Preliminary Study on the Immunostimulatory and Antitumor Mechanisms of Dictyophora Polysaccharides

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Abstract: This study aims to investigate the immunostimulatory and antitumor effects of Dictyophora polysaccharides and preliminarily analyze their effects on myeloid-derived suppressor cells (MDSCs). An in vitro cell culture approach was employed to evaluate the immunoproliferative and tumor-killing effects of Dictyophora polysaccharides. Additionally, the effects on MDSCs in the spleen of tumor-bearing mice were observed to explore the potential mechanisms underlying the antitumor immune response. The results showed that Dictyophora polysaccharides enhanced both cellular and humoral immunity, promoting the proliferation of CD4+, CD8+, and B cells in a dose-dependent manner. In T-cell-mediated tumor cell-killing assays, Dictyophora polysaccharides did not directly enhance the tumor-killing activity of immune cells. However, Dictyophora polysaccharides demonstrated a trend of reducing the proportion of MDSCs. These findings suggest that Dictyophora polysaccharides enhance the proliferation of immune cells and may exert antitumor effects by decreasing the proportion of immunosuppressive MDSCs.

Keywords: Dictyophora polysaccharides, immune cells, proliferation, antitumor, myeloid-derived suppressor cells (MDSCs)

Introduction: In recent years, research into natural compounds with immunomodulatory and antitumor properties has gained increasing attention. Dictyophora, a basidiomycete fungus known for its high nutritional value, contains polysaccharides that have shown promise for their health benefits. These include enhancing the immune system, regulating lipids, inhibiting microbial growth, and scavenging free radicals. Polysaccharides from Dictyophora are also being recognized for their potential in antitumor therapies, but their precise mechanisms of action remain unclear.

Many studies have demonstrated the ability of fungal polysaccharides to regulate the immune system. For example, Yang Hailong et al. found that short-skirt Dictyophora polysaccharides could effectively neutralize free radicals and protect red blood cell membranes from oxidative damage. Zhao Kai and colleagues extracted polysaccharides from Dictyophora rubrovolvata and confirmed that the component DRVP1 had inhibitory effects on murine S180 sarcoma cells.

Other researchers, such as Wang Jiatang and Lin Yuman, have conducted detailed structural analyses of Dictyophora polysaccharides, identifying key chemical compositions and chain conformations. Additionally, Yaw-Bee Ker's work highlighted the antioxidant properties of water-soluble Dictyophora components, attributing to them anticancer, anti-inflammatory, and anti-infective effects.

However, most current studies focus on the chemical structure of Dictyophora polysaccharides or their general impact on immune cells. Few have investigated their effects on specific immune cell populations, especially in the context of tumor immunity. Recent research has shown that myeloid-derived suppressor cells (MDSCs) play a crucial role in immune suppression in cancer patients, where they inhibit the tumor-killing activity of CD8+ T cells. An increase in MDSCs correlates with weakened immune function, making it essential to understand how to regulate these cells to improve antitumor responses. Despite this, the potential impact of Dictyophora polysaccharides on MDSCs has not yet been explored.

This study aims to fill this gap by examining the effects of Dictyophora polysaccharides on different immune cells, with a focus on their influence on MDSCs. Flow cytometry (FACS) was used to assess the proliferation of immune cells and the tumor-killing efficacy of cytotoxic T cells. Additionally, for the first time, the effects of Dictyophora polysaccharides on the proportion of MDSCs in the spleens of tumor-bearing mice were evaluated. This research seeks to provide new insights into the immunomodulatory properties of Dictyophora polysaccharides and their potential as adjuvants in cancer therapy.

1 Materials and Methods

1.1 Materials

1.1.1 Reagents and Samples

1. Wild-type C57 mice
2. Tumor-bearing C57 mice (tumor type: hepatic metastatic adenocarcinoma, MAC)
3. Crude Dictyophora polysaccharides
4. RPMI 1640 medium

1.1.2 Instruments

1. Hemocytometer
2. Flow cytometer (BD Biosciences)
3. GUAVA microcapillary cell analyzer

1.2 Methods

1.2.1 Preparation of 1640+/+ Medium

RPMI 1640−/− medium was purchased from Invitrogen. To prepare 1640+/+ medium, 10% fetal bovine serum (FBS) and 100 U/mL of a dual-antibiotic mixture (streptomycin and penicillin) were added to the 1640−/− medium.

