Advances in Life Science for Adolescents

RESEARCH ARTICLES VOL 1 | Apr 2025 | doi:

Effect of CDAP on myeloid-derived suppressor cells

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Abstract:

The aim of this study is to investigate the influence of Cistanche deserticola polysaccharide (CDAP) on myeloid-derived suppressor cells (MDSCs) within the tumor microenvironment. MTT assays were used to assess the viability of MDSCs, normal fibroblasts (NFs), and cancer associated fibroblasts (CAFs) following exposure to various concentrations of CDAP. Additionally, RT–qPCR was used to measure the expression levels of VEGFA and TNF-γ in treated cells. Our results indicate that CDAP not only facilitates the growth of NFs while suppressing the occupancy of CAFs in the microenvironment but also diminishes the activity of MDSCs. Furthermore, CDAP-treated MDSCs exhibit upregulated TNF-γ expression alongside downregulated VEGFA expression. In conclusion, the traditional Chinese medicine polysaccharide CDAP exhibits a multifaceted capacity to enhance the tumor tissue microenvironment through its influence on various targets and pathways.

Keywords: *Cistanche deserticola* polysaccharide (CDAP); Myeloid-derived suppressor cells (MDSCs); Tumor microenvironment (TME); Cancer-associated fibroblasts (CAFs)

1. Background:

1.1 Tumor Microenvironment and Tumor Immunity

The tumor microenvironment has consistently been a focal point of oncological research. It serves to sustain the vigorous metabolism, proliferation, and differentiation of tumor cells as well as provide a microenvironment unfavorable for immune cell action. Nonneoplastic cells within the tumor microenvironment offer an aberrant nutritional milieu to nourish tumor cells and suppress the activity of immune cells, including NK cells and effector T cells, by secreting inflammatory factors and immunosuppressive agents. Thus, the cells in tumor tissues are essential, as they provide a conducive environment for the growth and proliferation of tumor cells [1].

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Received: Feb. 23, 2025.
Accepted: Apr. 09, 2025.

According to the cancer immune-editing hypothesis, the interplay between the immune system and tumor cells occurs in three phases: elimination, equilibrium, and escape. The establishment of a stable tumor microenvironment that favors tumor growth marks the transition to the second phase. Research suggests that disrupting such an aberrant environment is beneficial for eliminating tumor cells and inhibiting tumor progression [2].

1.2 The Role of Cancer-Associated Fibroblasts in the Tumor Microenvironment

Cancer-associated fibroblasts (CAFs) are an essential component of tumor tissues, providing mechanical support for tumor cells and shaping the entire tumor microenvironment. In comparison to normal fibroblasts, CAFs are spindle-shaped fibroblasts with a larger volume. CAF activity is regulated by growth factors secreted by tumor cells, and CAFs themselves can secrete growth factors such as fibroblast growth factor (FGF), stromal cell-derived

factor 1 (SDF1), matrix metalloproteinases (MMPs), and insulin-like growth factor 1 (IGF1). These factors promote further tumor growth, angiogenesis, and metastasis. Research has shown that the removal of fibroblasts from tumor tissue disrupts the tumor's immune escape mechanism, leading to a significant reduction in tumor size and cell death [3].

1.3 The Role of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment

Myeloid-derived suppressor cells (MDSCs) are a type of cell widely present in the infiltrated and noninfiltrated tumor microenvironment of cancers such as liver cancer, breast cancer, and ovarian cancer. MDSCs are recruited from the bone marrow by various cytokines, such as VEGF, GM-CSF, M-CSF, IL-6, IL-1β, and β-fibroblast growth factor (β-FGF). [4] MDSCs migrate from the vascular and lymphatic systems to the tumor microenvironment, where they proliferate and secrete cytokines such as TGF-β, which inhibits the immune response mediated by T cells and B cells. Additionally, MDSCs promote tumor proliferation, infiltration, and metastasis and enhance tumor survival and migration. Existing research has shown a correlation between high levels of MDSCs in the tumor microenvironment and poor prognosis and shorter survival in cancer patients. Specific removal of MDSCs from the tumor microenvironment in mice has led to significant reductions in tumor volume, highlighting the importance of MDSCs in tumor survival and proliferation. Targeting MDSCs with drugs such as anti-GT-1 antibodies, anti-GM-CSF antibodies, PGE2/COX-2 inhibitors, CXCR2/4 antagonists, and RA190 has been demonstrated to enhance 'chemotherapy effectiveness to eradicate tumor cells. Notably, immunotherapies that target CXCR4 and IL-10, which are characteristic of MDSCs, have significantly improved the effectiveness of PD-1 monoclonal antibody therapy for ovarian cancer. However, mature targeted therapies with COX-2 inhibitors such as nimesulide, meloxicam, and celecoxib exhibit strong gastrointestinal side effects and kidney toxicity. Therefore, the discovery of effective, natural compounds with low toxicity to suppress MDSCs in tumors is of great significance [5].

