Preliminary Investigation into the Anti-Tumor Mechanism of Ganoderma lucidum Crude Polysaccharides

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Abstract: This study aims to explore the potential anti-tumor mechanisms of Ganoderma Lucidum Polysaccharide (GLP). Flow cytometry was employed to investigate the activation effects of GLP on different immune cell subsets. The direct inhibitory effects of GLP on tumor cells were evaluated using CCK assay, MTT assay, and real-time cell monitoring (Cell Index) methods. Additionally, the cytotoxic effects of GLP on cancer-associated fibroblasts (CAF) were examined. A hypothesis was proposed that GLP inhibits tumor development by improving the tumor microenvironment. The results indicate that GLP exerts anti-tumor effects not only by directly killing tumor cells but also by inhibiting the growth of CAFs and primarily promoting CD4+ T cell proliferation.

Keywords: Ganoderma Lucidum Polysaccharide, Cancer-Associated Fibroblasts, Immune Cells, Activation

Introduction

Ganoderma lucidum, traditionally known as the "immortal herb," has been widely recognized for its medicinal properties since ancient times. Modern scientific research has demonstrated that Ganoderma Lucidum Polysaccharide (GLP) is one of the key components responsible for its immunomodulatory and anti-tumor effects. GLP exerts broad regulatory effects on the immune system, including enhancing the activity of T cells, B cells, and natural killer (NK) cells; promoting the production of immune cytokines such as IL-2, TNF, and IFN; stimulating DNA synthesis in T lymphocytes; and modulating the number and function of T cell subsets [1].

Previous studies have shown that GLP effectively induces cytotoxicity against various tumor cell lines, such as MDA human breast cancer cells and HepG2 human liver cancer cells [2]. Additionally, GLP has been reported to promote the in vitro proliferation of both T and B cells [3].

Building upon prior research, this study compares the inhibitory effects of crude GLP on five tumor cell lines: LLC, A549, J558, CT26, and MDA. Furthermore, the activation effects of GLP on five immune cell subsets—CD4+ T cells, CD8+ T cells, B cells, dendritic cells, and macrophages—were systematically evaluated. Preliminary findings suggest that the anti-tumor mechanism of GLP is primarily attributed to its promotion of CD4+ T cell, CD8+ T cell, and B lymphocyte

proliferation, with CD4+ T cells showing the most significant response.

This study proposes that GLP may exert three major effects: selectively inducing cytotoxicity in specific tumor cells, activating certain lymphocyte subsets, and inhibiting the growth of cancer-associated fibroblasts (CAFs). Our findings provide a novel perspective on the role of microenvironment modulation in the anti-tumor activity of GLP and offer experimental evidence for the development of GLP-based anti-tumor therapeutics.

1 Materials and Methods

1.1 Materials and Equipment

1.1.1 Experimental Materials and Reagents

1. C57 wild-type mice
2. LLC (Lewis lung carcinoma) cells
3. A549 human lung cancer cells
4. MDA human breast cancer cells
5. J558 murine myeloma cells
6. CT26 murine colon carcinoma cells
7. CT26-CAF (cancer-associated fibroblasts derived from CT26 cells)
8. Crude Ganoderma lucidum polysaccharide (GLP)
9. DMEM+/+ culture medium
10. CCK-8 cell counting kit
11. Phosphate-buffered saline (PBS)
12. Flow cytometry fluorescent dyes

1.1.2 Equipment

1. BD flow cytometer
2. Microplate reader
3. Hemocytometer
4. Centrifuge
5. Optical microscope
6. xCELLigence real-time cell analysis system (model SP)

1.2 Methods

1.2.1 Extraction of Ganoderma lucidum Polysaccharides by Water Extraction

Weigh 50 g of Ganoderma lucidum and add 750 mL of water using a graduated cylinder. Boil the sample at 100°C for 1 hour. After cooling, filter the extract through a mesh. Slowly add ethanol (three times the volume of the extract) to the filtrate while stirring continuously. Balance the solution and centrifuge at 10,000 rpm for 5 minutes. Discard the supernatant and dry the precipitate in a 37°C incubator. Scrape the dried polysaccharide powder from the tube walls and collect it in a 50 mL centrifuge tube. Weigh the resulting polysaccharide powder.

