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Research article

Immune-boosting effect of *Yookgong-dan* against cyclophosphamide-induced immunosuppression in mice

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ABSTRACT

Immune responses must be strictly regulated to prevent autoimmune and infectious diseases and to protect against infectious agents. As people age, their immunity wanes, leading to a decrease in lymphocyte production in bone marrow and thymus and a decline in the efficacy of mature lymphocytes in secondary lymphoid organs. This study explores the immune-boosting potential of *Yookgong-dan* (YGD) in enhancing the immune system by activating immune cells. In our *in vitro* experiments, cyclophosphamide (Cy) treatment led to a significant decrease in primary splenocyte viability. However, subsequent treatment with YGD significantly improved cell viability, with doses ranging between 1 and 25 μg/mL in Cy-treated splenocytes. Flow cytometry analysis demonstrated that the Cy group exhibited reduced positivity of $CD3^+$ T cells and $CD45^+$ leukocytes compared to the blank group. In contrast, treatment with YGD led to a notable, dose-responsive increase in these immune cell types. In our *in vivo* experiments, YGD was orally administered to Cy-induced immunosuppressed mice at 20 and 100 mg/kg doses for 10 days. The results indicated a dose-dependent elevation in immunoglobulin (Ig)G and IgM levels in the serum, emphasizing the immunostimulatory effect of YGD. Furthermore, the Cy-treated group showed decreased T cells, B ($CD19^+$) cells, and leukocytes in the total splenocyte population. Yet, YGD treatment resulted in a dose-dependent reversal of this pattern, suggesting its ability to counter immunosuppression. Notably, YGD was found to effectively stimulate T ($CD4^+$ and $CD8^+$) lymphocyte subsets and natural killer cells, along with enhancing Th1/Th2 cytokines in immunosuppressed conditions. These outcomes correlated with the modulation of BCL-2 and BAX expression, which are critical for apoptosis. In conclusion, YGD has the potential to bolster immune functionality through the activation of immune cells, thereby enhancing the immune system's capacity to combat diseases and improve overall health and wellness.

Abbreviations: YGD, Yookgong-dan; Cy, cyclophosphamide; GJD, Gongjin-dan; YMJ, Yukmijihwan; PBS, phosphate-buffered saline; RBC, red blood cell; CCK-8, Cell Counting Kit-8; H&E, hematoxylin and eosin; ELISA, Enzyme-linked immunosorbent assay; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; Ig, immunoglobulin; Th, T-helper; NK, natural killer; DC, dendritic cell.

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1. Introduction

The immune system has a protective function, and aging leads to increased susceptibility to infection, poor antibody response, increased disease prevalence, and exacerbation of the pro-inflammatory state, such as autoimmune/inflammatory disease [1,2]. Therefore, maintaining immune homeostasis is of immense importance for preventing and recovering from immune-mediated diseases [3]. Cyclophosphamide (Cy), an alkylation inducer commonly used in clinical practice as an immunosuppressant and anticancer drug, is used in chemoimmunotherapy for patients with rheumatoid arthritis, bone marrow transplants, and hematologic malignancies, such as multiple myeloma, lymphoma, and leukemia [4,5]. However, Cy treatment causes side effects with respect to physiological functions, including weight loss, liver toxicity, hemorrhagic cystitis, amenorrhea, myelosuppression, hair loss, nausea, and vomiting [6–9].

Natural supplements are physiologically active substances that can reportedly alleviate changes in the body's immune environment caused by drugs used for anticancer treatment [10]. Previously, probiotic supplements, natural foods, or herbal plants have been used to facilitate healthy immune homeostasis and promote immune recovery after administration in immunodeficient models [11–13]. However, additional analysis is required, and functional studies on medicinal herbs that can strengthen the immune system to alleviate stress caused by viruses are being conducted. Reportedly, bioactive food components from plant sources have lower toxicity and fewer harmful side effects on humans than those of chemicals and synthetic drugs [14,15].

Yookgong-dan (YGD) is a herbal supplement produced from *Gongjin-dan* (GJD) and *Yukmijihwan* (YMJ) to increase energy, vigor, and stamina and alleviate stress, fading memory, insomnia, and oversensitivity. GJD, a well-established anti-fatigue and anti-aging multi-herbal drug used in Korea and China for centuries, has demonstrated minimal side effects [16,17]. Scientific validation supports its diverse pharmacological activities, including antioxidant, anti-inflammatory, hepatoprotective, neuroprotective, immune-boosting, and reproductive recovery properties [18,19].

YMJ has been an integral component of traditional herbal medicine in Korea, China, and Japan. The efficacy is derived from a synergistic blend of six herbal plants: *Rehmanniae radix* preparata, *Dioscoreae* rhizoma, *Corni* fructus, *Poria* sclerotium, *Moutan cortex* radicis, and *Alismatis* rhizome [20]. Scientific evaluations have highlighted its efficacy in renal ischemia/reperfusion, enhancing memory, stimulating spermatogenesis, curbing bone loss, alleviating asthma, and managing diabetes [21,22]. Traditionally, YMJ has been devoted to treating conditions such as renal disorders, diabetes mellitus, neurosis, and osteoporosis while activating immune function and promoting recovery from weakness [22]. For centuries, Korea, China, and Japan have utilized used GJD and YMJ as herbal medicines for their ability to boost immunity and vitality [17,22].

Despite the long-standing traditional use of GJD and YMJ, emphasizing their potential, the specific synergistic effects of the combined YGD formulation await conclusive substantiation through rigorous scientific research.

In this study, we administered YGD to Cy-induced immunodeficient mice, hypothesizing that it possesses immune-stimulating properties capable of strengthening the immune system following immunodeficiency.

2. Materials and methods

2.1. Preparation of YGD

YGD was prepared from 10 medicinal herbs: *Rehmannia glutinosa* (Gaertn.) Steud. (0.260 g/1 g), *Cervi pantotrichum* (0.065 g), *Angelicae* radix (0.130 g), *Dioscorea polystachya* Turcz. (0.130 g), *Cornus officinalis* Siebold & Zucc. (0.130 g), *Wolfiporia extensa* (Peck) Ginns (0.065 g), *Alisma canaliculatum* Braun & Bouché (0.065 g), *Moutan cortex radices* (0.065 g), musk, and *Aquilaria agallocha* Roxburgh (0.026 g). The mixture underwent a 24-h drying process at 70 °C within a dryer, and subsequently, it was comminuted using a grinder. The resulting YGD powder was then meticulously preserved at −20 °C until requisitioned for the experiment. Just prior to the experiment commencement, phosphate buffered saline (PBS, Gibco BRL) was used to dissolve the YGD powder, resulting in a concentrated solution with a 10 mg/mL. Thorough filter sterilization was performed with this solution using a 0.45 μm syringe filter from Advancec Co. Tokyo, Japan.

2.2. Mouse primary splenocytes

Primary splenocytes from mice were obtained through humane euthanasia using CO_2 asphyxiation. The spleen was carefully dissected from the abdominal cavity and put on a nylon cell strainer (40 μm, Falcon, NY, USA) positioned over a 6-well plate filled with PBS. Gentle pressure was applied to the isolated spleen tissue using a 5 mL syringe plug in a circular motion. After rinsing the cell strainer with PBS, the resulting cell suspension was collected in a 50 mL tube placed on ice. Red blood cell (RBC) lysis buffer (Invitrogen, Carlsbad, CA, USA) was added to the cells for 15 min at room temperature to remove RBCs, followed by centrifugation at 1500 rpm for 3 min at room temperature. The purified splenocytes were then deposited onto 6-well plates previously coated with CD3 (Bioxcell, New Hampshire, USA) at a 2 μg/mL concentration (overnight at 4 °C). The cells were stabilized in RPMI medium that was enhanced with 1 % penicillin/streptomycin (P/S), 10 % fetal bovine serum (FBS), and 2 μg/mL CD28. This culture was maintained for 6 h at a cell density of 1×10^7 cells/2 mL.