1.4 The Bioactivity of Cistanche deserticola Polysaccharide

Traditional Chinese medicine offers descriptions of tumors and cancer that are similar to those in Western medicine, and it has developed a unique system for understanding the etiology, pathogenesis, and treatment of malignant tumors since ancient times. Many diseases are closely related to immune system dysfunction, and tumor development due to weakened immune surveillance is one such association. Most traditional Chinese medicines do not have direct toxic effects to tumor cells, but they can inhibit tumor growth and development by enhancing and improving the immune functions of patients [6].

Chinese herbal polysaccharides are natural extracts of traditional Chinese medicines that are characterized by natural ingredients, minimal toxicity and side effects, and stable and long-lasting therapeutic effects. The primary effective ingredients of Chinese herbal polysaccharides function mainly by increasing host antibody concentrations and boosting immune cell activity for immunomodulation. Empirical evidence has demonstrated that different doses of Astragalus polysaccharide can upregulate the thymus and spleen indices in mice and increase the concentrations of interleukin-2 (IL-2), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in the serum, enhancing cell-mediated immunity and improving the immune surveillance capacity to inhibit tumor growth and development [7].

Cistanche deserticola (Fig. 1.a) is a yang-tonifying herb, and various historical texts, including Ben Jing and Yao Xing Ben Jing, have highlighted its efficacy in nourishing the viscera and inhibiting cancer development[8] .CDAP mainly containing active ingredients such as fucose, rhamnose, arabinose, et.[9] ,as shown in Fig. 1.a) Researches have proved those ingredients with natural anti-inflammatory, antifatigue effects[10] .We consulted the clinical database of traditional Chinese medicine and found that CDAP has been applied to various tumors, as shown in Fig. 1.c). Through bioinformatics analysis, CDAP has an influence in the breast cancer associated pathways, as shown in Fig. 1.b). However, the mechanism, dosage, and effectiveness of *C. deserticola* polysaccharide (CDAP) in anticancer treatment have not been verified by modern research. By Thus, in this experiment, the effects of CDAP on tumor microenvironment cells are studied from an immunological perspective.

2. Materials and Methods

2.1 Cell Lines: Myeloid-Derived Suppressor Cells (MSC2) and Hepatic Stellate Cells (LX-2)

The cellular resources employed in this investigation were procured from the Cell Repository at the Institute of Biophysics, Chinese Academy of Sciences. These resources encompassed myeloid-derived suppressor cells (MSC2), human hepatic stellate cells (LX-2), and human hepatic fibroblasts associated with breast cancer (ME-iLX-2). The LX-2 cell line, originated from hepatic stellate cells isolated from the livers of healthy adults. ME-iLX-2 cells were methodically generated within our laboratory by inducing hepatic stellate cells (LX-2) with TS/A breast cancer cells.

The cell culture medium was meticulously maintained utilizing high-glucose DMEM (Gibco, USA) supplemented with a 1:10,000 mixture of penicillin and streptomycin (Gibco) and enriched with 10% fetal bovine serum (FBS, Pan Biotech, Germany). Cultivation was carried out in a 37 °C incubator under a 5% CO2 atmosphere. After reaching confluence, the cells were detached with trypsin (Gibco). This was succeeded by a series of washes, centrifugation steps, and subsequent passages.

