow cytometry; fluorescence data were collected on 10,000 cells and analyzed using the BD CellQuest Pro software (BD Biosciences, San Jose, CA, USA) as previously described (16).

Immunofluorescence staining for surface markers from leukemic mice after exposed to chrysin. For the measurement of surface markers, blood samples of 1 ml from all experimental mice were collected before mice were sacrificed. Collected red blood cells were lysed through exposure to 1×Pharm Lyse™ lysing buffer (BD Biosciences). All samples were centrifuged for 15 min at 1500 ×g at 4°C. The isolated white blood cells were stained by the FITC-labeled anti-mouse CD3, PE-labeled anti-mouse CD19, FITC-labeled anti-mouse CD11b and PE-labeled anti-mouse Mac-3 antibodies (BD Biosciences Pharmingen Inc., San Diego, CA, USA) for 30 min before being analyzed for levels of the cell markers by flow cytometry as previously described (16, 18).

Quantification of NK cell cytotoxic activity. For the measurement of NK cell cytotoxic activity, approximately 1×10⁵ leukocytes from the spleens of animals from all treatments in 1 ml of RPMI-1640 medium were cultured in each well of 24-well culture plates for 24 h. YAC-1 target cells, at a density of 2.5×10⁷ cells per ml were maintained in 15-ml tubes with serum-free RPMI-1640 medium and then the PKH67 Fluorescent Cell Linker Kits (Sigma-Aldrich, St. Louis, MO, USA) was used to the cells, mixed thoroughly for 2 min at

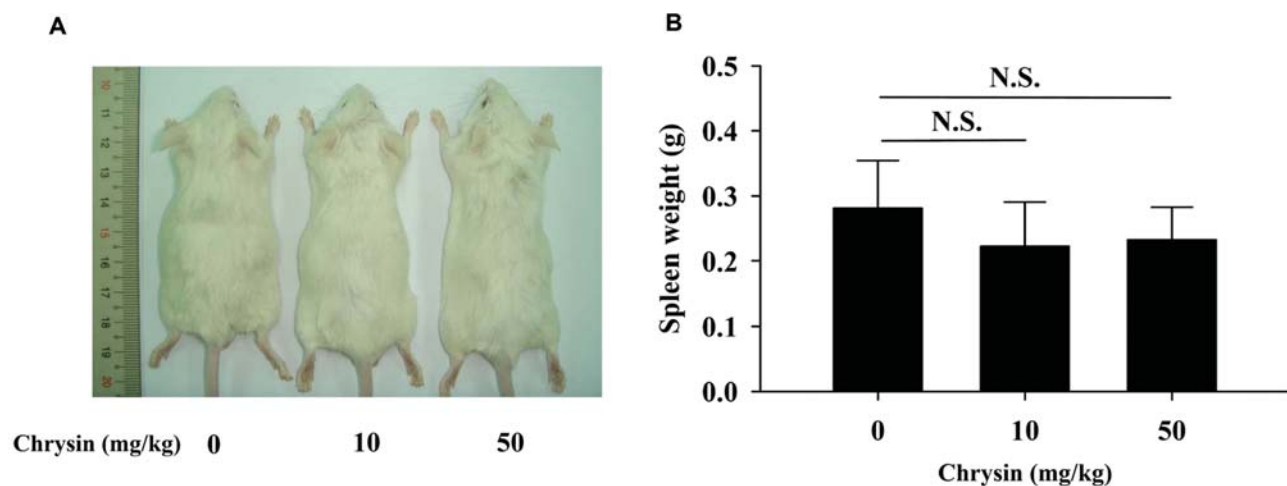


Figure 2. Representative mice after exposure to chrysin. Thirty male BALB/c mice were individually injected with WEHI-3 cells then randomly separated into three groups. Group I mice were treated with olive oil. Group II mice were treated with chrysin (10 mg/kg) in olive oil. Group III animals were treated with chrysin (50 mg/kg) in olive oil as described in the Materials and Methods. After sacrifice time, the blood and the spleen from all the animal was collected. NS: Not significant.

25°C then 2 ml PBS was added for 1 min. Subsequently, 4 ml of RPMI-1640 medium was added for a 10 min-incubation, and the mixture was centrifuged at 1200 \times g of 25°C. For the determination of NK cell cytotoxic activity, YAC-1 cells were placed on 96-well plates before the addition of the leukocytes from each treatment to the wells for 12 h and were analyzed by flow cytometry (BD Biosciences, FACSCalibur) as previously described (19, 20).

