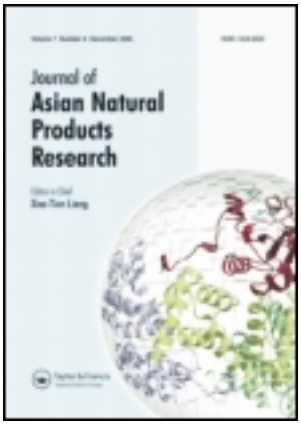

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The objective of this study was to evaluate the immunomodulatory effects of cinobufagin (CBG) isolated from Chan Su (*Venenum Bufonis*) *in vitro*. In this paper, our results show that CBG significantly stimulated cell proliferation of splenocytes and peritoneal macrophages (PMΦ) and markedly enhanced the phagocytic activation of PMΦ. CBG also significantly increased $CD4^+CD8^+$ double-positive T-cell populations and the percentage of S-phase cells of splenic lymphocytes. The levels of several Th1 cytokines, including interferon-γ and tumor necrosis factor-α, are significantly increased after CBG treatment, whereas the levels of the Th2 cytokine interleukin-4 and interleukin-10 are significantly decreased. As a result, the ratio of Th1/Th2 also increased. Taken together, these results indicated that CBG had potential immune system regulatory effects and suggested that this compound could be developed as a novel immunotherapeutic agent to treat immune-mediated diseases such as cancer.

Keywords: cinobufagin; immunoenhancement; cytokines

1. Introduction

Natural products are becoming increasingly important as sources of herbal drugs for the treatment of chronic diseases. It is reported that more than 60% of newly approved anticancer and anti-infection drugs are obtained from natural sources [1]. Chan Su (*Venenum Bufonis*), a traditional Chinese medicine (TCM) obtained from the dried white secretion of is auricular and skin glands of Chinese toads (*Bufo melanostictus* Schneider or *Bufo bufo gargarzinas* Gantor) [2]. It has been an important TCM in China and other Asian countries for centuries and has been used to treat a number of diseases, including sore throat, edema, pains, heart failure, skin problems, and cancers [3]. Cinobufagin (CBG; Figure 1) is one of the major active components of Chan Su. Some studies have shown that CBG induced apoptosis in some human carcinoma cells, such as prostate cancer cells [4] and HeLa cells [5]. However, there have been few reports related to immunomodulatory effects of CBG.

Immunoenhancement therapy has become a part of routine medical practice, though the development of pharmacological immunostimulation is at an early stage. Nowadays, immunomodulators are being developed and some might possess the effectiveness of immunopotentiation [2]. Immunoenhancement therapy could assist immunosuppressed patients to prevent infection and to deal with a variety of serious diseases, including cancer. Any disease is often associated with a depression of the immune system. Thus, the need for drugs to enhance the function of immune

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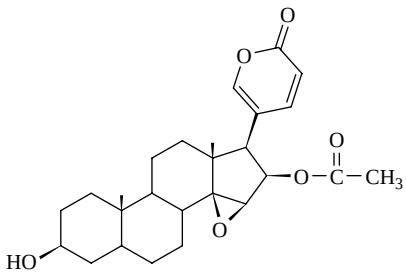

Figure 1. The chemical structure of CBG.

system is important. The objective of this study is to carry out preliminary preclinical immunization studies to determine whether CBG has the potential utility as adjuvant or additive for the stimulation of the cell-mediated immune response.

2. Results

2.1 CBG enhanced the proliferation of splenocytes

CBG promoted a small but significant effect on splenocyte proliferation ($p < 0.05$) when stimulated with 1 or 2.5 mg/l CBG alone or combined with Con A (Figure 2). When used in combination with LPS, CBG at concentrations of 0.5, 1, and 2.5 mg/l enhanced the proliferation of splenocytes significantly ($p < 0.05$; Figure 2).

All somatic cells proliferate via a mitotic process determined by progression through the cell cycle. To further verify the effect of CBG on immune function, the cell cycle profiles of these lymphocytes after CBG stimulation were analyzed by flow cytometry. The results indicated that the S-phase population increased for cells treated with CBG alone (Table 1). These effects were amplified significantly in cells treated with CBG combined with either LPS or Con A (Table 1).

