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Effects of Shenling Baizhu Powder on the Proliferation and Invasion of Tumor Cells: Regulating Polarization of Macrophages in Co-Culture System

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WT, JH and WH conducted the experiments in this study. JW and HW contributed to the design and interpretation of the current study and wrote the article. All authors read, revised, and approved the final manuscript.

Key words
Shenling Baizhu Powder, Co-culture system, Macrophage polarization, Tumor cells, Proliferation, Invasion of tremor cells

ABSTRACT

The objective of this study was to explore the effects of Shenling Baizhu powder on the proliferation and invasion of tumor cells by regulating macrophage polarization in the co-culture system. Raw264.7 macrophages were added with IL-424h to lead M2-like macrophages, and the activation phenotype was identified. The Lewis lung cancer (LLC) mouse lung cancer cell line was used for experimental studies. The cultured cells were separated into six groups. RT-PCR was used to test the mRNA expression of iNOS and M2 type I arginase (ArgI) genes in M1 macrophages at 12h, 24h, 36h, and 48h. Using ELISA technology, the contents of cytokines IFN-γ and TNF-α in the co-culture supernatant were tested at 12h, 24h, 36h, and 48h. RT-PCR and Western blot were used to test PD-L1 mRNA and protein expression in lung cancer tumor cells of LCC mice. CCK-8 method was used to test the multiplication of LCC mouse lung cancer tumor cells, and flow cytometry was used to test cell apoptosis. Transwell chamber was used to test cell invasion ability. The cell viability of the co-cultured drug-added group was significantly lower than the co-cultured blank group at 12h, 24h, and 48h. Cell apoptosis in the co-cultured drug group was significantly higher than the co-cultured blank group at both 12h and 24h. The number of cell invasion in the co-culture blank group increased compared to the LLC blank group and LLC drug-added group, while the co-culture drug-added group showed reduction in the number of cell invasion compared to the co-culture blank group. Alteration in macrophage phenotype greatly hampers the growth and infiltration of lung cancer cells. The discovery not only enhances our comprehension of the functioning of Shenling Baizhu Powder, but also presents novel molecular indicators and possible treatment objectives.

INTRODUCTION

Lung carcinoma is a prevalent form of cancer globally, responsible for a significant number of fatalities (Li *et al*., 2021; Feng *et al*., 2021; Chen *et al*., 2023). Despite the considerable advancements in the governance of lung cancer, encompassing surgical interventions, radiation therapy, chemotherapy, and immunotherapy, the success rate in achieving a complete cure remains relatively low. Hence, it is crucial to investigate and advance novel therapeutic approaches and medications, specifically those capable of enhancing the tumor microenvironment. Tumor microenvironment is a complex system, including various cell types, cytokines, signal pathways and so on. Macrophages, being one of the key immune cells, have a crucial function in this particular setting (Stadler *et al*., 2021; Gu *et al*., 2022). In addition to their involvement in inflammation and tissue healing, they also have a significant effect on the advancement, expansion, and spread of tumors. Macrophages can be categorized into two phenotypes: M1 and M2. M1 macrophages exhibit anti-tumor properties, whereas M2 macrophages are commonly believed to promote tumor growth and facilitate immune evasion (Li *et al*., 2021; Min *et al*., 2022). Traditional Chinese medicine, with its extensive historical background and distinctive therapeutic principles, holds significant significance in the treatment of diverse ailments, encompassing cancer. Shenling Baizhu powder, a compound medication frequently employed in traditional Chinese medicine, possesses numerous

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biological properties such as immunomodulatory, and anti-cancer effects. Nevertheless, the precise mode of operation of this drug, particularly its impact on the tumor micro environment, remains incompletely comprehended. In recent research, there has been an increasing emphasis on investigating the impact of macrophages in the tumor microenvironment and exploring strategies to enhance the efficacy of tumor treatment through modulation of macrophage polarization. Considering the possible function of Shenling Baizhu Powder in immune modulation, it holds immense clinical importance to discuss its ability to control the growth and infiltration of cancer cells through its impact on macrophage polarization. This study aims to investigate the impact of Shenling Baizhu Powder on macrophage polarization and its subsequent effects on the multiplication of lung cancer cells using a co-culture system. Our objective with this study is to offer fresh perspectives and objectives for the holistic management of lung cancer, particularly in the field of immunotherapy.