Statistics analysis. The results are expressed as the mean \pm S.D. and the difference between control and chrysin treated groups was analyzed by Student's *t*-test. *p*-Values of 0.05 or less to were considered as significant.

Results

Chrysin reduced the percentage of viable WEHI-3 cells. WEHI-3 cells were treated with different concentrations of chrysin for 24 and 48 h then all cells from each treatment were harvested by centrifugation and in order to determine the percentage of viable cells by flow cytometric assay. The results are shown in Figure 1, which indicates that chrysin reduced the percentage of viable WEHI-3 cells in a dose-dependent manner. The longer treatment of chrysin at 48 h led to higher inhibition than that of the 24 h treatment.

Growth effect of chrysin on leukemic mice. Thirty male BALB/c mice were intraperitoneally injected with WEHI-3 cells then were randomly separated into three groups. Group I mice were treated with olive oil alone. Group II mice were treated with chrysin (10 mg/kg) in olive oil. Group III animals were treated with chrysin (50 mg/kg) in olive oil for 3 weeks. Then all animals were photographed and are shown

in Figure 2A. The body weight in all groups was not significantly altered (data not shown). Figure 2B shows that spleen weight in all groups did not differ significantly.

Chrysin affected surface markers on whole blood cells from WEHI-3-leukemic BALB/c mice. In order to investigate whether chrysin affects the levels of cell surface markers, leukocytes from chrysin-treated and untreated (control) groups were isolated and levels of CD3, CD19, CD11b and Mac-3 were measured. The data from each treatment indicated that chrysin significantly increased the levels of CD3 (Figure 3A), CD19 (Figure 3B) and Mac-3 (Figure 3D) but did not have a significant effect on CD11b (Figure 3C) when compared to the control group.

Chrysin promoted macrophage phagocytosis by peripheral blood mononuclear cells in WEHI-3 leukemic BALB/c mice. In order to investigate whether chrysin affects phagocytosis, the leukocytes from chrysin-treated or untreated groups were isolated and the phagocytic activity was measured. The data in Figure 4 demonstrate that chrysin (10 and 50 mg/kg/day) significantly promoted the activity of phagocytosis from peripheral blood mononuclear cells (Figure 4A) but did not significantly affect phagocytosis of macrophages from the peritoneal cavity (Figure 4B) from leukemic mice.

Chrysin promoted NK cell activity of splenocytes in WEHI-3 leukemic BALB/c mice. To investigate whether chrysin acts on NK cell activity, leukocytes from chrysin-treated and untreated (control) groups were isolated and NK cell activity was measured, the results shown in Figure 5 indicate that the