2.3. Cell Counting Kit-8 (CCK-8) assays

To evaluate the viability of splenocytes, the Cell Counting Kit-8 assay from Dojindo, Kumamoto, Japan was utilized. The process

began with the seeding of cells in a 96-well plate, using a concentration of 2×10^4 cells per 100 μL. These cells were then pre-treated with YGD at five concentrations (1, 10, 25, 50, and 100 μg/mL), under both Cy-exposed and non-exposed conditions. After incubating for 24 h, 10 % of the total volume was supplemented with the CCK-8 solution and the mixture was incubated for 4 h at 37 °C. The absorbance at 450 nm was then measured using an Epoch microplate reader from BioteK, Winooski, VT, USA. A comprehensive timeline detailing the steps involved in determining the viability of splenocytes is presented in Scheme 1.

2.4. Animals and experimental groups

Six-week-old male C57BL/6 mice were used in accordance with the Use Committee guidelines (JSR-2022-03-003-A) and Jaseng Animal Care. All mice were housed under standard conditions, including standard chow diet, access to water, and a controlled environment. The mice were randomly allocated into six groups, each consisting of 20 individuals, as follows: (1) Normal group (Normal), (2) YGD administered at 20 mg/kg (YGD20), (3) YGD administered at 100 mg/kg (YGD100), (4) Cyclophosphamide (Cy), (5) Cyclophosphamide co-administered with YGD at 20 mg/kg (Cy + YGD20), and (6) Cyclophosphamide co-administered with YGD at 100 mg/kg (Cy + YGD100). Scheme 2 provides detailed information regarding the route, concentration, and frequency of both cyclophosphamide and YGD administration.

2.5. Body weights, food intake, and immune organ index

The body weight of each mouse was documented every 2 days for 10 days, and alterations in food intake were evaluated by measuring the remaining feed provided to all experimental animals. On day 10, tissue weight was determined by computing the immune organ index, as defined: thymus index = thymus weight (mg)/body weight (g); spleen index = spleen weight (mg)/body weight (g) [12].

2.6. Histology

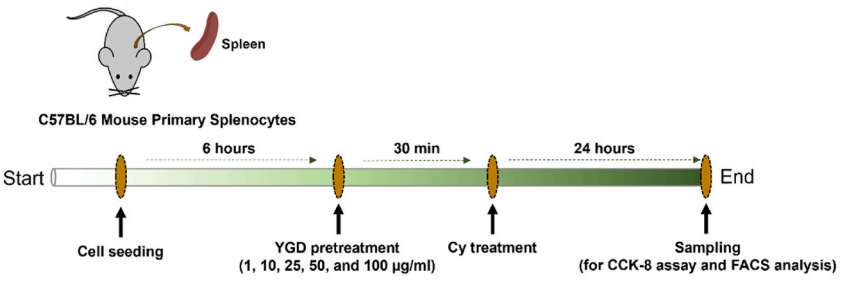
Following perfusion through the coronary artery for hematoxylin and eosin (H&E) staining, the thymus and spleen were meticulously isolated and fixed in 4 % paraformaldehyde at 4 °C for an overnight period. Thereafter, the tissues underwent dehydration through an ethanol gradient, were embedded in paraffin blocks, and sectioned in the coronal plane to generate 10 μm thick sections. H&E staining, following established protocols, was performed to evaluate the degree of immune cell infiltration on the 10th day post-administration. Photomicrographs were taken using an inverted microscope (Nikon, Tokyo, Japan).

2.7. Flow cytometry

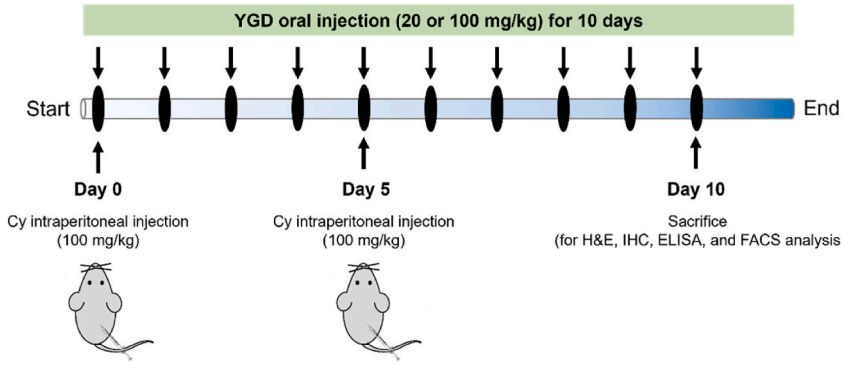
Flow cytometric assays were employed to evaluate various immune populations in splenic cells from mice *in vitro* and *in vivo*. In brief, splenic cells were prepared at a concentration of 1×10^7 cells and incubated with specific antibodies, including anti-$CD3^+$ (FITC), anti-$CD19^+$ (APC), anti-$CD45^+$ (PerCP), anti-$CD4^+$ (PerCP), anti-$CD8^+$ (PE), anti-natural killer (NK)1.1^+ (FITC), and anti-$CD11c^+$ (APC), for 30 min at 4 °C in the absence of light. Following this, the cells were fixed using cell fixation buffer (BD Biosciences, Franklin Lakes, NJ, USA; Abcam, Cambridge, UK) for 10 min. Subsequently, 1 mL of PBS was introduced for fluorescence-activated cell sorting analysis, which was performed using an Accuri C6 plus flow cytometer (BD Biosciences). The percentages of positive cells, as determined by flow cytometry, are reported relative to the Cy group.

2.8. Immunoglobulins, serum, and blood analysis

Immunoglobulins (Ig) and cytokines were analyzed at serum levels in mice blood using ELISA. Retro-orbital blood collection was performed by piercing the retro-orbital sinuses of mice with a sterile hematocrit capillary tube. The whole blood was incubated at room temperature for a duration of 30 min and then subjected to centrifugation at 3000 rpm for 5 min. Subsequently, the serum concentrations of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-10, immunoglobulin (Ig)M, and IgG were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits (IL-10: BD Bioscience, IFN-γ, TNF-α, IL-4, IgM, and IgG: Invitrogen,



Scheme 1. Schematic of *in vitro* experimental procedures.



Scheme 2. Schematic of *in vivo* experimental procedures.

Grand Island, NY, USA) in accordance with the manufacturer's instructions. The complete blood count (CBC) including hemoglobin (HGB), hematocrit (HCT), reticulocyte (Reti), white blood cell (WBC), lymphocyte (LYM), and eosinophil (EOS) was assessed on the hematology using blood analyzer XN-10 ® (Sysmex, Kobe, Japan).