1.2.2 Preparation of DMEM+/+ Medium

Prepare DMEM-/– medium supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin.

1.2.3 Preparation of Phosphate-Buffered Saline (PBS)

Prepare 1×PBS by dissolving 8 g NaCl, 0.2 g KCl, 3.63 g $Na_2HPO_4 \cdot 12H_2O$, and 0.24 g KH_2PO_4 in 1,000 mL ultrapure water.

1.2.4 Preparation of Immune Cell Suspension

Euthanize C57 wild-type mice by cervical dislocation and collect the spleen. Disinfect the spleen with ethanol and place it in PBS. Grind the spleen in PBS and filter through a 200-mesh sieve. Centrifuge at 1,500 rpm for 3 minutes and discard the supernatant. Add 1 mL of red blood cell lysis buffer (RLB) and mix thoroughly. After 1 minute of lysis, add 9 mL of PBS to stop the reaction. Filter and centrifuge at 1,500 rpm for 3 minutes, discard the supernatant, and resuspend the cells in 5 mL DMEM+/+ medium. Count the cells using a hemocytometer to obtain the single-cell suspension.

1.2.5 Flow Cytometry Staining of Immune Cells

Seed 5×10^6 splenocytes per well in a 24-well plate and add GLP at final concentrations of 0.5 mg/mL and 1.0 mg/mL. Add the same volume of DMEM+/+ medium to the negative control wells to reach a final volume of 1 mL per well. Incubate at 37°C for 2 days.

After 2 days, collect the supernatants and wash the wells with 200 μL of PBS to remove residual serum. Add 500 μL of trypsin to each well and incubate for 15 minutes. Stop the digestion by adding 500 μL DMEM+/+ medium and transfer the detached cells to centrifuge tubes. Count the suspended and adherent cells. Centrifuge at 40,000 rpm at 4°C for 5 minutes, discard the supernatant, and resuspend the cells in 50 μL PBS containing 2% NCS. Add flow cytometry dyes in proportion. Incubate for 30 minutes (stain with 7-AAD for the last 5 minutes), centrifuge again at 40,000 rpm at 4°C for 5 minutes, discard the supernatant, and resuspend the cells in PBS containing NCS. Transfer the cell suspension to flow cytometry tubes for analysis.

1.2.6 Real-Time Cell Analysis of GLP-Induced Tumor Cell Inhibition

The xCELLigence Real-Time Cell Analysis (RTCA) system (Roche Diagnostics, Shanghai) was used to evaluate the inhibitory effects of GLP on tumor cells by detecting changes in cell status based on electronic impedance. The system measures the interaction between cells and microelectrodes to generate electrical signals. Upon addition of GLP, changes in tumor cell morphology and adhesion affect the impedance signals. A decrease in the signal indicates cell death.

In this experiment, MDA tumor cells were seeded at a density of 1×10^4 cells/well in DMEM+/+ medium and incubated for 30 hours. The experimental group received 10 mg/mL GLP, while the control group received the same volume of DMEM+/+ medium. Five replicate samples were prepared for both groups. Background signals were recorded before cell seeding, followed by measurements every 15 seconds for 1 minute and every 15 minutes for 25 hours after cell seeding.

1.2.7 CCK-8 Assay for Evaluating GLP-Induced Tumor Cell Inhibition

The experiment included control and experimental groups, with three replicates per group. Seed 1×10^4 LLC cells (or other tumor cells) per well in a 96-well plate. Add 10 mg/mL GLP to the experimental group and an equal volume of DMEM+/+ medium to the control group. Add 100 μL PBS to the edge wells to prevent edge effects. Incubate the cells at 37°C with 5% CO_2 for 24 hours. Add 10 μL CCK-8 reagent to each well and incubate for 1 hour. Measure absorbance at 250 nm using a microplate reader.