2.2 CBG enhanced the peritoneal macrophage activation

To determine the effect of CBG on macrophage proliferation, macrophages were exposed to CBG and then subjected to a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At 0.5 and 2.5 mg/l of CBG alone or combined with LPS or Con A, a significant increase in macrophage proliferation was noted when compared with controls (Figure 3(a)). However, 0.5 mg/l of CBG with LPS did not significantly

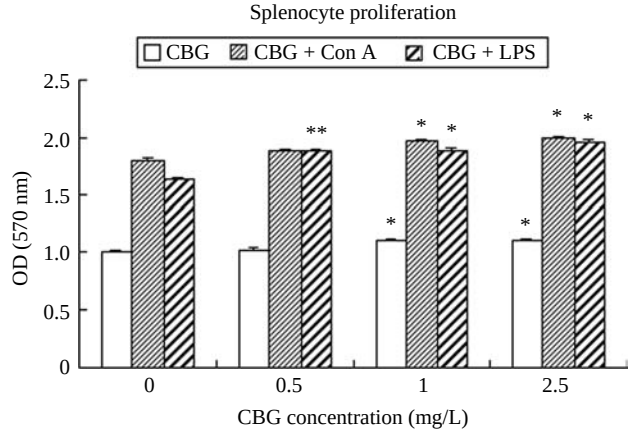

Figure 2. CBG enhanced the proliferation of splenocytes. Splenocytes were cultured with different concentrations of CBG alone and combined with either Con A or LPS for 44 h. Splenocytes proliferation was assessed by MTT assay. Each result was derived from three independent experiments and presented as the mean ± SD. Significant differences from control were indicated by $*p < 0.05$ and $**p < 0.01$.

Table 1. The effect of CBG on the cell cycle of splenocytes.

Group	Dose (mg/l)	G1 (%)	S (%)	G2/M (%)
Control	0.0	94.16 ± 0.363	4.96 ± 0.315	0.88 ± 0.264
CBG	0.5	93.82 ± 0.734	5.75 ± 0.659	0.43 ± 0.169
CBG	2.5	92.82 ± 0.906	5.70 ± 0.932	1.48 ± 1.076
CBG + Con A	0.0 + 5.0	86.44 ± 1.061**	11.10 ± 0.567**	2.47 ± 1.161
CBG + Con A	0.5 + 5.0	81.03 ± 1.684**	15.86 ± 1.060**	3.11 ± 0.809*
CBG + Con A	2.5 + 5.0	82.20 ± 1.365**	16.39 ± 1.803**	1.41 ± 0.991
CBG + LPS	0.0 + 20.0	85.22 ± 0.763**	12.54 ± 0.451**	2.24 ± 1.113
CBG + LPS	0.5 + 20.0	79.51 ± 0.633**	17.55 ± 1.428**	2.95 ± 0.881*
CBG + LPS	2.5 + 20.0	75.35 ± 1.039**	21.01 ± 0.523**	3.65 ± 0.516*

Note: Splenocytes were treated with CBG alone or in combination with LPS (20 mg/l) or Con A (5 mg/l) for 48 h. The cells were then stained with propidium iodide and the cell cycle distribution was analyzed by flow cytometry. Each result was derived from three independent experiments and presented as the mean ± SD. Significant differences from control were indicated by $*p < 0.05$ and $**p < 0.01$.

interfere with macrophage viability. Taken together, these results demonstrated that *in vitro* exposure to CBG significantly elevated the energy metabolism of macrophages (Figure 3(a)).

To determine the effect of CBG on the phagocytic function of macrophages, macrophages were exposed to CBG and subjected to the phagocytic function assay. Treatment of macrophages with 0.5 and 2.5 mg/l of CBG alone or combined with LPS or Con A led to significant increases in macrophage phagocytic function compared to the controls. Moreover, 0.5 mg/l of CBG with Con A did not significantly interfere with phagocytosis function of proliferation with peritoneal macrophages (PMΦ; Figure 3(b)).