2.9. Immunohistochemistry

Immunohistochemistry procedures were conducted on thymus and spleen tissues utilizing the following methods: Tissues were

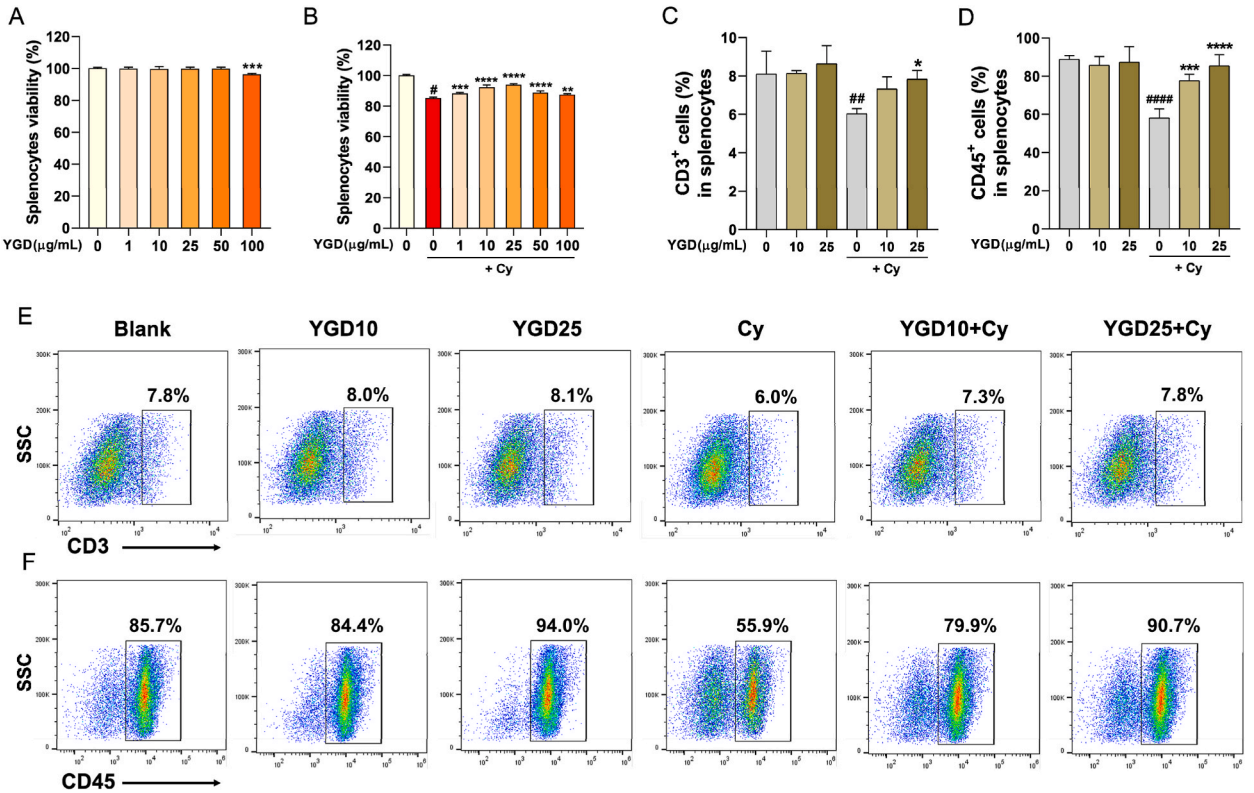
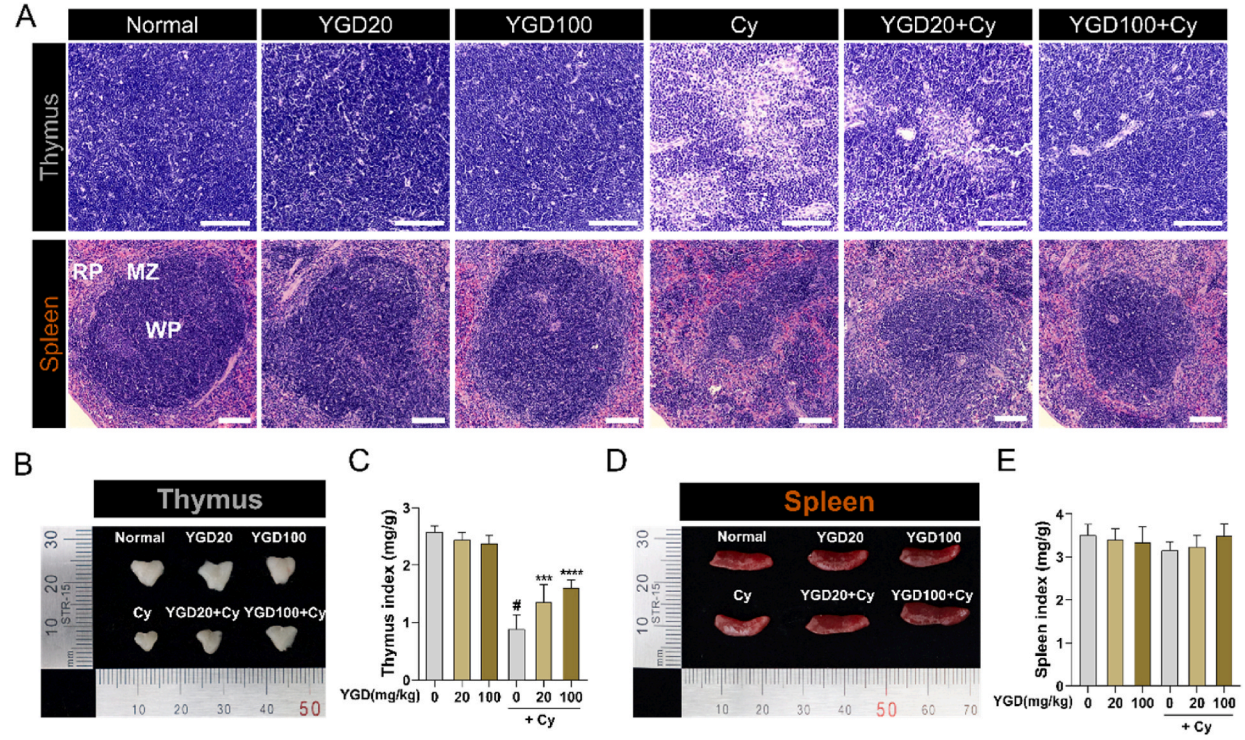
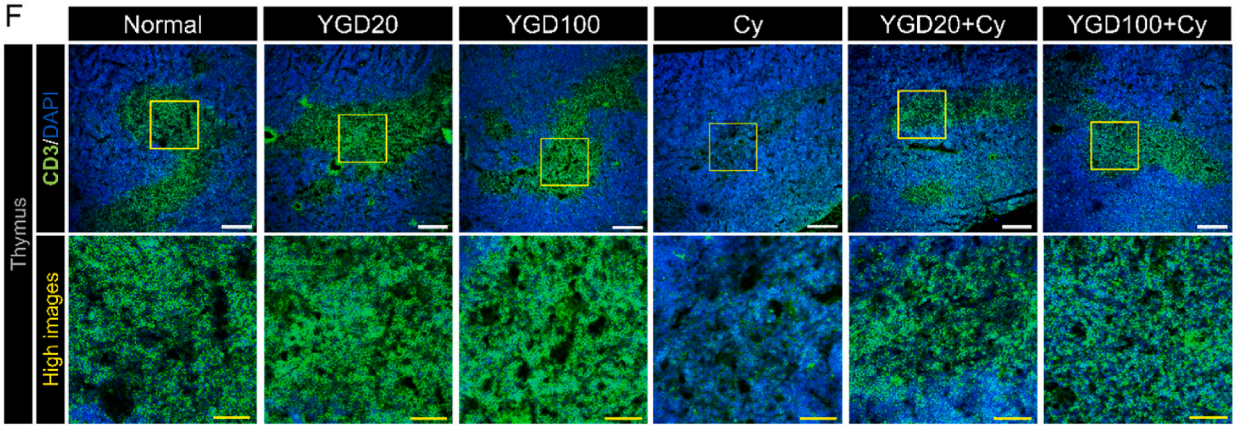


Fig. 1. Protective effect of *Yookgong-dan* (YGD) pre-treatment on cyclophosphamide (Cy)-treated primary splenocytes. (A) Cell Counting Kit (CCK)-8 assay results for YGD-only toxicity in primary splenocytes (n = 4) administered a dose range of 1–100 μg/mL for a 24-h duration. (B) CCK-8 assay for YGD pre-treatment on Cy-treated primary splenocytes (n = 4) within the same dose range. (C, D) Quantitative flow cytometric data for the positive populations of $CD3^+$ T cells and $CD45^+$ leukocytes, highlighting distinctions among the blank, YGD, Cy, and YGD + Cy groups (n = 4). (E, F) Representative flow cytometric plots visually present the distribution of $CD3^+$ T cells and $CD45^+$ leukocyte within each group. All data are expressed as the mean ± SEM. Statistical significance is denoted as $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#\#}p < 0.0001$ vs. the blank group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. the Cy group. The results were analyzed using a one-way analysis of variance (ANOVA) with Tukey's post hoc analysis.