1.2.8 MTT Assay for Evaluating GLP-Induced Tumor Cell Inhibition

The experiment included control and experimental groups, with five replicates per group. Seed 1×10^4 CT26 cells per well

in a 96-well plate. Add 10 mg/mL GLP to the experimental group and an equal volume of DMEM+/+ medium to the control group. Add 100 μL PBS to the edge wells to prevent edge effects. Incubate the cells at 37°C with 5% CO_2 for 48 hours. Add 10 μL MTT reagent to each well and incubate for 4 hours. Add solubilization solution (10% SDS, 5% isobutanol, 0.012 mol/L HCl, distilled water) and incubate for 24 hours. Measure absorbance at 490 nm using a microplate reader.

1.2.9 Statistical Analysis

Student's t-test was used for comparisons between two groups, and one-way ANOVA was used for comparisons among multiple groups. Homogeneity of variance was tested using Levene's test. If variances were homogeneous, multiple comparisons were performed using LSD and Dunnett's tests. If variances were not homogeneous, Dunnett's T3 correction was applied. Statistical analysis was conducted using SPSS 19.0 software, with a significance level of $\alpha = 0.05$.

2 Results and Analysis

2.1 Anti-Tumor Effects of Crude Ganoderma lucidum Polysaccharide (GLP)

2.1.1 Inhibition of Tumor Cells by GLP

Previous studies have shown that crude Ganoderma lucidum polysaccharide (GLP) exerts cytotoxic effects against various tumor cell lines [1]. In this study, the cytotoxic effects of 10 mg/mL GLP on five tumor cell lines, including LLC (murine lung carcinoma), A549 (human lung carcinoma), HepG2 (human liver carcinoma), J558 (murine myeloma), CT26 (murine colon carcinoma), and MDA (human breast carcinoma), were compared. The CCK-8 assay was used to measure cell viability. The CCK-8 reagent contains WST-8, which is reduced by dehydrogenases in viable cells to form a yellow formazan dye. The amount of formazan produced is proportional to the number of viable cells; a deeper color indicates a higher optical density (OD) value, corresponding to stronger cell viability. The experimental results are shown in Figure 1 (n = 3).

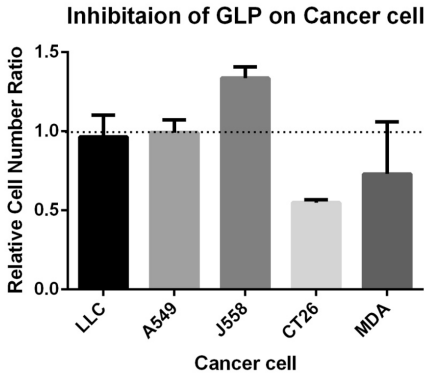

Fig. 1 Inhibition rate of GLP on five tumor cell lines.

In the figure, it can be observed that GLP shows no inhibitory effect on lung cancer cells (LLC, A549) but demonstrates growth-inhibitory effects on CT26 and MDA cells. Interestingly, GLP promotes the growth of J558 cells. For the three cell lines showing significant effects, the experiments will be repeated using different methodologies for further validation.

2.1.2 Cell Index Analysis of MDA Tumor Cell Inhibition by GLP

As shown in Figure 2, Ganoderma lucidum polysaccharide effectively inhibited the growth of MDA human breast cancer cells. Within the observation period (25 hours), the Cell Index value of the GLP-treated group (10 mg/mL) was 1.38 ± 0.31 (mean ± standard deviation), whereas the control group maintained robust tumor cell growth, with a Cell Index value of 1.93 ± 0.15 (mean ± standard deviation).