2.3 CBG increased the proportions of $CD4^+CD8^+$ T cell populations

To investigate whether CBG has an effect on the $CD4^+$ and/or $CD8^+$ T-cell population, cells were incubated with specific antibodies against T-cell surface markers and analyzed by flow cytometry following CBG treatment. The results showed that the $CD4^+CD8^+$ double-positive T-cell populations and the percentange of $CD4^+$-$CD8^-$ + $CD4^+CD8^+$ T-cell population were significantly increased after treatment with CBG alone or in combination with Con A (Table 2).

2.4 CBG increased the levels of IFN-γ and TNF-α and decreased the levels of IL-4 and IL-10

The results have revealed that CBG affects cell proliferation and activation of PMΦ and increases the $CD4^+CD8^+$ T-cell populations; therefore, CBG might also stimulate cells to secrete cytokines that could promote cell proliferation, activate PMΦ, and increase $CD4^+CD8^+$ T-cell populations. The protein levels of several cytokines in cell-free supernatants of splenocytes were analyzed after a 48 h treatment with CBG and Con A. The results showed that CBG treatment elevated the levels of including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) after sensitization with Con A (Figure 4(a,b)). In contrast, the levels of interleukin-4 (IL-4) and interleukin-10 (IL-10) were decreased after CBG treatment (Figure 4(c,d)). Taken together, these results indicated that CBG elevated Th1/Th2 cytokine production *in vitro.*

3. Discussion

In this study, the results showed that CBG can exhibit immunomodulatory activities *in vitro*. A number of research groups reported the potentiation of certain compounds in Con A or LPS-induced proliferation of immunocyte, suggested their

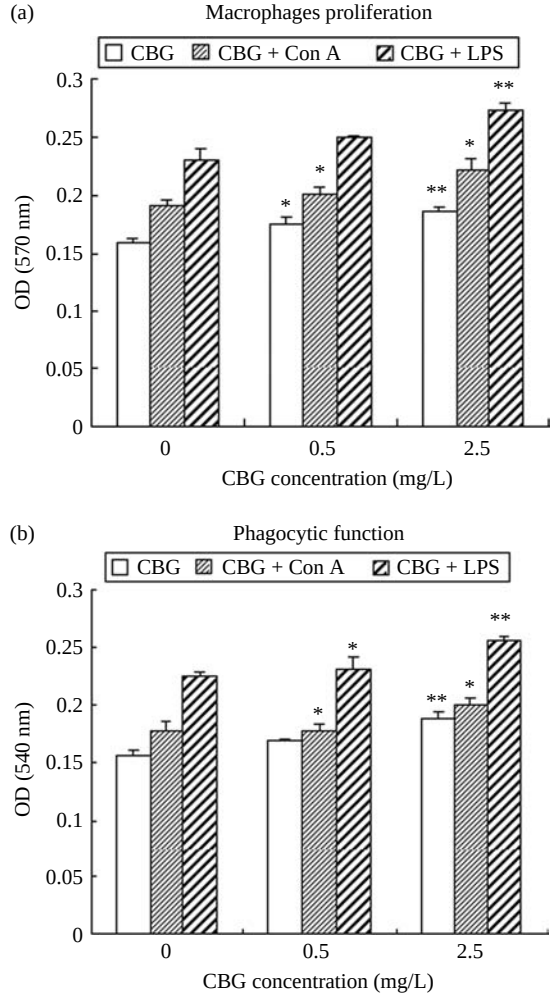

Figure 3. Effect of CBG on peritoneal macrophage proliferation and phagocytic function. PMΦ were cultured for 24 h with different concentrations of CBG (0, 0.5, or 2.5 mg/l). (a) Energy metabolism was assessed by MTT assay. (b) The neutral red measurement was prepared as described in 'Materials and methods'. Each result was derived from three independent experiments and presented as the mean ± SD. Significant differences from control were indicated by $*p < 0.05$ and $**p < 0.01$.

important effects in immune function [6]. In this study, the results indicated that CBG significantly increased splenocyte proliferation in response to Con A and LPS (Figure 2). And Con A acts directly on T-cells, whereas LPS acts on B cells. Therefore, CBG could enhance both cellular immunity and humoral immune response in cultured immune cells [7].