1.2.2 Preparation of Phosphate-Buffered Saline (PBS)

The 1× PBS buffer contained the following components:

1. 8 g NaCl
2. 0.2 g KCl
3. 3.63 g $Na_2HPO_4•12H_2O$

4. 0.24 g KH_2PO_4

5. 1,000 mL ultrapure water

1.2.3 Preparation of Dictyophora Polysaccharide Stock Solution

Dictyophora polysaccharide powder was dissolved in 1640+/+ medium and incubated in a 50°C water bath for 2 hours to obtain a suspension with a final concentration of 20 mg/mL. The suspension was centrifuged at 10,000 rpm for 15 minutes, and the supernatant was filtered through a funnel for 12 hours, followed by another centrifugation at 10,000 rpm for 15 minutes. The supernatant was further filtered through 0.45 μm and 0.20 μm filters to obtain a sterile Dictyophora polysaccharide stock solution.

1.2.4 Preparation of Immune Cell Suspension

Mouse spleens were homogenized in PBS buffer, and the homogenate was filtered through a 100-mesh strainer and centrifuged at 1,500 rpm for 3 minutes. The supernatant was discarded, and 1 mL of red blood cell lysis buffer (RLB) was added for 1 minute. Subsequently, 5 mL of PBS was added to resuspend the cells, which were then filtered and centrifuged at 1,500 rpm for 3 minutes. After discarding the supernatant, the cells were resuspended in 1640+/+ medium. The cell concentration was determined using a hemocytometer and adjusted to the required final concentration using 1640+/+ medium.

1.2.5 CFSE Fluorescent Labeling of Cells

The CFSE stock solution was diluted at a 1:3000 ratio, and 1 mL of the diluted solution was used to label 2×10^6 cells. The mixture was incubated at 37°C for 10 minutes, followed by the addition of 5 mL of pre-cooled 1640+/+ medium and incubation on ice for 5 minutes. The cells were centrifuged at 1,500 rpm for 8 minutes and washed twice with pre-cooled medium. The final cell suspension was prepared for further use, and the tubes were wrapped in aluminum foil to protect them from light.

1.2.6 In Vitro Immune Cell Proliferation Assay with Dictyophora Polysaccharides

The immune cell types studied included T cells, B cells, macrophages, and natural killer (NK) cells. Immune cells were directly stimulated by Dictyophora polysaccharides in vitro, and their proliferation was assessed using cell counting and flow cytometry with antibody staining.

Spleen cells from wild-type C57 mice were plated in 24-well plates at 5×10^6 to 1×10^7 cells per well in 500 μL of cell suspension. Then, 500 μL of Dictyophora polysaccharides was added to achieve final concentrations of 2 mg/mL, 3 mg/mL, 5 mg/mL, and 8 mg/mL. Control wells received an equal volume of 1640+/+ medium. Each group was tested in triplicate. Additional wells were set for single staining controls (four wells) and a blank control (one well), with equal amounts of cells and 500 μL of medium.

The plates were incubated in a 37°C incubator for 5–7 days. After incubation, the supernatant was collected into 1.5 mL centrifuge tubes. Each well was treated with 200 μL of trypsin for 1 minute, and the detached cells were transferred to the corresponding centrifuge tubes. Cell counts were performed using a hemocytometer. The cells were centrifuged at 4,500 rpm for 3 minutes and stained with CD4, CD8, F4/80, and B220 antibodies at a 1:300 dilution on ice for 50 minutes. The cells were washed with PBS, centrifuged again, and resuspended in 300–400 μL PBS for flow cytometry analysis.

1.2.7 In Vitro Tumor Cell Killing Assay with Cytotoxic T Cells

Spleen cells from wild-type C57 mice were plated in 24-well plates with the following experimental groups: (1) control group, (2) tumor cells with polysaccharides, (3) tumor cells with lymphocytes, and (4) tumor cells with polysaccharides

and lymphocytes. Each well was seeded with 2 × 10^5 tumor cells and 4 × 10^6 lymphocytes, with a final Dictyophora polysaccharide concentration of 8 mg/mL.