MATERIALS AND METHODS

Experimental cells

American type culture collection (ATCC) provided Raw 264.7 macrophages, which were cultivated in DMEM enriched with high glucose. The medium was supplemented with 100μg/mL penicillin, 100mg/mL streptomycin sulfate, and 10% bovine serum. After exposing the cells to IL-4 for 24 h, we examined the resulting phenotype of the macrophages, which resembled M2-like characteristics. The Lewis lung cancer (LLC) cell line, derived from mouse lung cancer, was acquired from the Japan Research Biological Resource Cell Bank, which is situated in Osaka, Japan.

M2 type macrophages

Prior to culturing, every type of cell was pre-incubated in RPMI-1640 solution at a concentration of $1x10^6$ cells/mL, comprising of 10% FBS and 1% antibiotics. The experiment utilized cell culture inserts with a 24-well plate and an aperture size of 0.4μm. To begin with, a volume of 1 mL of culture solution containing $1x10^5$ LCC mouse lung cancer cells was introduced into the lower section of a 24-well plate. Then, 1 milliliter of culture medium containing 100,000 M2 macrophages was added to each cell culture insert. Next, the M2-type macrophage insert was placed into a 24-well plate with LCC mouse lung cancer cells.

Experimental groups

In accordance with the principles of medical laboratory animal science, the serum with Shenling Baizhu Powder was formulated and gathered by following the conversion of dosages between humans and rats. To prepare the medicated serum medium and control medium, the medium was diluted with medicated serum and normal serum, respectively. The experimental groups were: M2 blank group (1×10^6 cells/ml of M2 macrophages were introduced), M2 dosing group (1×10^6 cells/ml of M2 macrophages were introduced in serum containing the drug), LLC blank group (1×10^6 cells/ml of lung cancer tumor cells were introduced in the blank serum), LLC dosing group (introducing 1×10^6 cells/mL lung cancer tumor cells in serum containing the drug), blank group (introducing $1x10^6$ M2 macrophages and lung cancer tumor cells in the blank serum), and dosing group (introducing $1x10^6$ M2 macrophages and lung cancer tumor cells in serum containing the drug).

RT-PCR

At specific time intervals (12h, 24h, 36h, 48h) during the experiment, samples were obtained, and RNA was isolated from the cells by an RNA extraction kit following the guidelines provided by the manufacturer. Combine 1 microgram of RNA, an appropriate quantity of RNase-free water, and the cDNA synthesis reagent in a micro centrifuge tube. cDNA was generated by conducting a reverse transcription reaction in a thermal cycler. For PCR reaction specific primers for iNOS, ArgI, and PD-L1 were used besides PCR master mix, and synthetic cDNA. The PCR thermal cycle comprised of a pre-denaturation at 95 °C for 5 min. The prepared PCR reaction mixture was loaded into a PCR plate or a PCR tube and placed in the PCR machine with the set PCR program. Electrophoresis was used to confirm the validity of the samples.

ELISA assay

An enzyme-labeled instrument was used to measure the absorbance or fluorescence intensity of each well at a wavelength of 450 nm. Quantitative determination of IFN-γ and TNF-α in the sample was conducted by comparing it to the control group, which typically consists of a standard with a known concentration and a blank control.

Western blot analysis

The appropriately diluted protein sample using Laemmli buffer were denatured by heating at 95°C for 5 min and then subjected to SDS-PAGE to segregate proteins based on their size. Following electrophoresis, the protein was transferred to a membrane made of polyvinylidene fluoride (PVDF) or nitrocellulose using either the wet or semi-dry transfer technique. Following the completion of the transfer, the membrane underwent incubation in a blocking solution, typically consisting of 5% milk or BSA in TBST buffer, for a duration of 1 hour

to inhibit any nonspecific binding. Following the blocking step, the primary antibody targeting PD-L1 was applied, and subsequently, the membrane underwent incubation with the secondary antibody against the primary antibody (typically labeled with fluorescence or enzyme) for a duration of 1-2 h. The detection and quantification of PD-L1 protein expression were accomplished using a chemiluminescence substrate or fluorescence scanner.