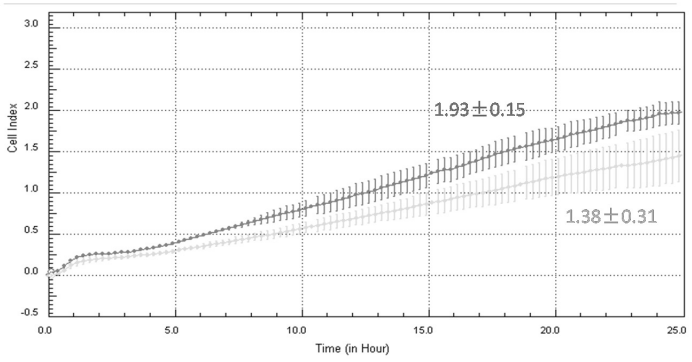

Fig. 2 Inhibition rate of GLP on MDA tumor cells.

2.1.3 MTT Assay Results for GLP Inhibition of CT26 Tumor Cells

Figure 3 shows the growth status of CT26 murine colon carcinoma cells after 3 days of incubation with GLP. In the MTT assay, viable cells reduce MTT to blue formazan crystals; therefore, a higher OD value corresponds to a higher number of viable cells. The mean OD value of the blank control group was 0.78, while the low-concentration GLP group had a mean OD value of 0.71, and the high-concentration GLP group had a mean OD value of 0.43. These results indicate that GLP exerts a significant inhibitory effect on CT26 cells, with statistically significant differences observed ($n = 3$, $p < 0.01$).

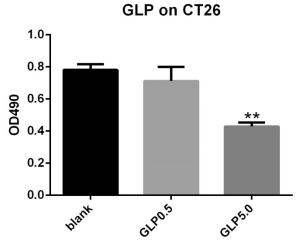

Fig. 3 Inhibitory Effect of GLP on CT26 Cells

2.1.4 CCK Assay Results for GLP Stimulation of J558 Tumor Cells

The stimulatory effect of GLP on J558 cells is shown in Figure 2 ($n = 3$, $p < 0.01$).

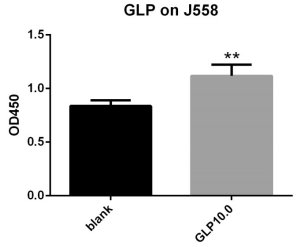

Fig. 4 Effect of GLP on J558 Tumor Cells

Interestingly, the mean OD value of the blank control group was 0.84, while that of the GLP-treated group reached 1.12. This indicates that GLP promotes the proliferation of J558 cells, with a statistically significant difference confirmed by analysis.

2.2 Stimulatory Effects of GLP on Immune Cells

2.2.1 Stimulation of Immune Cells by GLP

The target cells for stimulation were splenocytes from mice, with 5×10^6 cells per well seeded in 24-well plates, including

both adherent and suspended cells. The GLP concentration was 1.0 mg/mL. Figure 5 shows the morphology of adherent splenocytes after 2 days of culture under a microscope. Figure 7 presents the absolute counts of various immune cell subsets obtained through flow cytometry and cell counting (n = 3).

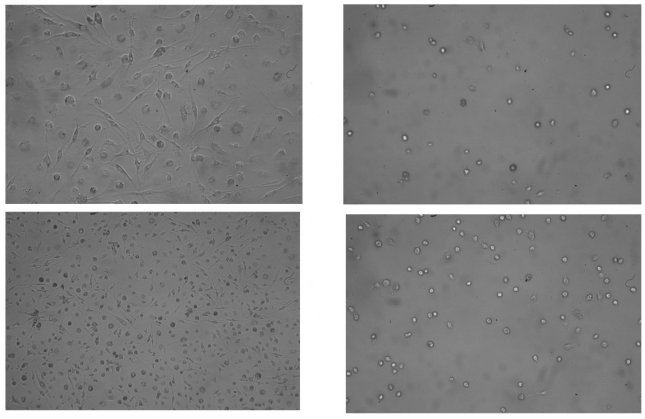

Fig. 5 Observation of Adherent Splenocytes Under Optical Microscope

The left side represents the GLP-treated group, and the right side represents the control group.The upper images are captured at 100× magnification, and the lower images are captured at 40× magnification.