Macrophages play an important role in the defense mechanism against host infection and the killing of tumor cells. The modulation of antitumor properties of macrophages by various biological response modifiers is an area of active interest for cancer chemotherapy, and it is closely related to immunomodulatory activity [8]. Our study showed that the treatment with CBG alone or combined with Con A and LPS could enhance the proliferation and the phagocytosis function of macrophages significantly. Macrophages are a part of innate immune system and play a major role as the first line of

Table 2. The effect of CBG on CD4 and CD8 expression of T-lymphocytes.

Group	Dose (mg/l)	$CD4^+CD8^-$%	$CD4^-CD8^+$%	$CD4^+CD8^+$%	$CD4^+/CD8^+$	$CD4^+CD8^-\% + CD4^+CD8^+\%$
Control	0	18.62 ± 0.607	9.92 ± 0.350	1.71 ± 0.165	1.75 ± 0.056	20.33 ± 0.753
CBG	0.5	18.65 ± 0.613	7.27 ± 0.471**	3.33 ± 0.483*	2.07 ± 0.035**	21.98 ± 0.799
CBG	2.5	17.79 ± 1.148	7.76 ± 1.029	5.60 ± 1.341*	1.75 ± 0.026	23.38 ± 0.359**
CBG + Con A	0 + 5	20.61 ± 0.388*	10.71 ± 0.607	2.37 ± 0.234*	1.76 ± 0.082	22.98 ± 0.462*
CBG + Con A	0.5 + 5	15.31 ± 0.540**	5.68 ± 1.098*	12.56 ± 1.979*	1.53 ± 0.091*	27.88 ± 1.517**
CBG + Con A	2.5 + 5	14.28 ± 0.622**	4.97 ± 0.515**	14.56 ± 0.633**	1.48 ± 0.046**	28.84 ± 1.194**

Note: CBG increased the percentage of $CD4^+CD8^+$ lymphocytes in the cultures. Splenocytes were cultured with CBG alone or in combination with Con A (5 mg/l) for 48 h. The cells were then labeled with PE-anti CD4/L3T4 and FITC-anti CD8/lyt-2 antibodies and analyzed by flow cytometry. Each result was derived from three independent experiments and presented as the mean ± SD. Significant differences from control were indicated by $*p < 0.05$ and $**p < 0.01$.

defense to protect the host from both tumors and virus-infected cells. These results indicate that CBG not only enhances adaptive immunity but also activates innate immunity.

In the past few years, the number and ratio of two main lymphocyte T-subsets ($CD4^+$ cells or T-helpers, and $CD8^+$ cells or T-cytotoxic/suppressors) have been recognized as the most meaningful parameters for evaluating the balanced state of immunomodulation and homeostatic responses of the intrinsic immune system [9]. CD4 T-cells recognize antigens presented by major histocompatibility complex class II (MHC II) and mediate both cellular immune response through Th1 cells and humoral immune response through Th2 cells. CD8 recognizes antigens presented by MHC I and mediates cellular immune responses through cytotoxic T-cells. In our study, a significant up-regulation of the $CD4^+CD8^+$ double-positive cell populations after CBG treatment was found. $CD4^+CD8^+$ double-positive T-cells represent a minor subpopulation of T-lymphocytes and it has been reported that the proportion of peripheral double-positive lymphocytes increases in certain disease conditions such as bacterial and viral infections, autoimmune disorders, and tumors [9]. Our results also showed that the percentange of $CD4^+CD8^- + CD4^+CD8^+$ T-cell population were significantly increased after CBG treatment. It is reported that rat peripheral $CD4^+CD8^+$ T-lymphocytes are partially immunocompetent thymus-derived cells that undergo post-thymic maturation to become functionally mature $CD4^+$ T-lymphocytes [10]. It means that CBG can promote the activation of T-lymphocyte and implement the functions.