The plates were incubated at 37°C for 5 days, after which the cells were stained with propidium iodide (PI) for flow cytometry. Supernatant was collected, and cells were detached using 200 μL of trypsin for 1 minute. After centrifugation at 4,500 rpm for 3 minutes, the cells were stained with PI (1:300 dilution) on ice for 30 minutes, washed, and resuspended in 300–400 μL PBS for flow cytometry analysis within 1 hour.

1.2.8 In Vitro MDSC Conversion Assay

Spleen cells from tumor-bearing mice were plated in 24-well plates at 2 × 10^6 cells per well in 500 μL of suspension. Dictyophora polysaccharides were added at final concentrations of 2 mg/mL, 3 mg/mL, 5 mg/mL, and 8 mg/mL. Control wells received an equal volume of 1640+/+ medium. Each group was tested in triplicate.

The plates were incubated at 37°C for 6 days. The cells were stained with GR1 and CD11b antibodies at a 1:300 dilution on ice for 50 minutes, washed with PBS, and fixed with formaldehyde. Flow cytometry analysis was performed.

1.2.9 In Vitro MDSC Conversion Assay

Quantitative data were analyzed using one-way analysis of variance (ANOVA). Levene's test was used to assess the homogeneity of variances. If variances were homogeneous, post-hoc multiple comparisons were performed using the LSD and Dunnett's tests. If variances were heterogeneous, Dunnett's T3 correction was applied. Statistical analysis was performed using SPSS 10.0, with a significance level of $\alpha = 0.05$.

2 Results and Analysis

2.1 In Vitro Immune Cell Proliferation Assay with Dictyophora Polysaccharides

Mouse spleen cells were stimulated with different concentrations of Dictyophora polysaccharides and cultured in vitro for 5 days. The results are shown in the figure below:

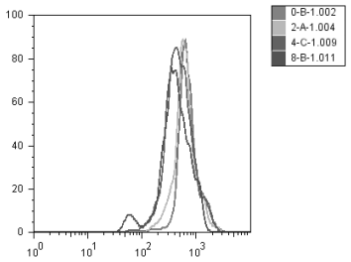

Fig. 1 Proliferation of CD4+ T Cells

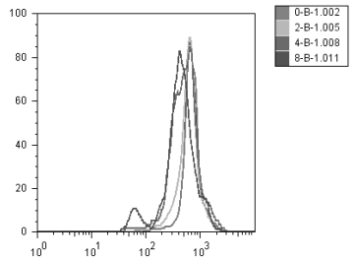

Fig. 2 Proliferation of CD8+ T Cells

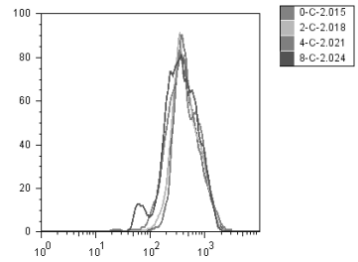

Fig. 3 Proliferation of B Cells

In Figures 1 to 3, the legends 0-c-2, 2-c-2, 4-c-2, and 8-c-2 correspond to Dictyophora polysaccharide concentrations of 0 mg/mL, 3 mg/mL, 5 mg/mL, and 8 mg/mL, respectively. The x-axis represents CFSE fluorescence intensity detected via the FITC channel, while the y-axis indicates the proportion of cells corresponding to each fluorescence intensity.

Figures 1 to 3 demonstrate that Dictyophora polysaccharides at concentrations ≤3 mg/mL exhibit minimal effects on immune cell proliferation. In contrast, a significant stimulatory effect on proliferation was observed at concentrations ≥5 mg/mL. At a concentration of 8 mg/mL, the proliferation-stimulating effect was most pronounced, as evidenced by a marked leftward shift of the fluorescence peak and the emergence of new subpopulations, indicating that a substantial proportion of

cells had completed division and proliferation.

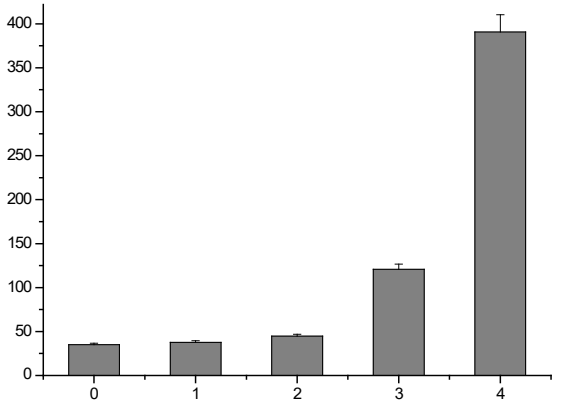

Fig. 4 Proliferation of Immune Cells

In Figure 4, the x-axis values 0, 1, 2, 3, and 4 represent Dictyophora polysaccharide concentrations of 0 mg/mL, 2 mg/mL, 3 mg/mL, 5 mg/mL, and 8 mg/mL, respectively, while the y-axis represents the total number of cells.