As shown in Figure 5, the number of adherent immune cells in the GLP-treated group is higher than that in the control group, and the adherence status is improved compared to the control group. This indicates that GLP promotes the proliferation of adherent immune cells.

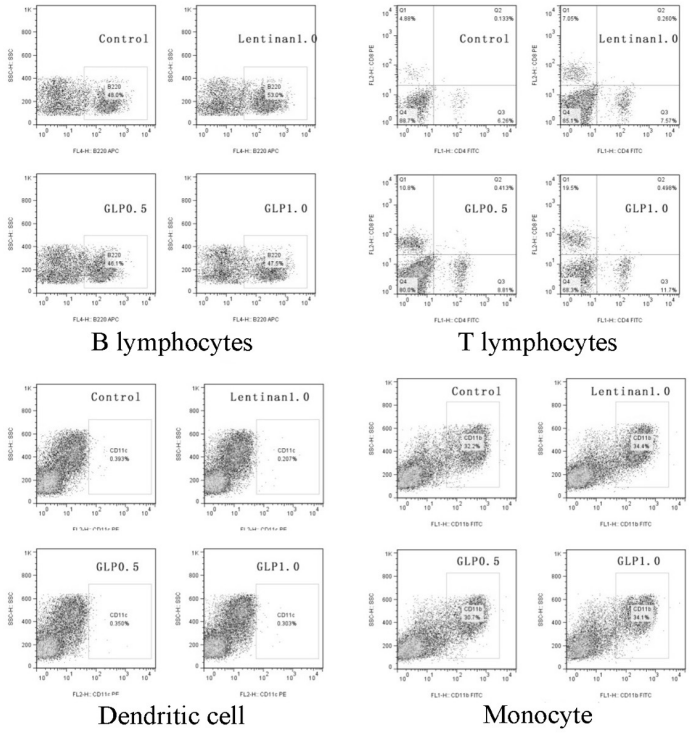

Fig. 6 Flow Cytometry Plots of GLP Effects on Immune Cell Subsets

Figure 6 shows the flow cytometry results for different immune cell subsets:

• B cell proportions: Control group: 48.0%, lentinan (1.0 mg/mL) group: 53.0%, GLP (0.5 mg/mL) group: 46.1%, GLP (1.0 mg/mL) group: 47.5%.

• CD8+ T cell proportions: Control group: 4.9%, lentinan (1.0 mg/mL) group: 7.0%, GLP (0.5 mg/mL) group: 10.8%, GLP (1.0 mg/mL) group: 19.5%.

• CD4+ T cell proportions: Control group: 6.3%, lentinan (1.0 mg/mL) group: 7.6%, GLP (0.5 mg/mL) group: 8.8%, GLP (1.0 mg/mL) group: 11.7%.

• Dendritic cell proportions: Control group: 0.39%, lentinan (1.0 mg/mL) group: 0.21%, GLP (0.5 mg/mL) group: 0.35%, GLP (1.0 mg/mL) group: 0.30%.

• Monocyte proportions: Control group: 32.2%, lentinan (1.0 mg/mL) group: 34.4%, GLP (0.5 mg/mL) group: 30.7%, GLP (1.0 mg/mL) group: 34.1%.