After activation, $CD4^+$ T-cells can be subdivided into either Th1-type cells secreting IL-2, IL-12, IFN-γ, and TNF-α or Th2- type cells secreting IL-4, IL-5, IL-10, and IL-13. Indeed, the ratio of Th1

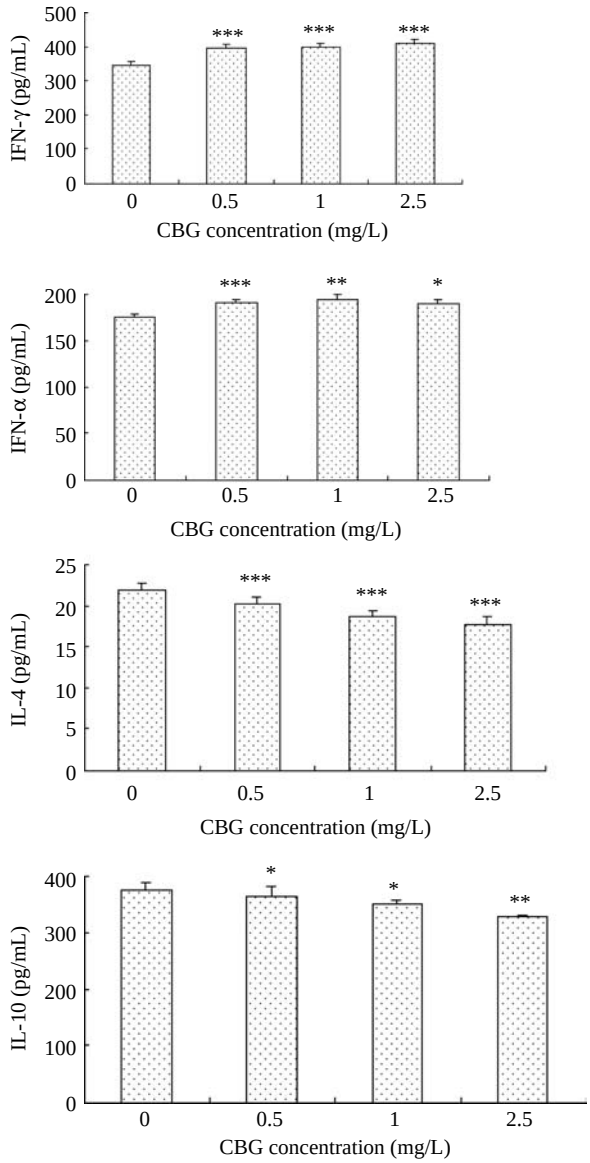

Figure 4. CBG regulated the cellular production of cytokines. The levels of cytokines IL-4, IL-10, TNF-α, and IFN-γ were analyzed after culturing the splenocytes in the presence of different concentration CBG (0, 0.5, 1, or 2.5 mg/l) and Con A (5 mg/l) for 48 h. The cytokine levels were quantified by ELISA. Each result was derived from three independent experiments and presented as the mean ± SD. Significant differences from control were indicated by $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$. (a) Level of IFN-γ, (b) level of TNF-α, (c) level of IL-4, and (d) level of IL-10 in the culture supernatants from the splenocytes.

to Th2 cytokines is critical for the orientation of the inflammatory response toward cell-mediated or humoral-mediated responses. Thus, any factor that can interfere with the Th1/Th2 axis might also affect the outcome of the body's immune state [11]. A variety of experiments have shown that excessive production of cytokines in many diseases has pathological consequences [12]. The immune balance,

controlled by Th1 and Th2 levels, is crucial for immunoregulation. An imbalance causes various immune diseases including allergic disorders and asthma, which results from excess production of Th2 cytokines relative to Th1 cytokines [13]. In this study, it showed that CBG had a significant stimulatory effect on inducing the release of Th1 cytokines (IFN-γ and TNF-α) and decreasing the release of Th2 cytokines (IL-4 and IL-10) and exerted the effect of immunological regulation by up-adjusting the Th1/Th2 arms of the immune system. These results suggested that Th1 might be one of the target cells of CBG and confirmed the general effect of CBG on the cell-mediated immune response. Th1 response supports protective immunity against intracellular infections, such as viruses, protozoa, and bacteria, and also against cancer cells [14]. These results also suggested that CBG might be useful for vaccine adjuvant [15] or for the correction of Th2-dominant pathological disorders [16].