The total cell counts corresponding to concentrations of 0 mg/mL, 2 mg/mL, 3 mg/mL, 5 mg/mL, and 8 mg/mL were 3.5×10^5, 3.8×10^5, 4.5×10^5, 1.2×10^5, and 3.9×10^5, respectively. After 7 days of in vitro culture, all concentrations of Dictyophora polysaccharides promoted immune cell proliferation, with the most significant effect observed at 8 mg/mL, where the total cell count reached 11.2 times that of the control group.

Statistical analysis showed that the overall variance among the five groups was significant, with $F = 2377.50$ and $P < 0.001$. Levene's test for homogeneity of variance yielded a value of 0.653 ($P = 0.638$), indicating that variances were homogeneous. Post-hoc multiple comparisons using Dunnett's T3 test showed significant differences between the 8 mg/mL group and all other groups. Pairwise comparisons using LSD tests also revealed significant differences between the control, 2 mg/mL, and 3 mg/mL groups and the 5 mg/mL and 8 mg/mL groups.

These findings suggest that Dictyophora polysaccharides significantly promote in vitro immune cell proliferation, supporting the conclusion that Dictyophora polysaccharides possess immunostimulatory properties.

2.2 In Vitro Cytotoxic T Cell Tumor-Killing Assay with Dictyophora Polysaccharides

Based on the results of the in vitro immune cell proliferation assay, 8 mg/mL Dictyophora polysaccharides, which demonstrated the most significant stimulatory effect, were selected for stimulating cytotoxic T cells in tumor-killing assays.

(1) Control Group: This group consisted of tumor cells cultured alone. Flow cytometry analysis indicated that approximately 15.00% of the tumor cells underwent cell death (Figures 5-1 and 5-2).

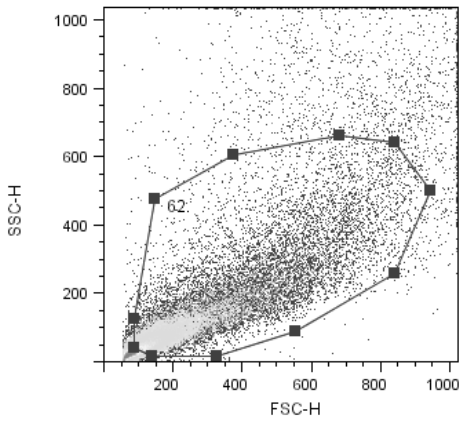

Fig. 5–1

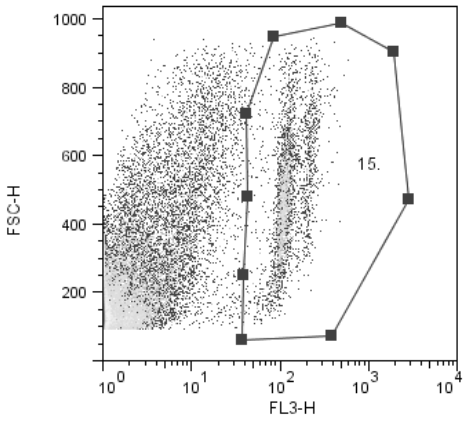

Fig. 5–2

(2) Polysaccharide Group: This group consisted of tumor cells cultured with 8 mg/mL Dictyophora polysaccharides. Flow cytometry analysis showed that approximately 7.00% of the tumor cells underwent cell death (Figures 6-1 and 6-2).