CCK-8 method

The cells were grown in 96-well dishes, followed by the addition of CCK-8 reagent. OD values were measured using a microplate reader at various time intervals (e.g., 12 h, 24 h, 36 h, 48 h) at a wavelength of 450 nm.

Apoptosis

Cells were collected and stained with flow cytometry dye (Annexin V), and then analyzed by flow cytometry.

Transwell chamber test

Following pretreatment, the cells were placed inside the transwell chamber, which was subsequently transferred into an incubator and subjected to incubation at a temperature of 37°C and a CO_2 concentration of 5% for 12 h. Following the incubation period, the cells that did not successfully traverse the upper membrane were eliminated using cotton swabs. Staining required incubation for 10-30 min. Following the dyeing process, it was necessary to completely rinse off the surplus dye, and subsequently, the cells that passed were enumerated using a microscope.

Statistical analysis

The data was analyzed by SPSS16.0 software. The data is presented as the average plus or minus the SD. The distinction between the two groups was examined using a t-test, while the variation among multiple groups was analyzed through one-way ANOVA, followed by the implementation of the LSD test. A P-value less than 0.05 was deemed to be statistically significant.

RESULTS

The levels of iNOS mRNA were higher in the co-culture group than M2 dosing group and M2 blank group at 12h, 24h, 36h, and 48h (P<0.05). The expression of iNOS mRNA in the co-culture group was higher at 12h, 24h, 36h, and 48h than control group (P<0.05), suggesting that Shenling Baizhu Powder facilitated the conversion of M2-type macrophages to M1-type cells in the co-culture system (Table I).

The expression of ArgI mRNA in cells at different time points was detected using RT-PCR. The levels of ArgI mRNA were higher in the co-culture group than M2 dosing group and M2 blank group at 12H, 24H, 36H, and 48H (P<0.05). The expression of ArgI mRNA in the co-culture group gradually decreased from 12h to 48h, according to the time trend (P<0.05) (Table I).

Table I. Effect of Shenling Baizhu powder on the expression of iNOS and ArgI expressions as determined by RT-PCR ($\bar{x}$±s).

Group	12h	24h	36h	48h
iNOS expression				
M2 dosing group	1.00±0.06	10.59±1.52	1.61±0.15	3.89±0.10
Co-culture and dosing group	3.93±1.19	31.03±0.59	2.80±0.07	17.23±0.34
M2 blank group	0.53±0.03	5.48±0.35	1.70±0.32	4.00±0.18
Co-culture blank group	1.85±0.42	18.00±2.85	2.00±0.09	9.11±0.44
Variance ratio	15.038	24.557	11.312	17.604
P value	0.003	0.012	0.027	0.004
ArgI expression				
M2 dosing group	1.00±0.07	0.36±0.00	0.08±0.07	0.33±0.07
Co-culture and dosing group	76.69±6.32	43.59±8.26	28.40±1.52	14.78±1.32
M2 blank group	1.04±0.06	0.42±0.11	0.01±0.01	0.46±0.10
Co-culture blank group	65.94±12.84	24.72±3.07	29.39±7.96	8.27±0.49
Variance ratio	13.118	10.397	17.254	11.245
P value	0.013	0.008	0.005	0.016

Table II. Effect of Shenling Baizhu powder on the levels of TNF-α and IFN-γ as determined by ELISA ($\bar{x}$±s, pg/ml).

Group	12h	24h	36h	48h
TNF-α level				
M2 blank group	85.97±5.33	68.27±3.49	28.85±2.63	21.71±1.88
M2 Chinese medicine group	96.67±6.48	77.95±4.26	36.91±3.04	30.28±2.35
Co-culture blank group	39.06±3.16	39.30±3.27	54.17±3.27	30.28±2.26
Co-culture and dosing group	43.92±3.37	44.14±2.33	39.89±3.27	37.59±2.10
Variance ratio	18.339	12.103	5.651	3.506
P value	0.024	0.016	0.103	0.312
IFN-γ level				
M2 blank group	64.29±5.88	96.38±10.55	43.75±4.19	60.71±6.23
M2 Chinese medicine group	71.18±6.34	99.20±9.53	29.49±3.75	77.95±6.55
Co-culture blank group	35.09±2.65	46.36±4.33	55.21±5.36	77.54±6.88
Co-culture and dosing group	44.65±3.79	74.59±4.26	85.75±7.23	94.18±6.33
Variance ratio	4.105	3.664	12.358	11.416
P value	0.518	0.662	0.014	0.006

Table III. Effect of Shenling Baizhu powder on the levels of PD-L1mRNA as determinded by by RT-PCR and expression of PD-LI as determined by western blot assay ($\bar{x}$±s).