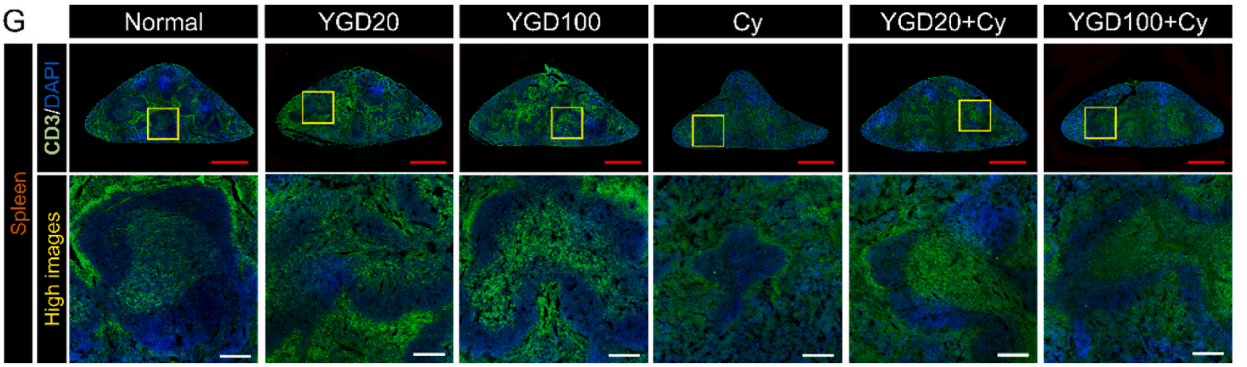


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Fig. 2. Effect of *Yookgong-dan* (YGD) on T cell infiltration while preventing damage in mice with cyclophosphamide (Cy)-induced immunosuppression. (A) Representative H&E images for the thymus and spleen in normal, YGD20, YGD100, Cy, YGD20+Cy, and YGD100+Cy groups. Scale bar = 100 μm. (B, C) Image and graph showing the thymus size and index for each group (n = 6). (D, E) Image and graph showing the spleen size and index for each group (n = 6). (F, G) Representative immunohistological images of $CD3^+$ T cells (green) and DAPI (blue) in the thymus and spleen tissues of the normal, YGD20, YGD100, Cy, YGD20+Cy, and YGD100+Cy groups. Red scale bar = 1000 μm, white scale bar = 200 μm, and yellow scale bar = 100 μm. All data are expressed as the mean ± SEM. Statistical significance is denoted as $^{\#}p < 0.05$ vs. the normal group; $^{***}p < 0.001$ and $^{****}p < 0.0001$ vs. the Cy group analyzed via one-way analysis of variance (ANOVA) with Tukey's post hoc analysis.

rendered permeable by exposure to 0.2 % Triton X-100 in PBS for a duration of 5 min, followed by rinsing with PBS and subsequent blocking with 10 % normal goat serum in PBS for 1 h. Subsequently, primary antibodies including anti-$CD3^+$ (FITC, BD Biosciences, Franklin Lakes, CA, USA), anti-Bax (Santa Cruz, CA, USA), and anti-Bcl2 (Santa Cruz, CA, USA) were incubated overnight at 4 °C. The sections underwent three 5-min washes with PBS, and then, the secondary antibody, FITC-conjugated goat anti-mouse IgG, was applied for 2 h at room temperature. The cell nuclei were stained with 4,6-diamidino-2-phenylindole (1:1000, TCI, Tokyo, Japan). Finally, the stained sections were visualized at 100 × magnification using a confocal microscope (Eclipse C2 Plus, Nikon).

3. Statistics

The standard error of the mean (SEM) was computed for the experimental findings. Statistical analyses were carried out using either one-way or two-way analysis of variance (ANOVA) along with Tukey's post hoc analysis, utilizing GraphPad Prism 8.0, Inc. (La Jolla, CA, USA). Significance levels were designated as follows: For comparisons to the normal group, significance levels were denoted as $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$, while for comparisons to the Cy group, significance levels were represented as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

4. Results

4.1. YGD exerted a protective effect on Cy-treated splenocytes via T ($CD3^+$) cell activation

To assess YGD's impact on cell viability in primary splenocytes obtained from spleen tissues, we conducted a CCK-8 assay to examine YGD-only toxicity. The results indicated no notable variance in relative cell viability up to a dosage of 50 μg/mL, with a significant decrease observed only at 100 μg/mL (Fig. 1A). We further investigated cell viability within the same dose range after YGD pre-treatment under Cy treatment conditions. The relative percentage of viable cells was significantly decreased after Cy treatment but was significantly increased and maintained at ~90 % in the YGD groups (Fig. 1B). Additionally, we conducted flow cytometry analysis to examine T cell and leukocyte populations in YGD- and Cy-treated splenocytes. The relative percentage of T cells in the blank and YGD groups exhibited no noteworthy difference. However, a significant contrast was observed in the Cy group compared to the blank group. In contrast, YGD at a concentration of 25 μg/mL significantly augmented the T cell population in Cy-treated splenocytes (Fig. 1C–E). In addition, the results for the leukocytes displayed no substantial differences in relative percentages between the blank and YGD groups in the absence of Cy treatment. However, these percentages underwent significant reduction after Cy treatment, followed by a noteworthy concentration-dependent increase following YGD pretreatment (Fig. 1D–F).

YGD mainly recruited $CD3^+$ T cells to the splenic white pulp and thymus of mice with Cy-induced immunosuppression.

To ascertain the safety of administering YGD, initial evaluations focused on body weight and food consumption, key indicators of general health. Compared to the Normal group, the Cy group showed a notable decline in body weight starting from the second day. In contrast, the YGD groups maintained consistent weight gain without significant losses. Over a two-day period, average food consumption, at about 35 g, did not show significant variations among the groups (Fig. S1). The study further investigated the degree of histological harm to the thymus and spleen in mice with suppressed immune systems 10 days following YGD treatment, and whether YGD could counteract the depletion of immune cells caused by Cy. This was done by conducting H&E staining on tissue sections of the thymus and spleen from each group (Fig. 2A). Post-Cy treatment, there was a noticeable reduction in the area of hematoxylin-stained nucleated cells. Conversely, YGD treatment led to an increase in these cells, which depended on the dose administered. In the spleen tissues of the normal group, a distinct arrangement of white medulla around the central vein and a clear delineation between white and adjacent red pulp were evident. On the other hand, the Cy group showed blurred boundaries of the marginal zone, significant shrinking of the white medulla, densely packed cells in the red pulp, and disorganized structure. These histological damages were significantly lessened in the YGD groups in a dose-responsive manner. Additionally, when assessing the size and index of the thymus in mice treated with YGD and immunosuppressed, it was observed that Cy treatment led to a substantial decrease in both aspects compared to the normal group. However, in the YGD group, both the size and index of the thymus increased significantly and in a dose-dependent manner relative to the Cy group (Fig. 2B and C). It is noteworthy that the spleen size and index showed no significant differences among the groups (Fig. 2D and E). $CD3^+$ T cell expression in the thymus and spleen was analyzed via immunohistochemistry. The thymus tissue of the normal group exhibited a high density of $CD3^+$ T cells, which experienced a decline in the Cy group but showed a subsequent increase following YGD administration (Fig. 2F). In the spleen's white pulp of the normal group, numerous $CD3^+$ T cells were evident, while their presence was notably scarce in the Cy-administered group. Conversely, concentrated T cells were observed in the splenic white pulp of the YGD groups (Fig. 2G).