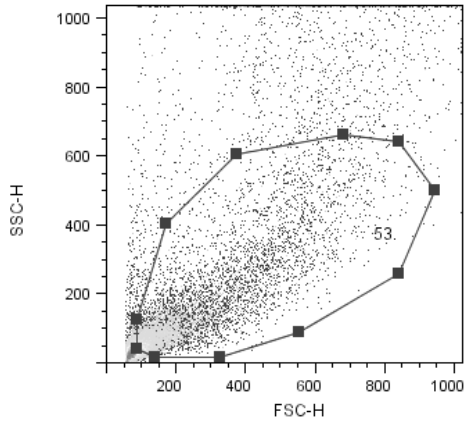

Fig. 6–1

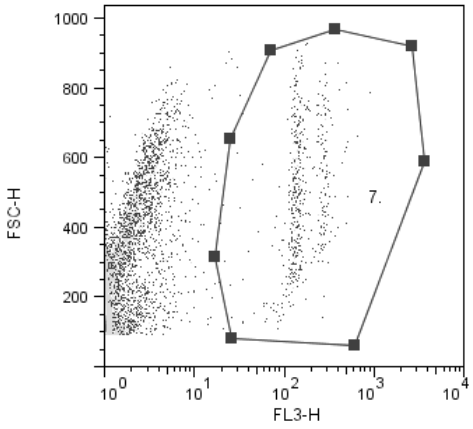

Fig. 6–2

(3) Lymphocyte Group: This group consisted of tumor cells co-cultured with lymphocytes at a ratio of 1:20. Flow cytometry analysis showed that approximately 1.00% of the tumor cells underwent cell death (Figures 7-1 and 7-2).

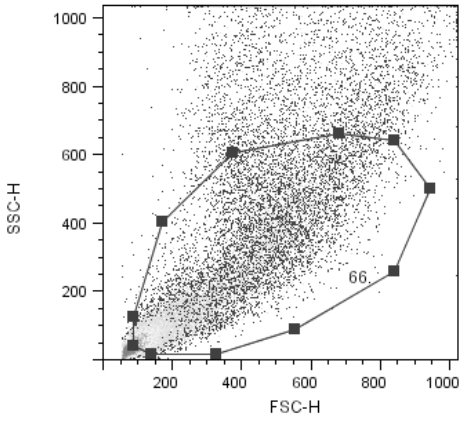

Fig. 7–1

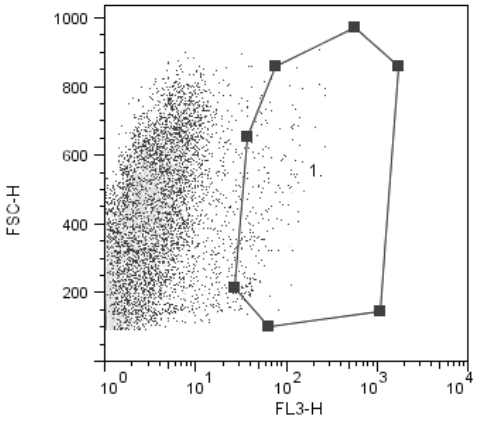

Fig. 7–2

(4) Polysaccharide and Lymphocyte Combination Group: This group consisted of tumor cells co-cultured with

lymphocytes at a ratio of 1:20, along with 8 mg/mL Dictyophora polysaccharides. Flow cytometry analysis showed that approximately 17.00% of the tumor cells underwent cell death (Figures 8-1 and 8-2).