Group	12h	24h	36h	48h
PD-L1mRNA expression by RT-PCR				
LLC dosing group	1.00±0.05	2.19±0.31	2.70±0.11	2.94±0.31
Co-culture and dosing group	1.38±0.01	2.63±0.10	1.97±0.28	2.57±0.10
LLC blank group	0.80±0.02	1.90±0.07	2.67±0.35	3.54±0.22
Co-culture blank	1.44±2.24	2.74±0.20	1.93±0.20	2.85±0.58
Variance ratio	14.683	25.396	12.001	18.594
P value	0.026	0.017	0.006	0.034
PD-L1 expression by western blot				
LLC cells+blank serum treatment group	1.00±0.00	1.13±0.02	1.29±0.03	1.35±0.07
M2+LLC cell co-culture+blank serum treatment group	0.68±0.01	0.77±0.01	0.83±0.02	1.54±0.02
LLC cells+medicated serum treatment group	1.00±0.01	1.23±0.03	2.49±0.19	2.70±0.20
M2+LLC cell co-culture+medicated serum treatment group	0.20±0.01	0.33±0.05	0.41±0.03	0.31±0.03
Variance ratio	16.309	18.114	10.257	12.355
P value	0.003	0.026	0.014	0.005

The levels of TNF-α in the M2 blank group and M2 traditional Chinese medicine group were higher than the co-culture group at 12 and 24 h ($P<0.05$). No significant distinguish in TNF-α levels was observed between the groups at both 36 and 48 h ($P>0.05$) (Table II).

The IFN-γ level in the co-culture group increased over time, but there was no significant distinguish than the M2 blank group and M2 traditional Chinese medicine group($P>0.05$). The IFN-γ level in the co-culture group was higher than the co-culture blank group at 36h and 48h ($P>0.05$) (Table II).

The levels of PD-L1 mRNA were elevated in the co-culture group compared to both the LLC dosing group and the LLC blank group ($P<0.05$). Additionally, the co-culture dosing group exhibited decreased PD-L1 mRNA expression in comparison to the co-culture blank group at 12h, 24h, and 48h ($P<0.05$) (Table III).

Table IV. Effect of Shenling Baizhu powder on cell viability (detected by CCK-8), apoptosis (detected by flow cytometry) and invasion of LLC cells ($\bar{x}$±s).

Group	12H	24H	36H	48H
Cell viability				
LLC blank 5 group	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
LLC dosing 2 group	1.05±0.02	1.15±0.03	1.17±0.02	1.02±0.01
Co-culture blank 6 group	0.58±0.05	0.71±0.03	0.72±0.03	0.59±0.03
Co-culture and dosing group 3	0.39±0.04	0.51±0.05	0.50±0.05	0.43±0.03
Variance ratio	11.660	13.274	9.335	12.152
P value	0.031	0.026	0.005	0.013
Apoptosis				
LLC blank 5 group	9.57±2.36	11.77±1.53	16.46±3.22	17.35±2.50
LLC dosing 2 group	9.94±1.88	10.98±2.06	13.87±4.05	16.36±1.43
Co-culture blank 6 group	21.56±2.27	25.67±3.19	32.11±3.26	49.90±3.15
Co-culture and dosing group 3	29.84±3.55	30.66±4.15	35.12±2.04	45.13±2.66
Variance ratio	10.251	9.276	3.108	1.445
P value	0.026	0.017	0.336	0.529
Cell invasion				
LLC blank 5 group	212.58±11.48	180.73±10.26	183.25±8.44	186.19±12.53
LLC dosing 2 group	97.44±8.55	122.45±14.27	123.30±10.55	104.26±7.29
Co-culture blank 6 group	228.44±13.26	223.41±10.44	242.33±16.59	251.36±14.59
Co-culture and dosing group 3	164.38±10.26	158.44±12.73	151.02±11.63	149.35±13.27
Variance ratio	15.051	9.446	13.289	15.304
P value	0.015	0.026	0.007	0.002