4.2. YGD promotes survival of splenocytes and thymocytes through regulation of Bax and Bcl-2 expression in immunosuppressed mice

To explain the pharmacological action of YGD and how it protects against immune cell depletion, we used immunohistochemical staining to examine Bcl-2-associated X protein (Bax) expression levels. Bax is known to induce apoptosis by facilitating early release of cytochrome *c* and activating caspase 3 in thymic and spleen tissues, exhibited increased expression in the Cy group. Contrastingly, the YGD groups displayed a decrease in Bax expression, with a noteworthy dose-dependent reduction in mean Bax intensity compared to the Cy group (Fig. 3A–C).

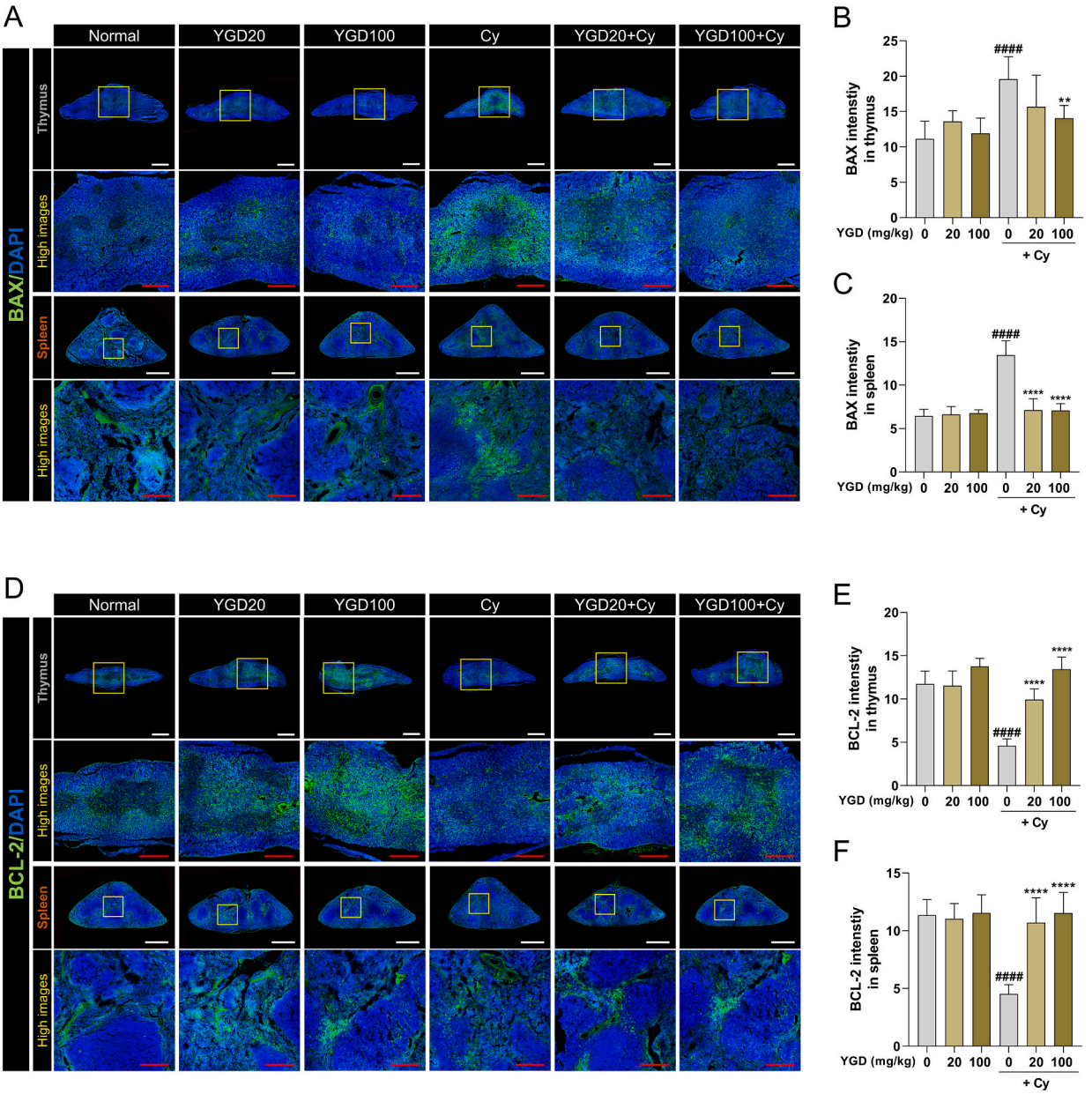

Fig. 3. Immune-protective effect of *Yookgong-dan* (YGD) against immune cell depletion through regulation of Bax and Bcl-2 expression in thymus and spleen tissues. (A) Representative immunohistological images of Bax (green) and DAPI (blue) in the thymus and spleen tissues of the normal, YGD20, YGD100, Cy, YGD20+Cy, and YGD100+Cy groups. Red scale bar = 1000 μm, white scale bar = 200 μm, and yellow scale bar = 100 μm. (B, C) Relative intensity of Bax expression in the thymic and spleen tissues of each group. (D) Representative immunohistological images of Bcl-2 (green) and DAPI (blue) in the thymic and spleen tissues of the Normal, YGD20, YGD100, Cy, YGD20+Cy, and YGD100+Cy groups. Red scale bar = 1000 μm, white scale bar = 200 μm, and yellow scale bar = 100 μm. (E, F) Relative intensity of Bcl-2 expression in the thymic and spleen tissues of each group. All data are expressed as the mean ± SEM. Statistical significance is denoted as $^{\#\#\#\#}p < 0.0001$ vs. the normal group; $^{**}p < 0.01$ and $^{****}p < 0.0001$ vs. the Cy group analyzed via one-way analysis of variance (ANOVA) with Tukey's post hoc analysis.

Furthermore, we confirmed low expression of B-cell lymphoma-2 (Bcl-2), a key anti-apoptotic protein, in the thymic and spleen tissues of the Cy group. However, this expression increased after YGD administration (Fig. 3D). The relative intensity of Bcl-2 in thymic and spleen tissues exhibited a substantial decrease in the Cy group when compared to the Normal group, and a dose-dependent increase was evident after YGD administration in comparison to the Cy group (Fig. 3E and F). These findings collectively highlight YGD's robust protective activity, indicating its capacity to inhibit cell death by modulating the expression of Bax and Bcl-2 in Cy-induced immunosuppressed mice.

4.3. YGD has been implicated in increased production of Th1 and Th2-type cytokines in immunosuppressed mice

We found that Th1-produced IFN-γ and TNF-α were significantly decreased and increased after Cy administration and when immunosuppressed mice were administered YGD, respectively (Fig. 4A and B). Furthermore, Th2-produced IL-4 and IL-10 were found to be considerably lower in the Cy group compared to the YGD group. Notably, these cytokines showed a significant rise in levels with YGD treatment (Fig. 4C and D). The ratio of Th1/Th2 levels in the YGD-treated group demonstrated a dose-dependent escalation, with a distinct change in cytokine levels (TNF-α, IFN-γ, IL-4, and IL-10) particularly evident in the group receiving 100 mg/kg of YGD. We also examined the impact of YGD on serum IgG and IgM concentrations. In the Cy group, the production of both IgG and IgM in the serum was significantly reduced, but showed a notable increase in a dose-dependent manner with YGD treatment (Fig. 4E and F). These observations suggest that the administration of YGD in mice with Cy-induced immunosuppression enhances the production of Th1 and Th2-type cytokines, underscoring its potential as an immunoenhancing agent.