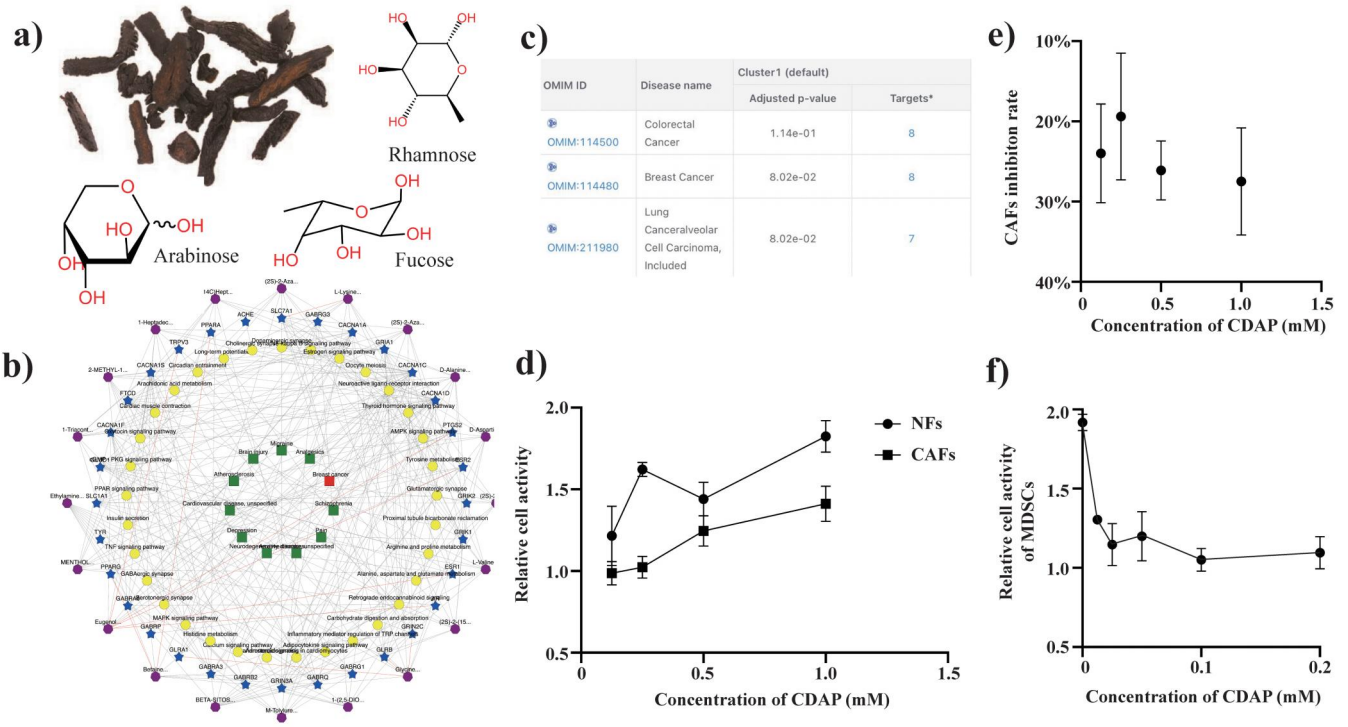

Fig. 1: a) Morphology of C. deserticola. **b)** pharmacological characteristics of Cistanche deserticola. **c)** Predicting targets of CDAP. **d)** Effects of CDAP on fibroblasts. **e)** CDAP suppresses CAF (ME-iLX-2 cell) viability. **f)** CDAP inhibits MDSC viability.

2.2 Extraction and Preparation of C. deserticola Polysaccharide (CDAP)

C. deserticola, a hallmark of traditional Chinese herbal medicine (depicted in Fig. 1.a), was used as the raw material for the extraction of CDAP. The preparation of CDAP was graciously performed by the State Key Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences. Extraction was performed by thermal water-ethanol precipitation, mainly include air-drying the C. deserticola at 40 ◦C and ground it into powder. Then treat the powder in ethanol(60◦C) for 3 h. Diluted the powder with water, refluxed at 90 ◦C, and centrifuged twice (1500× g) to separate the supernatant. Subsequently, 95% ethanol was added. 4◦C overnight and centrifuge (1500× g) twice. Repeat the cycle twice, resulting in 0.15% yield as well as 97.3% purity of polysaccharide. Following this, the resultant extract was subjected to phosphate-buffered saline (PBS) solution and subsequently passed through a 0.22 μm cell filter, culminating in the formulation of a 1 mg/mL stock solution that we denoted as CDAP.