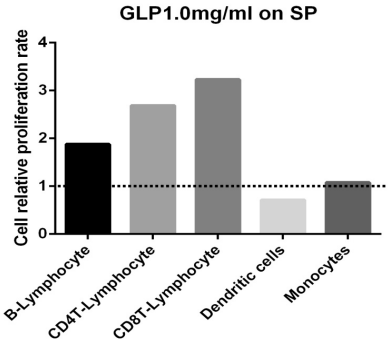

Fig. 7 Proliferation Rate of Immune Cell Subsets Induced by GLP (1.0 mg/mL)

The absolute counts of immune cell subsets were obtained using flow cytometry and cell counting methods, with the results shown in Figure 7. The horizontal axis represents the different immune cell subsets, and the vertical axis represents the relative proportions of each cell subset in the experimental group treated with GLP (1.0 mg/mL) compared to the blank control group. The relative proportions are as follows: B lymphocytes: 1.87, CD4+ T lymphocytes: 2.69, CD8+ T lymphocytes: 3.23, dendritic cells: 0.72, and monocytes: 1.08. These results indicate that GLP promotes the proliferation of B lymphocytes, CD4+ T lymphocytes, and CD8+ T lymphocytes.

2.2.2 Stimulatory Effects of GLP on CD4+ T Cells, CD8+ T Cells, and B Lymphocytes

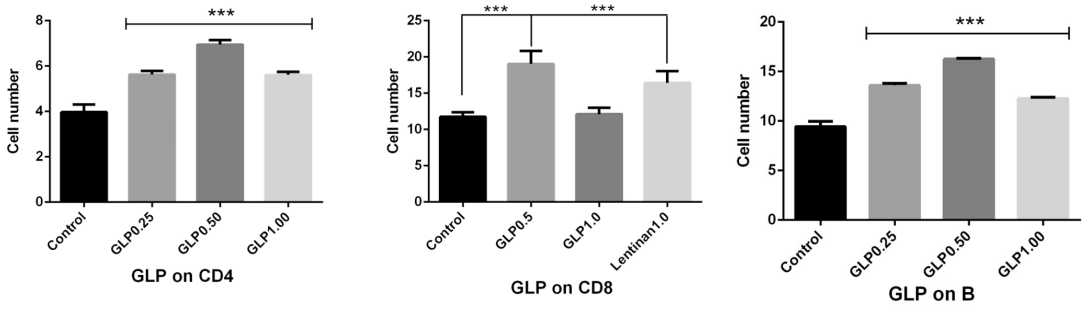

Fig. 8 Stimulatory Proliferation Effect of GLP on CD4+ T Cells, CD8+ T Cells, and B Lymphocytes

To validate the stimulatory proliferation effects of GLP, experiments were repeated using the three lymphocyte subsets

that showed significant responses. The results are presented in Figure 8, where the vertical axis represents the cell count (unit: 10^4). The data indicate that GLP significantly promotes the proliferation of CD4+ T cells, with statistically significant differences observed.

Subsequently, an extended experiment was performed on CD4+ T cells with four concentration groups: the control group (no GLP), the low-concentration group (0.25 mg/mL GLP), the medium-concentration group (0.5 mg/mL GLP), and the high-concentration group (1.0 mg/mL GLP), with six replicates per group.

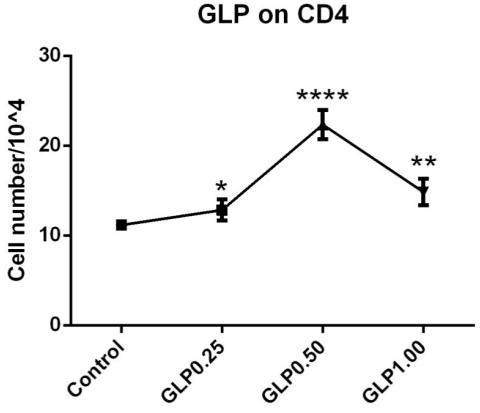

Fig. 9 Stimulatory Proliferation Effect of Different Concentrations of GLP on CD4+ T Cells

The stimulatory proliferation effects of different concentrations of GLP on CD4+ T cells are shown in Figure 6. It can be observed that all three concentration groups (low, medium, and high) significantly promoted CD4+ T cell proliferation. A dose-dependent effect was evident within the 0 mg/mL, 0.25 mg/mL, and 0.50 mg/mL concentration range. However, the effect at 1.0 mg/mL was weaker than at 0.50 mg/mL, suggesting that an excessively high concentration may inhibit CD4+ T cell proliferation.