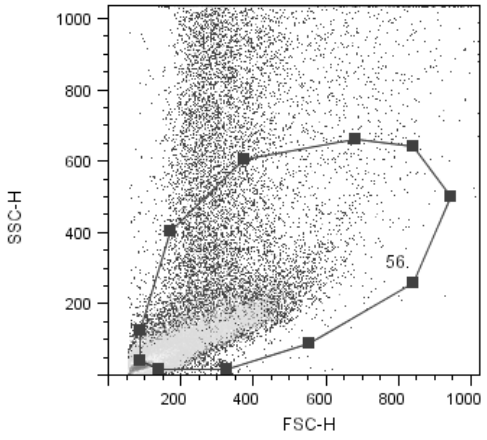

Fig. 8–1

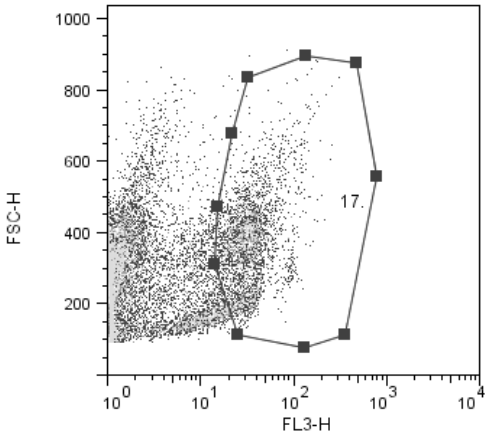

Fig. 8–2

In Figures 5 to 8, the FSC-H axis represents cell size, the SSC-H axis represents cell granularity, and the FL3-H axis represents the fluorescence intensity of PI antibody staining detected in channel 3 (PerCP). In Figures 5-1, 6-1, 7-1, and 8-1, the gated population represents tumor cells, while in Figures 5-2, 6-2, 7-2, and 8-2, the gated population represents dead tumor cells.

From Figures 5 to 8, it can be observed that the addition of whole spleen cells alone, Dictyophora polysaccharides at 8 mg/mL alone, or Dictyophora polysaccharides combined with lymphocytes had no significant effect on enhancing the tumor-killing activity of cytotoxic T cells.

When Dictyophora polysaccharides were used alone, they may have been utilized as a carbohydrate source by the tumor cells, thereby exhibiting no notable effect on inducing tumor cell apoptosis. When Dictyophora polysaccharides were combined with lymphocytes, the proportion of dead tumor cells increased from 15.0% (control group) to 17.0%. However, this increase from 15.0% to 17.0% was minimal and statistically insignificant, indicating that the addition of Dictyophora polysaccharides did not enhance the tumor-killing ability of cytotoxic T cells.

Based on these findings, it can be preliminarily concluded that “Dictyophora polysaccharides do not exhibit a significant in vitro effect on enhancing the tumor-killing ability of cytotoxic T cells.”

2.3 In Vitro MDSC Conversion Assay with Dictyophora Polysaccharides

(1) Control Group: This group consisted of spleen cells from tumor-bearing mice cultured alone. Flow cytometry analysis showed that MDSCs accounted for approximately 25.8% of the total cells (Figures 9-1 and 9-2).

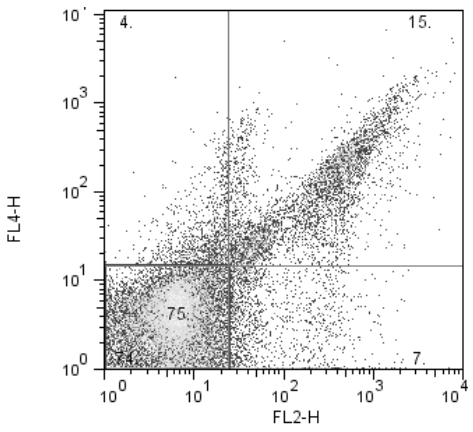

Fig. 9–1

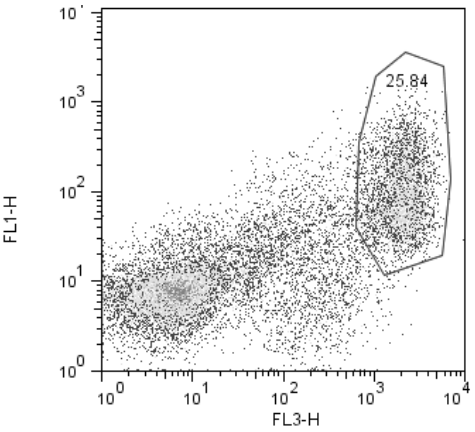

Fig. 9–2

(2) 3 mg/mL Polysaccharide Group: This group consisted of spleen cells from tumor-bearing mice co-cultured with 3 mg/mL Dictyophora polysaccharides. Flow cytometry analysis showed that MDSCs accounted for approximately 4.5% of the total cells (Figures 10-1 and 10-2).