2.3 MTT Cell Viability Assays

MTT experiments were executed with MSC2, ME-iLX-2, and LX-2 cells as the subjects of interest. Given the reduced proliferation of these cells, an augmented cell density was adopted for the initial seeding process. Specimens were judiciously sown within 96-well plates at a seeding density of 5×103 cells per well. Following a 12-hour incubation period at 37 °C under 5% CO2, the supernatant medium was meticulously aspirated. Then, 100 μL of the appropriate concentration of CDAP solution was introduced, and for an additional 24 hours. Following this incubation period, MTT solution was added, and the plates were subjected to an additional 4-hour incubation period at 37 °C. The formed formazan crystals were solubilized by the addition of lysis buffer (comprising 10 g of SDS, 5 mL of isobutyl alcohol, and 0.1 mL of 10 M HCl diluted to a final volume of 100 mL with double distilled water). After undisturbed incubation overnight to ensure complete dissolution, absorbance measurements were taken at a wavelength of 570 nm. The results obtained were normalized by PBS treatment group to determine relative cell viability, thereby offering insights into the cellular proliferation rates.

2.4 RT–qPCR analysis to Determine Relative mRNA Expression

After 72 hours of cocultivation of CDAP and cells in 6-well plates, total RNA was extracted with TRIzol reagent. The extracted RNA underwent reverse transcription into cDNA with the M-MLV reverse transcriptase system. Subsequently, primers targeting TNF-γ, VEGF, and GAPDH were used to perform real-time quantitative PCR (RT–qPCR). The housekeeping gene GAPDH was used as an internal reference. Subsequent data analysis was performed by determining the Ct values of the samples with Rotor Gene 5 software. These data allowed calculation of the relative VEGFA mRNA levels in the specimens through the utilization of the ΔΔCt method.

2.5 WB for protein expression analysis

LX-2, ME-iLX-2, MDSCs cells treated with CDAP for 72 hours were lysed on ice using RIPA lysis buffer (Biyuntian, China), and the collected proteins were quantified

using the BCA assay. Five times loading buffer was added to the remaining protein samples and incubated at 95°C in a metal bath for 10 minutes. Protein separation was performed by electrophoresis on a 12.5% polyacrylamide gel at 150V for 50 minutes. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore, USA) using the wet transfer method at 100V for 70 minutes. After transfer, the membrane was blocked with 3% BSA in PBS-T (containing 0.5‰ Tween-20) for 1 hour and washed five times with PBS-T (five minutes each). The membrane was then incubated overnight at 4°C with the primary antibody. After washing with PBS-T, an HRP-conjugated goat anti-rabbit IgG (Abclonal, USA) was used as the secondary antibody (dilution 1:3000) and incubated at room temperature for 1.5 hours. Following PBS-T washing, the membrane was exposed using a chemiluminescent substrate. b-actin (Abclonal, USA) was used as an internal reference, diluted at 1:6000, and VEGFA (Bioss, China) was diluted at 1:2000.

2.6 Data Analysis and Graphical Representation

Statistical analysis was performed by IBM SPSS version 22. To visually display the findings, GraphPad PRISM 5 software was used. Batman V2.0 was involved for pharmacological research. Furthermore, Adobe Illustrator was used for image annotation.

3. Results

3.1 CDAP inhibits ME-iLX-2 cell growth

Different concentrations of CDAP solution was added to cells in 96-well plates, and relative cell viability was determined using the MTT reagent. The results showed that under the influence of CDAP, the viabilities of both CAFs (ME-iLX-2 cells) and NFs (LX-2 cells) normalized to the control group(PBS treated) increased, indicating that CDAP did not exhibit significant toxicity to fibroblasts, as shown in Fig. 1.d).

Furthermore, CDAP had a greater effect on promoting NF (LX-2 cell) proliferation than on CAF (B-iLX-2 cell) proliferation (22.62%). After normalizing the relative activities of NFs at the same concentration, the calculated CAF relative inhibition rate is shown in Fig. 1e).

3.2 CDAP Suppresses MDSC Growth

Similarly, different concentrations of CDAP solution were added to the culture system with MDSCs (MSC2 cells), and after treatment with the MTT reagent and measuring the light absorbance, it was found that CDAP had a significant, concentration-dependent inhibitory effect on MDSC activity, as shown in Fig. 1.f).