4.4. YGD effectively stimulated splenogenic immune cells in immunosuppressed mice

We next performed a complete blood cell count (CBC) test using a blood analyzer to assess the impact of YGD on blood components in a Cy-induced immunosuppressed mice. Post-Cy treatment, the levels of hemoglobin, hematocrit, and reticulocytes in the blood were

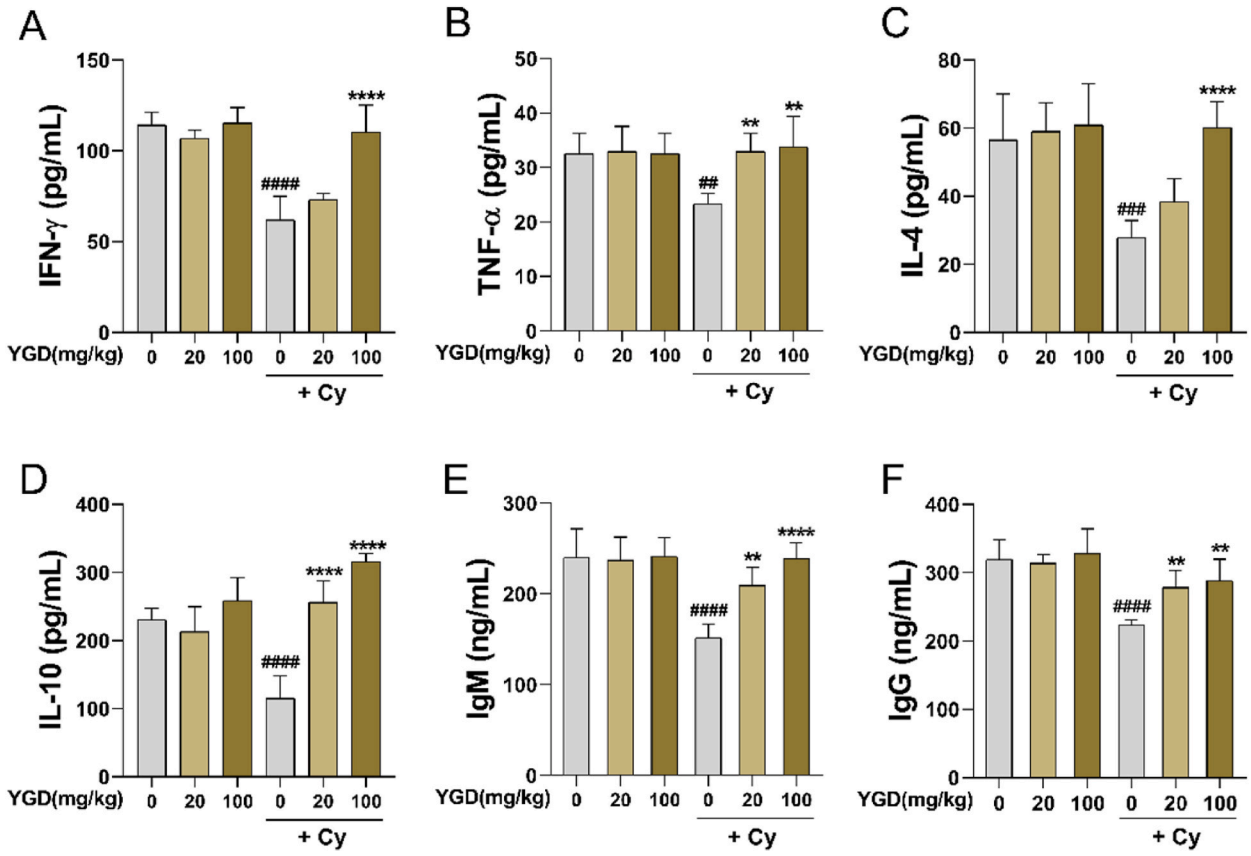

Fig. 4. Potential role of *Yookgong-dan* (YGD) in strengthening the immune system via T-helper (Th)1/Th2 cytokines production at serum levels in mice blood. (A, B) Serum levels of interferon (IFN)-γ and tumor necrosis factor (TNF)-α produced by Th1 cells were measured by enzyme-linked immunosorbent assay (ELISA) in each group (n = 6). (C, D) Serum levels of interleukin (IL)-4 and IL-10 produced by Th2 cells were measured by ELISA in each group (n = 6). (E, F) Serum levels of immunoglobulin (Ig)G and IgM were measured by ELISA in each group (n = 6). All data are expressed as the mean ± SEM. Statistical significance is denoted as $\#\#p < 0.01$, $\#\#\#p < 0.001$, and $\#\#\#\#p < 0.0001$ vs. the normal group; $^{**}p < 0.01$ and $^{****}p < 0.0001$ vs. the Cy group analyzed via one-way analysis of variance (ANOVA) with Tukey's post hoc analysis.

significantly lower compared to those in the normal group. However, in groups treated with both YGD and Cy, there was a notable increase in these levels (Figs. S3A–C). Moreover, the administration of Cy led to decreased levels of white blood cells, lymphocytes, and eosinophils, while YGD administration caused a significant, concentration-dependent increase in these cells. This suggests YGD's capability to restore hematopoietic function that was impaired by Cy (Figs. S3D–F). Specifically, we employed a flow cytometer to analyze T cells, B cells, and leukocytes in spleen-derived splenocytes to assess changes in immune cell populations following YGD and Cy administration (Fig. 5A and B). The percentages of T cells and B cells in the YGD-only group did not show significant differences. Conversely, splenocytes from mice treated with Cy exhibited significantly lower percentages of T and B cells compared to those from untreated, normal mice. In contrast, YGD treatment led to significantly higher percentages of T and B cells than those in the Cy group. Notably, a dose-dependent increase in the proportion of CD3 cells was observed following YGD administration in the immunosuppressed mice (Fig. 5C and D). Furthermore, a similar pattern was seen in the average percentage of leukocytes, which were considerably reduced in the Cy group but showed a significant, dose-dependent increase in the YGD-treated group (Fig. 5E).

4.5. YGD activated major subsets of $CD4^+$, $CD8^+$, NK1.1, and $CD11c^+$ cells in immunosuppressed mice

We assessed the relative percentages of $CD4^+$ and $CD8^+$ T cells between the groups using flow cytometry (Fig. 6A and B). The percentage of $CD4^+$ and $CD8^+$ cells after Cy administration significantly decreased. YGD at the dose of 100 mg/kg was found to significantly increase the percentage of CD4 cells (Fig. 6E). We further quantified the relative percentage of $CD8^+$ cells associated with YGD administration in Cy-induced immunosuppressed mice. The relative percentage of $CD8^+$ cells was significantly higher in the YGD groups compared with that in the Cy group and was dose-dependent (Fig. 6F). Furthermore, both dendritic cells (DCs) and natural killer (NK) cells are part of the innate immune system and play important roles in the regulation of adaptive immune responses [23,