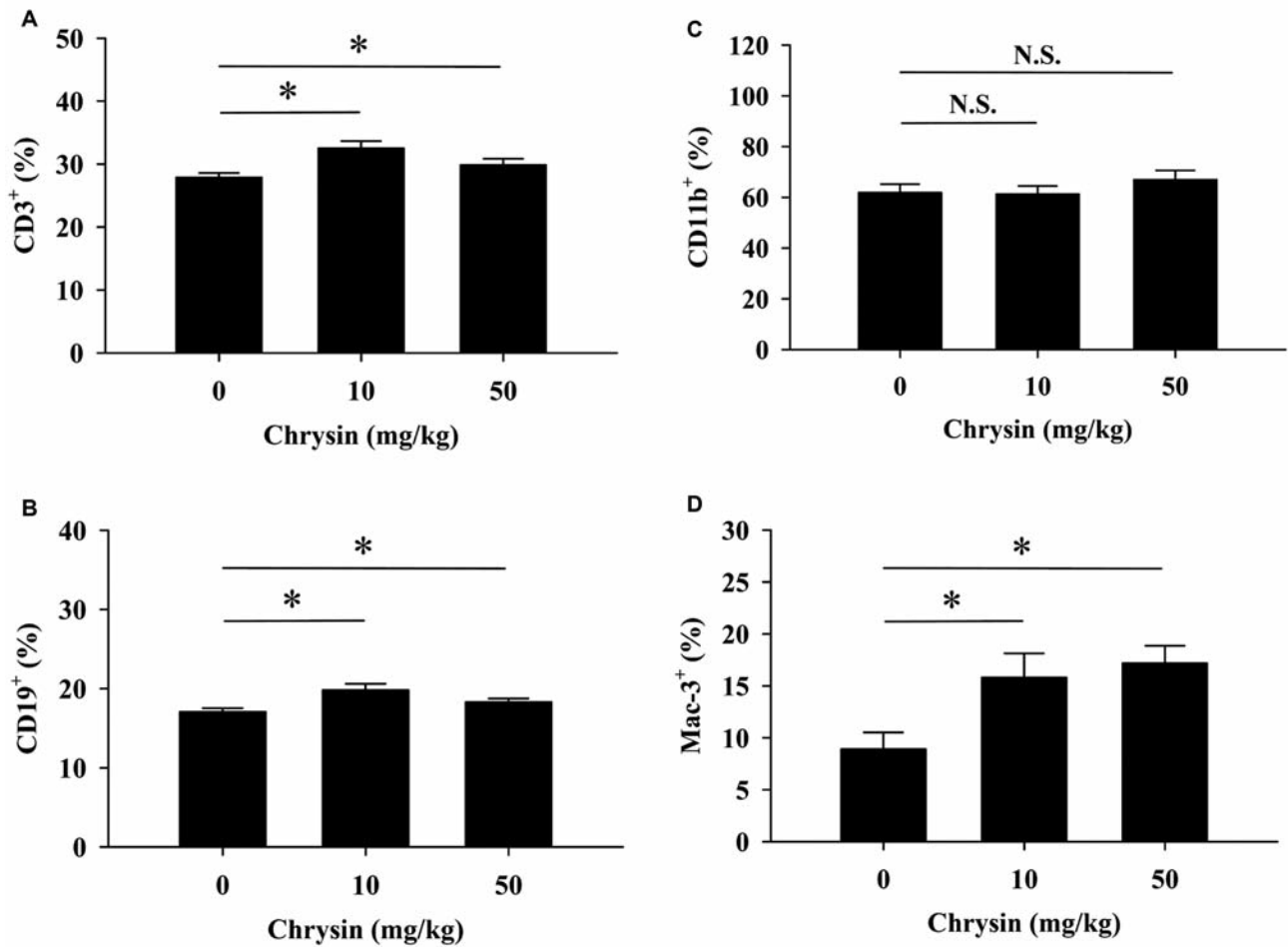


Figure 3. Chrysin affected the levels of cell markers in white blood cells from leukemic BALB/c mice. All mice were intraperitoneally injected with WEHI-3 cells for 2 weeks, followed by oral treatment with or without chrysin for 3 weeks. Blood was collected from each animal and was analyzed for cell markers (A: CD3; B: CD19; C: CD11b and D: Mac-3) 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 Significant by different between control and chrysin-treated groups. NS: Not significant.

YAC-1 target cells were only significantly killed by NK cells from the group treated with chrysin at 10 mg/kg/day at target cells ratio of 25:1 and 50:1.

Discussion

Numerous studies have demonstrated that chrysin induces cell cycle arrest and apoptosis in many human cancer cell lines (9, 10, 21) including leukemia cells (9, 11), and in mouse leukemia cells (12). Furthermore, chrysin can enhance apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL), and the apoptosis is caspase dependent and related to the activation of caspase-8 in CNE1 cells (13). Although other reports have shown that chrysin induced apoptosis in human and mouse leukemia cells, there is no available information to show the effect of chrysin on immune

responses in animal models *in vivo*. Herein, we investigated the effect of chrysin on the immune response in WEHI-3 cell-generated leukemia mice *in vivo*.

Our results indicated that chrysin induced *in vitro* cytotoxic effects and reduced the percentage of total viable WEHI-3 cells in a concentration-dependent effect (Figure 1). This is in agreement with another report which indicated that chrysin induced cytotoxic in L1210 mouse leukemia cells (12). Our results demonstrated that chrysin did not significantly affect the weights of animals, nor the weights of the liver and spleen in leukemic mice after comparison to leukemic mice without treatment with chrysin (control) (Figure 2).