3.3 CDAP Upregulates TNF-γ Expression in MDSCs

The MDSCs (MSC2 cells) that survived after culture with different concentrations of CDAP for 72 hours were

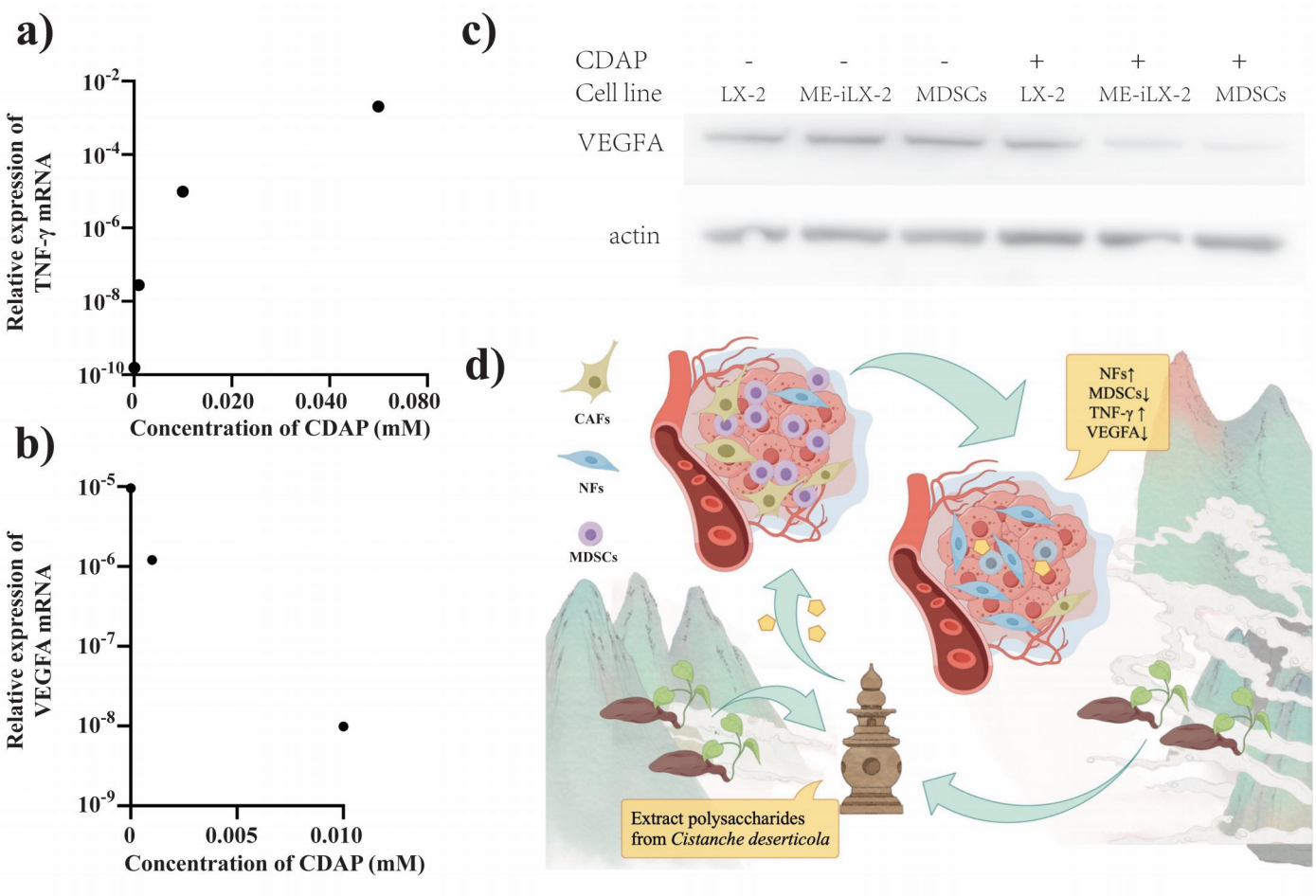

Fig. 1: a) Upregulation of TNF-γ by CDAP in MDSCs. **b)** Downregulation of VEGFA mRNA by CDAP in MDSCs. **c)** Downregulation of protein VEGFA by CDAP in MDSCs. **d)** The ability of CDAP to improve the tumor microenvironment.

used for this experiment. Total RNA was extracted from the samples using TRIzol total RNA extraction reagent, reverse-transcribed, and subjected to qPCR fluorescence analysis, which provided the cycle threshold (Ct) values for the corresponding gene in the samples. After processing, it was found that CDAP significantly upregulated TNF-γ mRNA in MDSCs in a concentration-dependent manner, as shown in Fig. 2a).