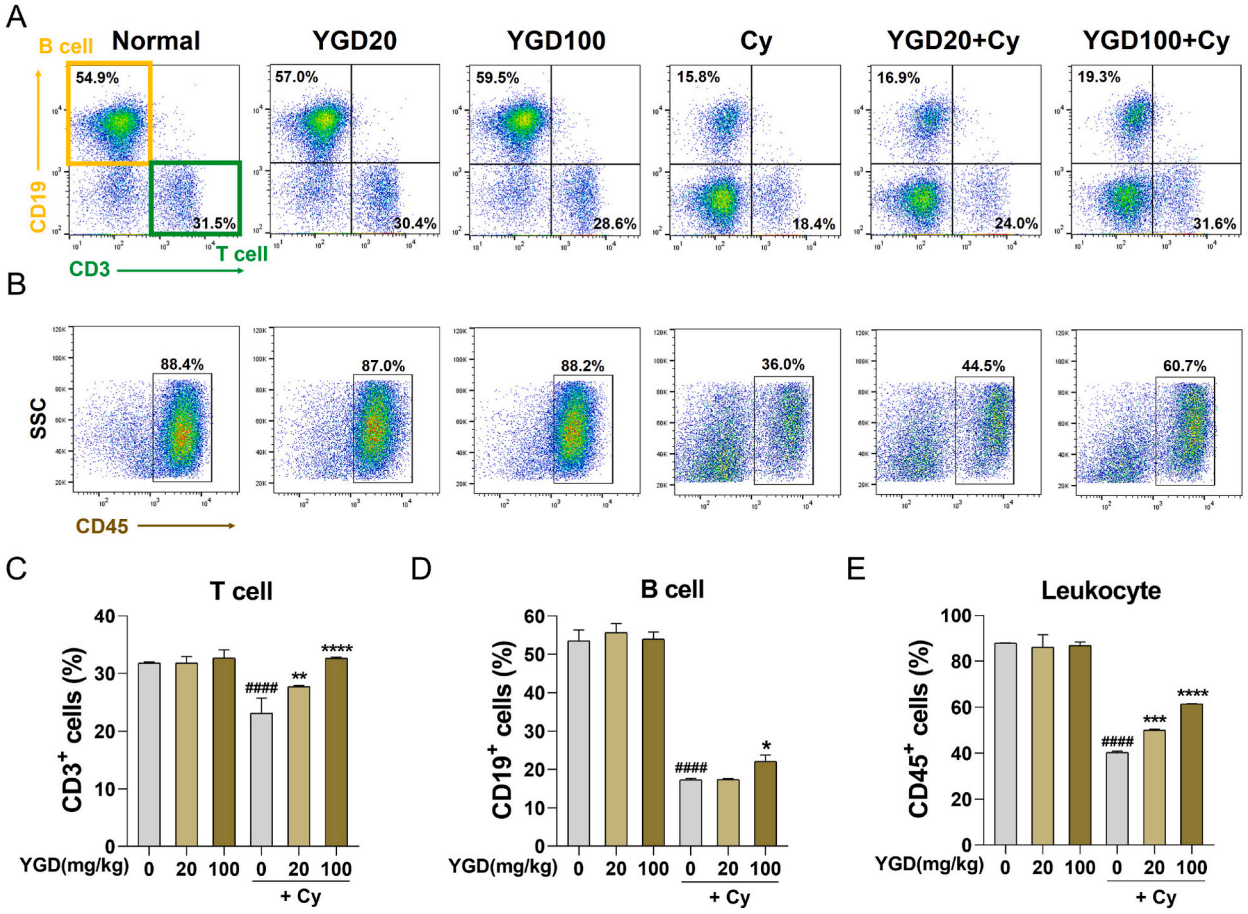

Fig. 5. Immune-boosting effect of *Yookgong-dan* (YGD) on T cells, B cells, and leukocytes in immunosuppressed mice. (A) Representative flow cytometric plot showing T cells and B cells in the normal, YGD20, YGD100, Cy, YGD20+Cy, and YGD100+Cy groups. (B) Representative flow cytometric plots showing leukocytes in each group. (C–E) Quantitative analysis via flow cytometry for the relative percentage of (C) T cells, (D) B cells, and (E) leukocytes in each group (n = 4). All data are expressed as the mean ± SEM. Statistical significance is denoted as ####$p < 0.0001$ vs. the normal group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs. the Cy group analyzed via one-way analysis of variance (ANOVA) with Tukey's post hoc analysis.

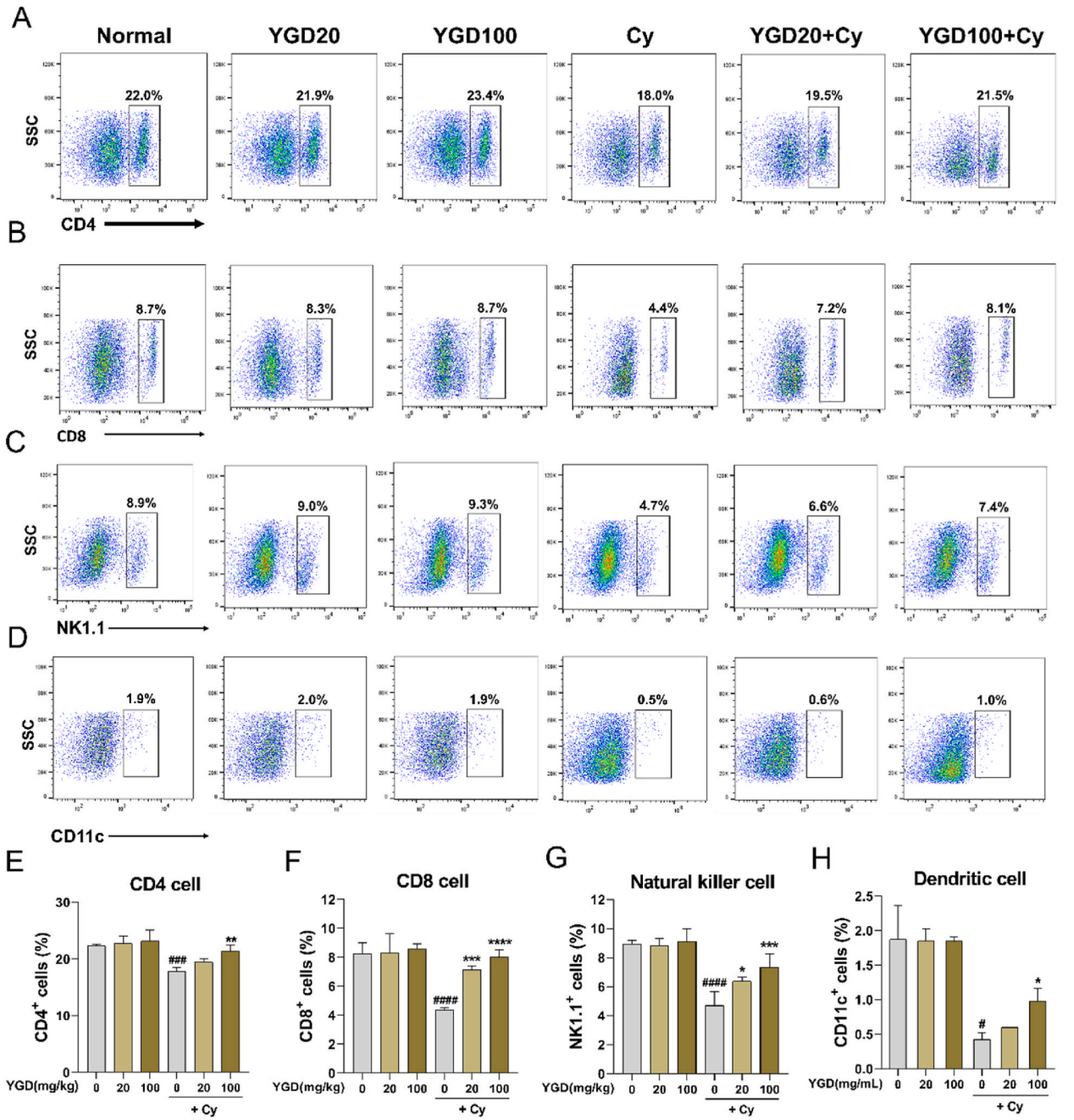

Fig. 6. Immune-boosting effect of *Yookgong-dan* (YGD) on T lymphocytes, natural killer (NK) cells, and dendritic cells (DCs). (A, B) Representative flow cytometry plot showing $CD4^+$ and $CD8^+$ T lymphocytes in the normal, YGD20, YGD100, Cy, YGD20+Cy, and YGD100+Cy groups. (C) Representative flow cytometry plots showing NK1.1 cells in each group. (D) Representative flow cytometry plots showing DCs in each group. (E–H) Quantitative analysis by flow cytometry for the relative percentage for $CD4^+$, $CD8^+$ T lymphocytes, $NK1.1^+$ cells, and $CD11c^+$ DCs in each group (n = 4). All data are expressed as the mean ± SEM. Statistical significance is denoted as $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. the normal group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. the Cy group analyzed via one-way analysis of variance (ANOVA) with Tukey's post hoc analysis.