The CD19 is an activated B-cell surface marker (16) and B-cell differentiation requires the interaction of various cytokines, which come from macrophages or T-cell secretions (22). In the present study, chrysin promoted the

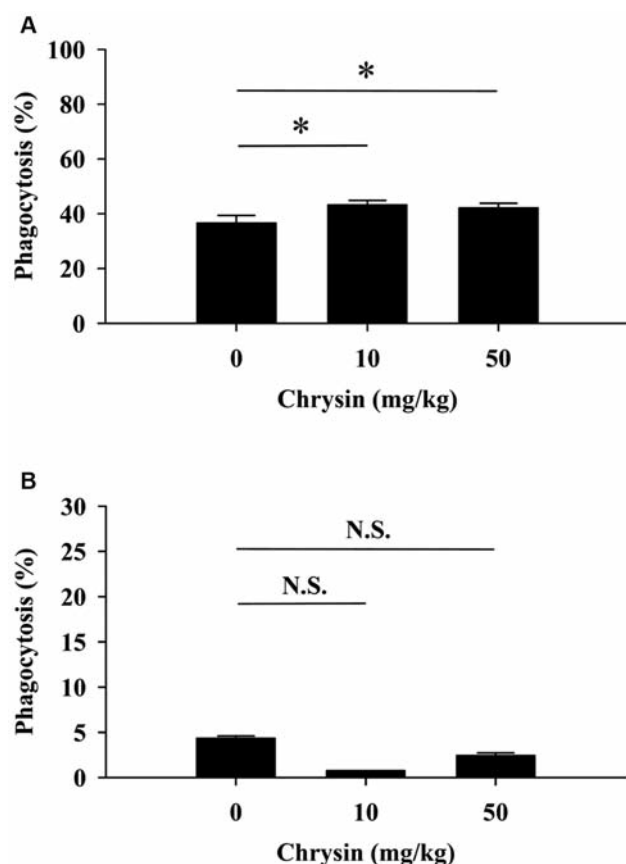


Figure 4. Chrysin stimulated phagocytotic activity of peripheral blood mononuclear cells (PBMCs) in leukemia BALB/c mice. Macrophages were isolated from PBMCs (A) and the peritoneal cavity (B) of each leukemic BALB/c mice after exposure to 0, 10 and 50 mg/kg/day of chrysin by oral administration for three weeks. The percentage of phagocytosis of green fluorescent particles (FITC-*E.coli.*) under chrysin treatment was determined by flow cytometric analysis as described in the Materials and Methods. Each point is mean \pm S.D (n=10). * p <0.05 Significant when compared with untreated leukemic mice. NS: Not significant.

B-cell population. Results from the flow cytometric assays indicated that chrysin promoted the phagocytosis by macrophages from blood samples (Figure 4A) but did not significantly affect the phagocytosis by macrophages from the peritoneal cavity (Figure 4B). However, chrysin did elevate the activity of NK cells (Figure 5). Thus, based on these observations, chrysin not only increased the humoral immune response (increased B-cell population and promotion of macrophage activities) but also affected the cellular immune response (T-cell) *in vivo*. Numerous studies have shown that macrophage phagocytosis and NK cell activity both played important roles in immune responses after mice were exposed to the antigen (23, 24).

Based on these observations, we may suggest that chrysin promoted the immune response and increased the activity of

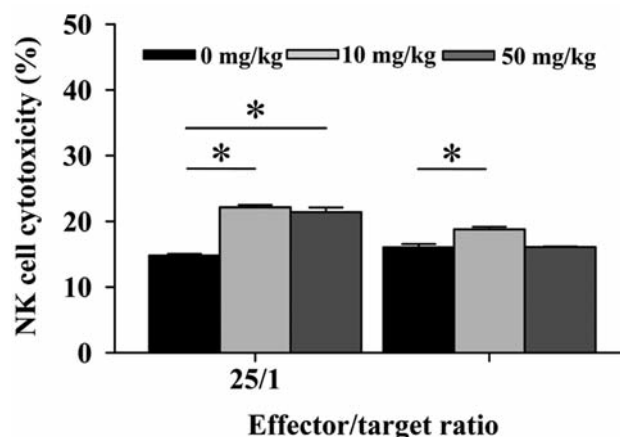


Figure 5. Chrysin affected the activity of natural killer (NK) cells in leukemic BALB/c mice. The YAC-1 target cells were killed by NK cells of splenocytes from the mice after treatment with chrysin (10 and 50 mg/kg/day) by oral administration in target cell ratio of 25:1 and 50:1. Each point is the mean \pm S.D.* p <0.05 Significant when compared with untreated leukemic mice. NS: Not significant. (n=10).

macrophages and NK cells in WEHI-3-generated leukemic BALB/c mice *in vivo*. This is a first finding showing that oral treatment of chrysin can promote immune responses in leukemic mice. Chrysin can act as a potent immunological adjuvant *in vivo*.

Declaration of Conflicting Interests

The Authors have no conflicts of interests.

Acknowledgements

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