In addition, since lentinan is known to promote immune cell proliferation, CD4+ T cells were stimulated with the same concentrations of GLP and lentinan (0.5 mg/mL). The proliferation results are shown in Figure 8. It can be seen that at 0.5 mg/mL, the stimulatory effect of GLP on CD4+ T cell proliferation was superior to that of lentinan.

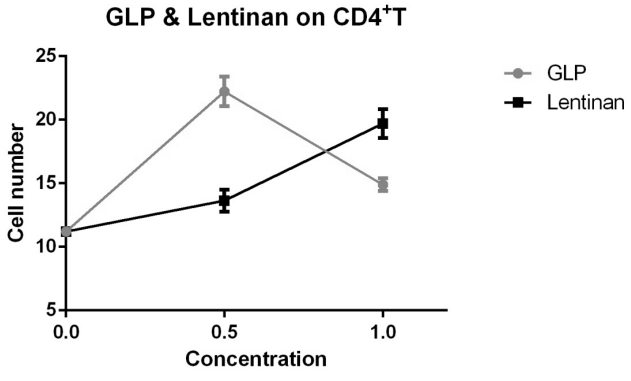

Fig. 10 Comparison of Stimulatory Proliferation Effects of GLP and Lentinan on CD4+ T Cells

2.3 Inhibitory Effects of GLP on CAF Cells

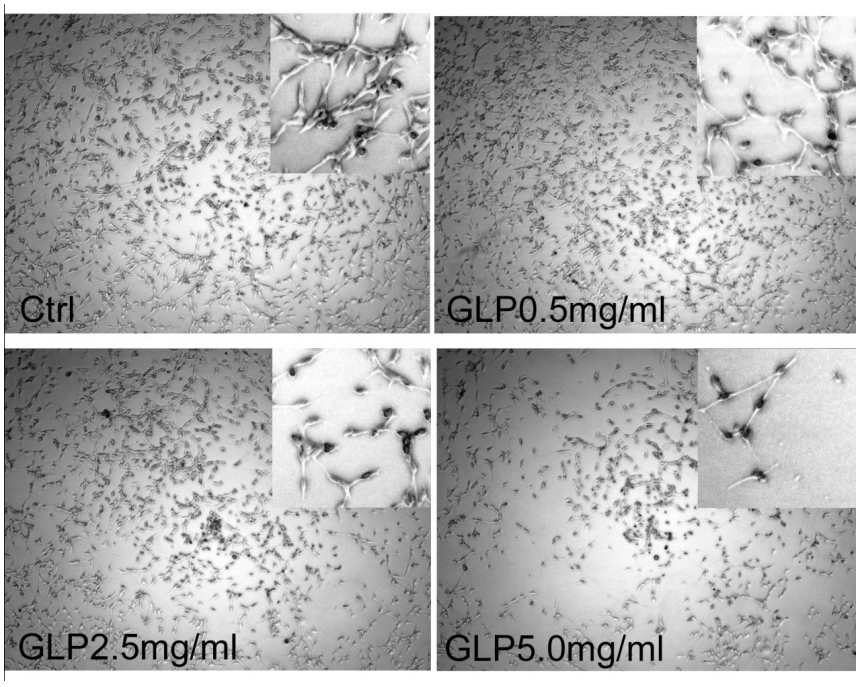

Fig. 10 Observation of GLP Inhibition on CT26-CAF Cell Growth

Figure 11 shows the effect of GLP on CT26-CAF cells observed under a microscope. A significant amount of cell death and poor growth status of CT26-CAF cells were observed under the influence of GLP. Moreover, as the GLP concentration increased, the number of CT26-CAF cells decreased.

The quantitative analysis of the inhibitory rate of GLP on CT26-CAF cells is shown in Figure 8. CT26-CAF cells were cultured in medium containing GLP at concentrations of 0.5 mg/mL and 5.0 mg/mL. After 3 days, the number of cells was measured using the MTT assay ($n = 3$, $p < 0.05$).