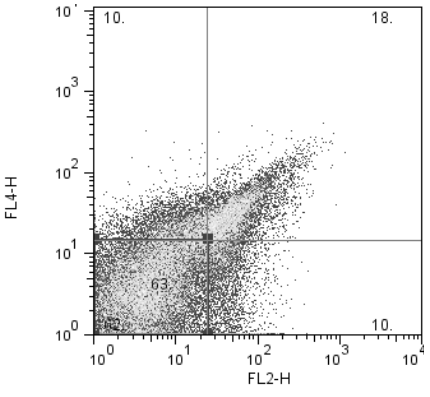

Fig. 10–1

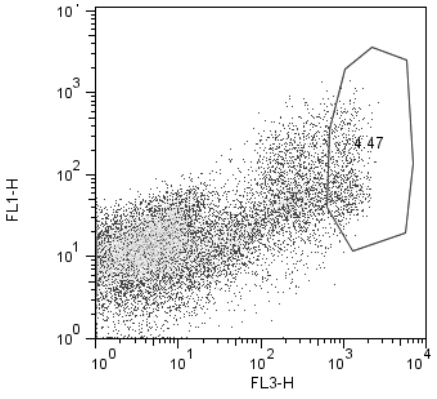

Fig. 10–2

(3) 5 mg/mL Polysaccharide Group: This group consisted of spleen cells from tumor-bearing mice co-cultured with 5 mg/mL Dictyophora polysaccharides. Flow cytometry analysis showed that MDSCs accounted for approximately 3.8% of the total cells (Figures 11-1 and 11-2).

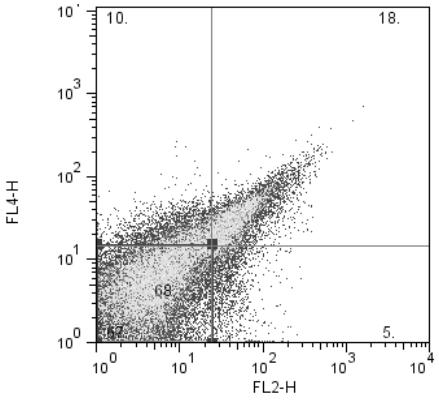

Fig. 11–1

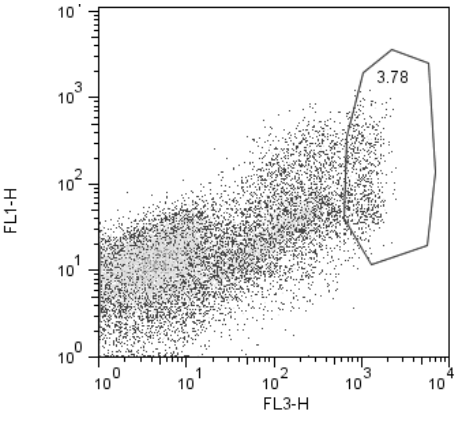

Fig. 11–2

(4) 8 mg/mL Polysaccharide Group: This group consisted of spleen cells from tumor-bearing mice co-cultured with 8 mg/mL Dictyophora polysaccharides. Flow cytometry analysis showed that MDSCs accounted for approximately 2.4% of the total cells (Figures 12-1 and 12-2).

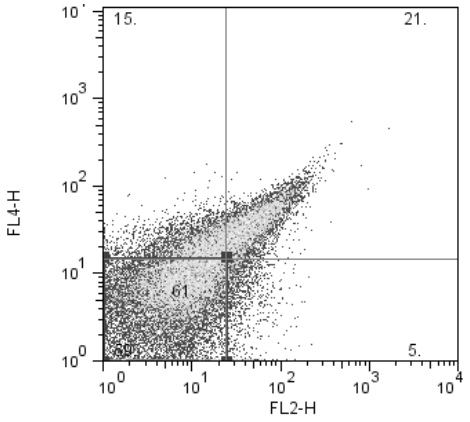

Fig. 12–1

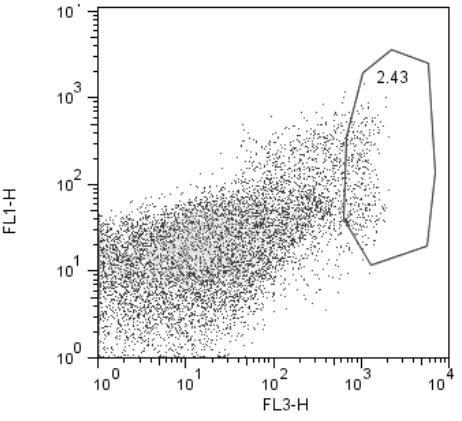

Fig. 12–2

In Figures 9 to 12, the FL2-H axis represents PE fluorescence intensity (channel 2), the FL4-H axis represents APC fluorescence intensity (channel 4), the FL1-H axis represents FITC fluorescence intensity corresponding to CD11b antibody staining (channel 1), and the FL3-H axis represents PerCP fluorescence intensity corresponding to GR1 antibody staining (channel 3). In Figures 9-1, 10-1, 11-1, and 12-1, the gated cells represent live spleen cells, while in Figures 9-2, 10-2, 11-2, and 12-2, the gated cells represent MDSCs.