In conclusion, this study demonstrated that CBG, isolated from Chan Su, could enhance both innate and adaptive immunities and elevate both cellular immunity and humoral immune response in cultured immune cells. These functions of CBG may be related to its ability to modulate the Th1/Th2 cytokine balance by inducing Th1 cytokines. Taken together, these results suggested that CBG may be developed as a novel immunotherapeutic agent to treat immune-mediated diseases such as cancer. However, more studies are required to reveal the exact mechanisms *in vivo*.

4. Materials and methods

4.1 Isolation and identification of CBG from Chan Su

Chan Su (640 g) was extracted three times with 95% EtOH and then concentrated to yield an EtOH extract. The extract was further suspended in H_2O (5000 ml) and extracted with $CHCl_3$ (5000 ml). Then the $CHCl_3$ extract (64.8 g) was subjected to column chromatography over silica gel with the gradient $CHCl_3$:MeOH (1:0–0:1) to afford 38 fractions, and the fractions 16–21 were again subjected to column chromatography over silica gel with $CHCl_3$:MeOH gradient elution to give a final yield of 33 mg of CBG. The purity of the compound is up to 98%. Its molecular formula is $C_{26}H_{34}O_5$ and relative molecular mass is 442, and the chemical structure is shown in Figure 1. The identity of the substance was determined by comparison with its spectral data with those reported in the literature [17]. The concentration of bacterial endotoxins (LPS) in the sample solution (10 mg/l) is 0.072 EU/ml assayed by chromogenic tachypleus amebocyte lysate Endpoint Kit (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd, Xiamen, China) with a standard curve from 0.01 to 0.1 EU/ml [18].

4.2 Proliferation measurement of splenocytes

Specific pathogen-free BALB/c mice (male, 6–8 weeks old, weighing 18–22 g) were obtained from the Experimental Animal Center of Jilin University (Changchun, China). Mice were housed in standard conditions of temperature, humidity, and light. Animal experiments were approved by Experimental Animal Center of Jilin University. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health.

Mice were sacrificed, and their spleens were aseptically removed and ground by passing through a sterile plastic strainer. After centrifuging the cells twice at 1000*g* for 5 min, erythrocytes were lysed in a hypotonic solution and the cell pellets were washed twice with RPMI-1640 medium (Invitrogen, Foster City, CA, USA). Complete RPMI-1640 medium was supplemented with 10% fetal bovine

serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were resuspended in complete RPMI-1640 and the cell concentration was adjusted to 1×10^6 cells/ml. Cell counts were performed using a hemocytometer and cell viability was determined using the trypan-blue dye exclusion technique with the results showing more than 95% of viable cells. The lymphocyte cell suspension was transferred into 96-well plates (100 μl/well) and stimulated by 0, 0.5, 1, or 2.5 mg/l CBG alone or combined with either Con A (Sigma, St. Louis, MO, USA) at 5 mg/l or LPS (Sigma) at 20 mg/l inside a humidified incubator with 5% CO_2 at 37°C for 44 h. Cell proliferation was measured using the MTT assay. Briefly, 20 μl of MTT solution (5 g/l) was added to each well after removing the medium. After a 4 h incubation, the purple formazan crystals were solubilized by adding 150 μl of dimethyl sulfoxide (DMSO). The optical density was measured at 570 nm using a microplate reader (TECAN, Grodig, Austria) [19].

4.3 Proliferation measurement and phagotrophic activity of peritoneal macrophage

Peritoneal fluid from male BALB/c mice was harvested from peritoneal cavities by infusing with 10 ml RPMI-1640. After centrifugation at 1000*g* for 5 min, the cell pellets were suspended in supplemented RPMI-1640 and seeded in 96-well plates at a cell density of 5×10^6 cells/ml. They were allowed to adhere for 3 h at 37°C in a humidified incubator with 5% CO_2. After a 3 h incubation, nonadherent cells were removed by washing twice with phosphate buffer saline (PBS). Freshly prepared medium was added. The viability of the adherent cells was assessed by the trypan blue exclusion test, and the proportion of macrophages was determined by cell morphology under a light microscopy [20]. To measure proliferation of PMΦ, the cell suspension of macrophages (1×10^6 cell/ml) was pipetted into 96-well plates (100 μl/well) and cultured in the presence of 0, 0.5, or 2.5 mg/l CBG alone or combined with either Con A or LPS at 37°C for 20 h in a humidified atmosphere containing 5% CO_2. After 20 h, 20 μl of MTT solution was added to each well followed by a 4 h incubation. The purple formazan crystals were solubilized by adding 150 μl of DMSO. The absorbance was measured at a wavelength 570 nm.