24]. The relative percentages of $NK1.1^+$ and $CD11c^+$ cells were also examined between the groups using flow cytometry (Fig. 6C and D). We also confirmed that the relative percentage of $NK1.1^+$ and $CD11c^+$ cells was significantly decreased after Cy administration. In contrast, YGD at doses of 20 and 100 mg/kg significantly increased the percentage of NK cells, and only 100 mg/kg of YGD significantly increased the percentage of DCs (Fig. 6G and H).

5. Discussion

Recent advancements have led to the development of various chemotherapeutic agents designed to directly eliminate cancer cells. However, the serious toxicity associated with these agents has shifted research focus toward utilizing natural ingredients found in natural products to mitigate side effects and enhance effectiveness [25–27]. Studies in this direction aim to increase the concentration of cytokines involved in immune enhancement or boost the activity of immune cells that can directly kill cancer cells [28,29]. These natural substances have demonstrated the ability to non-specifically stimulate various immune cells, promoting overall body homeostasis and reducing the incidence of diseases [30].

In our study, we administered a herbal formula called YGD, composed of ten medicinal herbs known to enhance immunity, to immunodeficient mice. The goal was to provide scientific evidence for cytokine production and the immune-enhancing efficacy resulting from YGD administration. Ultimately, our aim is to propose the potential application of YGD as an immune-boosting agent with the capacity to replace immunostimulants associated with numerous side effects.

Cy is well known as a potent immunosuppressant that can reduce immune responses by inducing DNA damage in cells [31]. This investigation is geared towards unraveling the impacts and potential mechanisms of YGD in counteracting immune apoptosis and enhancing activity within the Cy-induced immunosuppressive environment.

The administration of a single dose of Cy aligns with previous studies, consistently demonstrating a significant reduction in spleen-resident lymphocytes, Th1 and Th2 type cytokine production, and immunoglobulin (Ig) levels [32–34]. Additionally, Cy has been reported to have apoptosis-inducing effects that significantly increase Bax mRNA expression and decrease Bcl-2 expression [35]. Typically, the Bax protein is located in the cytoplasm of cells. However, when cells are under stress, for instance, due to DNA damage, Bax undergoes a process of relocation to the outer mitochondrial membrane. This movement of Bax to the mitochondria initiates the release of cytochrome-c, a critical step that leads to the promotion of cell apoptosis [36,37]. In contrast, Bcl-2, a representative anti-apoptotic protein, inhibits Bax action by inhibiting the formation of Bax protein oligomers on the mitochondrial membrane, ultimately promoting cell survival [38,39].

Remarkably, YGD not only significantly reduces the increased expression of BAX protein induced by Cy but also effectively increases the expression of BCL-2 protein. This observation suggests that YGD might harbor a potential protective influence against Cy-induced cell death by regulating BAX/BCL-2 expression in spleen and thymus tissues.

Cy is recognized for its capacity to suppress the immune response, particularly impacting T cells. This results in the inhibition of both Th1 and Th2 immune responses [40]. Our observations showed that in a Cy-induced immunodeficient model with reduced Th1 and Th2 cytokine production, administration of 20 or 100 mg/kg YGD promotes dose-dependent Th1 and Th2 cytokine production. Specifically, the two principal categories of immune cells in this context are Th1 cells, which are known for producing IFN-γ, and Th2 cells, characterized by their production of IL-4. These cell types play critical roles in orchestrating different aspects of the immune response [41]. IFN-γ is an important cytokine for host defense as it activates humoral and cellular immunity against diseases and acts as a primary activator of macrophages [42], whereas IL-4 plays a crucial role in regulating allergic immune responses [43]. Our findings indicate that YGD is involved in enhancing Th1 and Th2 cytokine production, crucial factors known to affect immune activity. Moreover, YGD increased the IgG and IgM levels in the serum of mice, which had decreased following Cy administration.

IgG, constituting about 75–80 % of all the immunoglobulins in human serum, is the most prevalent class of antibodies within the human body [44]. Present in both blood and bodily tissues, IgG is notable for its ability to cross the placenta, thereby providing passive immunity to newborns [45]. This class of antibodies is highly versatile and plays a crucial role in various immune responses. These include neutralization of pathogens, opsonization (marking pathogens for destruction), activation of the complement system (a part of the immune response), antibody-dependent cellular cytotoxicity (destruction of targeted cells), and contributing to the establishment of long-lasting immunity [46].

Conversely, IgM, another class of antibodies akin to IgG, is particularly important in the early stages of the immune response, where the immune system is encountering a specific antigen for the first time [47]. Overall, we confirmed the effective immune activity of YGD in a mouse model of Cy-induced immunosuppression. Specifically, we screened for the immune cell activity of YGD in spleen tissue-derived splenocytes. The $CD3^+$ T lymphocytes, $CD19^+$ B lymphocytes, $CD45^+$ leukocytes, T lymphocyte subsets ($CD3^+$ and T $CD4^+$), and total NK cells were efficiently activated by YGD administration in Cy-induced immunosuppressed mice. Although most immune cells were affected by Cy and YGD administration, we have not presented more specific immune cells associated with YGD efficiency. Our data showed that the size and index of the thymus were more affected than the spleen in YGD-administered immunosuppressed mice. The thymus is a primary lymphoid organ in which T cells develop and the T cell receptor repertoire is formed [48]. Histologically, very few $CD3^+$ T cells were found in the thymus after Cy administration, whereas a relatively large number of $CD3^+$ T cells were observed after YGD administration in immunosuppressed mice.

Seeing that YGD is a complex multi-herbal formula, limitations of our study included the difficulty of identifying a single immunomodulatory target. Although the precise chemical makeup of YGD has not been thoroughly researched, numerous studies have identified its individual components and explored their respective pharmacological properties. These investigations contribute to a better understanding of how YGD functions as an herbal remedy. A series of studies have already demonstrated the immunopotentiating and modulating effects of catalpol against *Rehmannia glutinosa* (Gaertn.) Steud [49–51], α-pinene against *Angelica gigas* Nakai [52–55] and loganin and moroniside for *Cornus officinalis* Siebold & Zucc [56,57].

In addition, among YGD herbal medicines, *Cervi* pantotrichum is attracting attention for its immunomodulatory activity [58] and *Wolfiporia* extensa (Peck) Ginns is currently being sold as a main ingredient in immunity supplements. While direct evidence of immune activity in other medicinal herbs is lacking, these herbs have been reported to offer a variety of pharmacological benefits. These include anticancer, anti-inflammatory, antioxidant, nerve-regenerative, and neuroprotective activities [59–64].

These observations collectively highlight the diverse and complex nature of the components found in YGD, each contributing to its broad spectrum of pharmacological effects. Despite limitations in the scope of the current study, they provide valuable insights into their potential as immune-boosting therapeutics through the enhancement of immune cells.

Moreover, we did not identify a specific pathway for interpreting the overall increase in immune cells by YGD. Therefore, we advocate for additional research to explore the individual targets of YGD. Identifying specific pathways involved in its immunomodulatory effects and immune enhancement is crucial to fully understand its mechanisms of action and potential therapeutic applications. Our findings indicate that YGD has immune-enhancing effects in mice with immunosuppression induced by Cy. It appears that T lymphocytes may play a more significant role in the immunomodulatory activity of YGD.

6. Conclusion

These findings suggest that YGD enhances immunity, including increased production of Th1 and Th2-type cytokines as well as the enhancement of immune cell activity, especially in $CD3^+$ T lymphocytes in mice with Cy-induced immunosuppression.

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Data availability

The data will be made available on request.

CRediT authorship contribution statement

Hyunseong Kim: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Jin Young Hong:** Visualization, Validation, Formal analysis, Data curation. **Junseon Lee:** Visualization, Validation, Formal analysis. **Changhwan Yeo:** Validation, Formal analysis. **Wan-Jin Jeon:** Validation, Formal analysis. **Yoon Jae Lee:** Writing – review & editing, Formal analysis. **In-Hyuk Ha:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24033.

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