From Figures 9 to 12, it can be observed that with increasing concentrations of Dictyophora polysaccharides, the proportion of MDSCs in cultured spleen cells from tumor-bearing mice gradually decreased from 25.8% to 2.4%. This indicates that the ability of Dictyophora polysaccharides to promote MDSC conversion in vitro follows a dose-dependent trend. The effect was most significant at the highest concentration (8 mg/mL), with the MDSC proportion decreasing by approximately 23.4% compared to the control group. The 3 mg/mL and 5 mg/mL polysaccharide groups also promoted MDSC conversion, with MDSC proportions reduced by 21.4% and 22.1%, respectively, compared to the control group.

These results suggest that Dictyophora polysaccharides have the potential to promote MDSC conversion in vitro.

3 Discussion

3.1 Future Directions

1. Additional experimental groups with Dictyophora polysaccharide concentrations exceeding 8 mg/mL should be included to determine whether 8 mg/mL is the optimal concentration or if a more effective concentration exists.

2. A concentration gradient of Dictyophora polysaccharides should be added to the tumor-killing assay to observe any potential dose-response relationship. Additionally, co-culture experiments using EGFP mice with green fluorescent protein-labeled immune cells and tumor cells transfected with red fluorescent protein could provide more precise flow cytometry analysis.

3. Future studies will focus on MDSC conversion assays, increasing the number of replicates and including additional antibody staining for dendritic cells (DCs) and granulocytes during flow cytometry to assess the proportion of downstream

cells. If the proportion of MDSCs decreases while granulocyte populations increase, this would indicate that Dictyophora polysaccharides promote MDSC differentiation, suggesting potential as an adjuvant in tumor therapy. Additionally, isolating MDSCs from spleen cells for targeted conversion assays would improve experimental accuracy.

4. If this study achieves consistent results after multiple replications, the in vivo effects of Dictyophora polysaccharides on the immune system should be investigated to determine the optimal in vivo concentration, thereby increasing the translational value of the findings.

5. Further theoretical research should be conducted to deepen the understanding of the mechanisms underlying the effects of Dictyophora polysaccharides.

3.2 Outlook and Applications

Traditional Chinese medicine (TCM) is a valuable asset of Chinese culture, with its efficacy established through centuries of empirical evidence accumulated by numerous practitioners. However, modern scientific validation through experimental studies is necessary. The isolation of active components from TCM is critical for the advanced development of these traditional therapies. If Dictyophora polysaccharides are proven to enhance immunity and support tumor therapy through experimental validation, this will significantly promote the comprehensive utilization of Dictyophora.

Polysaccharides, as essential biological molecules with diverse bioactivities, hold great promise for use in health supplements and functional foods. The demonstrated effects of Dictyophora polysaccharides in stimulating immune cell proliferation and supporting tumor therapy suggest their potential as key active ingredients in health products such as oral liquids and medicinal tonics.

4 Conclusion

This study employed flow cytometry to perform immune cell classification analysis, which had not been conducted in previous studies, and investigated the immunomodulatory effects of Dictyophora polysaccharides through an MDSC conversion assay for the first time. The key findings are as follows:

1.The ability of Dictyophora polysaccharides to promote cell proliferation increased with concentration. A significant increase in cell count was observed at concentrations ≥5 mg/mL, and at 8 mg/mL, the polysaccharides stimulated cell proliferation to 11.16 times that of the control group, showing the strongest effect. In contrast, low-concentration groups exhibited minimal effects.

2.The addition of Dictyophora polysaccharides did not enhance the tumor-killing ability of cytotoxic T cells during lymphocyte-mediated tumor cell killing.

3.The promotion of MDSC conversion by Dictyophora polysaccharides showed a dose-dependent trend. At 8 mg/mL, the effect was most pronounced, with the proportion of MDSCs reduced by 23.4% compared to the control group. Other concentration groups also demonstrated a promoting effect. This conclusion requires further validation with larger sample sizes.

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