3.4 CDAP Downregulates VEGFA in MDSCs

Concurrently, the MDSCs and Me-iLX-2 that survived treatment with CDAP showed a significant decrease in VEGFA mRNA as well as protein expression, indicating that vascular endothelial growth factor A (VEGFA) was downregulated in these cells, but not in normal hepatic cells LX-2. This inhibitory effect was concentration dependent, as shown in Fig. 2b) & Fig. 2c).

4. Discussion

4.1 CDAP May Inhibit Self-Recruitment of MDSCs

Studies suggest that VEGFA is one of the important cytokines involved in the recruitment of MDSCs, and highly expressed VEGFA in MDSCs in the tumor microenvironment can create a positive feedback loop by recruiting more MDSCs and accelerating the deterioration of the tumor microenvironment. The results of this study suggest that CDAP may have a significant effect on breaking this cycle. On the one hand, CDAP can inhibit the proliferation of MDSCs, leading to a reduction in the number of MDSCs in the cell population. On the other hand, the MDSCs that survive treatment with CDAP lose their ability to express VEGFA, further reducing the recruitment effect of the VEGFA secreted outside the tumor tissue and reducing the recruitment of MDSCs.

Moreover, the decrease in the secretion of VEGFA can alleviate excessive angiogenesis in the tumor microenvironment and slow tumor proliferation, differentiation, and migration. It has been well documented that due to the high metabolic demands of tumor cells, tumors highly express VEGFA to increase the rate of angiogenesis, enhance the nutritional density in the local tissue, and promote rapid growth. By inhibiting the secretion of VEGFA by MDSCs, CDAP can somewhat alleviate these phenomena. Furthermore, VEGFA is associated with tumor cell survival and proliferation and acts downstream of VEGFR, regulating the ERK and MAPK pathways to ensure cell survival and promote cell proliferation. CDAP's ability to reduce VEGFA expression in the cells in the tumor microenvironment can slow these processes. Finally, VEGFR is associated with the reorganization of the cytoskeleton, the promotion of cancer cell infiltration and migration and acceleration of tumor development. CDAP may slow these processes by reducing the expression of VEGFA.

4.2 CDAP Suppresses Tumor Growth by Acting on MDSCs

Under the actions of CDAP, the density and activity of myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment significantly decrease, as does the recruitment of new cells from the bone marrow. The above pharmacological effects indicate that the function of the MDSC population decreases along with the concentration of immune inhibitory factors (TGF-β, IL-10, etc.) in the tumor microenvironment, allowing the immune system to no longer be restrained by suppressive cells and increasing the toxicity of immune cells to tumor cells, thus inhibiting tumor growth. Moreover, under the action of CDAP, TNF-γ expression in MDSCs is significantly increased, initiating the tumor cell TRADD protein complex and transmitting signals to caspase 3/7 via caspase 8/10 to initiate tumor cell apoptosis. Therefore, MDSCs that survive treatment with CDAP may not possess their original protumor growth function but may instead exhibit antitumor growth and anticancer functions.

4.3 CDAP Acts on Fibroblasts to Suppress Tumor Development

On the other hand, normal fibroblasts (NFs) in the tumor microenvironment, when under the influence of CDAP, showed increased activity, indicating that CDAP had no significant toxicity to normal cells. This ensures that under the influence of CDAP, normal human tissues can still grow and proliferate normally. However, CDAP displayed a weaker effect on promoting tumor-associated fibroblasts (CAFs), considering the competitive transformation relationship between NFs and CAFs. It can be concluded that CDAP indirectly inhibits the biological activity of CAFs, helping normal fibroblasts (NFs) occupy and improve the tumor microenvironment.

In summary, CDAP not only significantly reduces the population density of MDSCs, causing cells to lose their protumor activity, but also inhibits CAF activity and promotes NF growth. This demonstrates that CDAP, a traditional Chinese medicine polysaccharide, can improve tissue microenvironments as a multitarget, multipath agent.

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