To measure phagotrophic activity, PMΦ were prepared and treated with CBG for 24 h as described earlier. And then the PMΦ were plated into 96-well flat-bottomed microplates and incubated in 5% CO_2 at 37°C for 24 h followed by centrifugation at 1800*g* for 10 min and careful removal of the medium by pipetting. One hundred microliters of neutral red (1 mg/ml, prepared in saline and filtered) were added into each well and then incubated for 30 min. The plate was washed twice with PBS and then 100 μl of cytolysate (1:1 mixture of acetic acid and dehydrated alcohol) was added to each well. The plate was then incubated for an additional 12 h at 25°C, and then the absorbance was measured at 540 nm [2].

4.4 Analysis of T-lymphocytes surface markers by flow cytometry

Splenocytes were prepared as described earlier and treated with CBG (0, 0.5, or 2.5 mg/l), or CBG plus Con A (5 mg/l). T-cell surface markers were determined by staining cells with R-Phycoerythrin (PE)-conjugated anti-CD4/L3T4 and Fluoresceine Isothiocyanate (FITC)-conjugated anti-CD8/Lyt-2 (Becton Dickinson and Company (BD), San Diego, CA, USA). Briefly, 1×10^6 cells were washed twice with wash buffer (PBS containing 0.1% NaN_3). The samples were then incubated with the conjugated antibodies for 30 min at 4°C. Each sample was resuspended in

0.5 ml of the fixing solution (PBS containing 2% formaldehyde and 0.05% NaN_3) and analyzed by a BD FACS Calibur flow cytometer (BD, San Jose, CA, USA). CellQuest software (BD) was used to identify and quantify distinct populations of cells by mean fluorescent intensity [21].

4.5 Analysis of lymphocyte cell cycle by flow cytometry

Splenocytes were prepared as described earlier and treated with CBG (0, 0.5, or 2.5 mg/l), CBG plus Con A (5 mg/l), or CBG plus LPS (20 mg/l). Subsequently, a total of 1×10^6 cells were washed twice with wash buffer and stained with propidium iodide at 4°C for 20 min. The samples were then resuspended in 0.5 ml of the fixing solution and analyzed with BD FACS Calibur flow cytometer. Data acquisition was performed with the Cell Quest software, and the percentages of nuclei at each phase of the cell cycle were calculated with the Modfit software (Verity Software House, San Jose, CA, USA) [22].

4.6 Detection of cytokines

Flat-bottomed microplates (24-well) were rinsed three times with PBS before use. A 500 μl volume of the splenocytes suspension was then transferred to each well. Different concentrations of CBG combined with Con A (5 mg/l) were added to wells. The cells were then incubated in a total volume of 1000 μl per well for 48 h. Culture supernatants were collected for measurement of cytokine levels. The concentration of each cytokine in the supernatants was determined by ELISA kit (BioLegend, Inc., (Headquarters), San Diego, CA, USA). Briefly, each well of the microplate was coated with 100 μl of the capture antibody and incubated overnight at 4°C. After washing with PBS and blocking with PBS plus 5% nonfat milk, culture supernatants and the standards were added to the wells. The plate was incubated for 2 h at room temperature and was then washed three times. Biotinylated monoclonal antibody was added to each well after washing and incubated at room temperature for 1 h. The plates were washed and further incubated with avidin-peroxidase for 30 min before detection using the TMB solution (Biolegend, San Diego, CA, USA). Absorbance was measured at 450 nm. The amounts of cytokines were calculated from the linear portion of the standard curve [19].

4.7 Statistical analysis

All data were analyzed by the SPSS 12.0 statistical software. Data were expressed as the mean ± SD. Statistical differences were examined using single-factor analysis of variance and Dunnett's *t* comparison. Statistical significance was accepted when $p < 0.05$.

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