The results indicate that high concentrations of GLP significantly inhibited the growth of CT26-CAF cells.

3 Discussion

3.1 Analysis and Discussion

The anti-tumor effects of Ganoderma lucidum polysaccharides (GLP) have gained increasing attention in recent years. This study aims to explore the potential mechanisms by which GLP exerts anti-tumor effects.

The first hypothesized mechanism is the direct cytotoxicity of GLP against tumor cells. Using CCK-8, MTT, and Cell Index assays, our experiments showed that GLP exhibited different effects on various tumor cell lines. Specifically, GLP had no significant effect on LLC and A549 lung cancer cells but displayed a clear inhibitory effect on the proliferation of MDA human breast cancer cells and CT26 murine colon cancer cells. Interestingly, GLP was found to promote the growth of J558 murine myeloma cells. These findings suggest that the application of GLP requires a case-by-case approach based on specific clinical conditions rather than a generalized approach.

On the other hand, immune modulation represents another important pathway in anti-tumor therapy. This study

compared the proliferation of five immune cell subsets after GLP stimulation. The results demonstrated that GLP promotes the proliferation of CD4+ T cells, CD8+ T cells, and B lymphocytes. Both the relative and absolute numbers of these cell subsets increased significantly, with CD4+ T cells showing the most pronounced proliferation. Subsequently, experiments focused on CD4+ T cells using four different concentrations of GLP. A dose-dependent response was observed within the range of 0.0 mg/mL to 0.5 mg/mL. However, at higher concentrations, the stimulatory effect decreased, suggesting that the optimal concentration of GLP for CD4+ T cell proliferation is approximately 0.5 mg/mL. At this concentration, GLP outperformed lentinan, a known immune-stimulating anti-tumor agent, in promoting CD4+ T cell proliferation.

Cancer-associated fibroblasts (CAFs) play a critical role in tumor progression by secreting various bioactive factors that influence tumor growth, angiogenesis, invasion, and metastasis. Previous studies have shown that eliminating CAFs can lead to tumor regression, even without direct cytotoxic effects on tumor cells [4]. Although extensive research has been conducted on the cytotoxicity of GLP toward tumor cells, few studies have investigated its effects on CAFs. This study explored the inhibitory effects of GLP on CT26-CAF cells and revealed a potential new anti-tumor pathway involving CAF suppression.

3.2 Future Perspectives and Applications

Due to the widespread use of Ganoderma lucidum preparations, the anti-tumor effects of GLP, one of its primary active components, have garnered significant interest. This study investigated three potential anti-tumor pathways of crude GLP and provided preliminary evidence supporting its multifaceted anti-tumor effects, thereby contributing to the future application of GLP in anti-cancer therapies.

Furthermore, this study proposes an innovative hypothesis that GLP inhibits the proliferation of CAFs and provides preliminary evidence supporting this hypothesis. This finding offers a new direction for future research on GLP and its potential applications in cancer treatment.

4 Conclusion

Building upon existing research on the anti-tumor effects of GLP, this study preliminarily explored the potential mechanisms through which GLP exerts anti-tumor effects and proposed that GLP may act through multiple pathways. Compared with other studies, this research is the first to investigate the inhibitory effects of GLP on CAFs. Within the scope of the experiments conducted, the following conclusions were drawn:

1. The direct effects of crude GLP on tumor cells vary among different cell lines: GLP showed no cytotoxic effects on LLC murine lung cancer cells or A549 human lung cancer cells but exhibited inhibitory effects on MDA human breast cancer cells and CT26 murine colon cancer cells. Conversely, GLP promoted the proliferation of J558 murine myeloma cells.

2. Crude GLP promoted the proliferation of lymphocytes, with CD4+ T cells showing the most significant response, demonstrating a dose-dependent relationship.

3. Crude GLP inhibited the proliferation of CAFs.

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