The findings indicated that the levels of PD-L1 protein were greater in the co-culture group than LLC dosing group and LLC blank group (P<0.05). Additionally, the horizontal of PD-L1 protein were lower in the M2+LLC cell co-culture + dosing serum treatment group than in the M2+LLC cell co-culture + blank serum treatment group at 12h and 24h (P<0.05) (Table III).

The cell viability of the co-culture group was significantly lower than the co-culture group at 12, 24, and 48 h (P<0.05) (Table IV). At 12 h and 24 h, the apoptosis of the co-culture group with drugs was higher than that of the co-culture blank group (P<0.05) (Table IV). At 12H, 24H, 36H and 48H, the number of cell invasion in co-culture blank group raised than LLC blank group and LLC dosing group (P<0.05), and the number of cell invasion in co-culture dosing group reduced than in co-culture blank group (P<0.05) (Table IV).

DISCUSSION

This study extensively examined the effects of Shenling Baizhu powder, a traditional Chinese medicine, on the multiplication and invasion of LLC. The main focus was on its ability to regulate macrophage polarization. Macrophages play an extremely complicated role in tumor microenvironment, because they can present different polarization states according to microenvironment conditions (Chen *et al*., 2022; Qin *et al*., 2022; Li *et al*., 2021). When it comes to the growth of tumors and evasion of the immune system, M2-type macrophages are commonly seen as providing assistance to other cells. Not only do they facilitate the proliferation and infiltration of cancerous cells, but they also impede the immune system's reaction, thereby exacerbating the gravity of the ailment (Hao *et al*., 2023; Chen *et al*., 2022; Li *et al*., 2023). The multiple sets of data from this experiment provide strong evidence that Shenling Baizhu Powder has the ability to alter the polarization direction of macro phages. In particular, the findings from the experiment indicated a notable rise in the mRNA levels of iNOS (Nitric Oxide Synthase), whereas the mRNA levels of ArgI (Arginase) exhibited a corresponding decline subsequent to the

introduction of Shenling Baizhu Powder to M2-type macrophages. This change is crucial because iNOS and ArgI are generally regarded as the symbolic molecules of the polarization state of M1 and M2 macrophages (Liu *et al*., 2021; Qian *et al*., 2022). By facilitating the shift from M2 to M1, it is suggested that Shenling Baizhu Powder has the potential to enhance the tumor micro environment. It should be noted that M1 macrophages exhibit enhanced anti-cancer properties and are capable of efficiently eradicating tumor cells (Liu *et al*., 2022; Wu *et al*., 2022; Gu *et al*., 2022; Sun *et al*., 2021). The potential impact of this experimental finding could be extremely significant. The tumor microenvironment has consistently posed a challenging issue in the field of cancer therapy, particularly concerning the evasion of the immune system by tumors (Kim *et al*., 2021; Pang *et al*., 2021; Tao *et al*., 2022; Song *et al*., 2022). The findings of this research demonstrated that Shenling Baizhu Powder has the ability to modify the polarization state of macrophages, potentially aiding in the resolution of this issue. When combined with contemporary anticancer medications, Shenling Baizhu Powder can serve as a supplementary approach to cancer treatment, offering a fresh perspective and potentially enhancing the overall therapeutic outcome.

Studying the relationship between the tumor microenvironment and immune cells is a crucial and evolving area of research (Tada *et al*., 2021; Han *et al*., 2023). In this study, the potential mechanism of Shenling Baizhu Powder, a traditional Chinese medicine, in regulating the interaction between LLC and macrophages was further investigated. This drug exhibits its distinctive and significant biological activity, particularly in the expression of cytokines and immune response. Regarding immune cytokines, while there was no notable disparity in the TNF-α levels among various experimental groups, there was a substantial rise in IFN-γ within the co-culture and drug-supplemented group. The importance of this observation cannot be overstated as IFN-γ is commonly considered the primary signaling molecule for initiating Th1 immune response. The activation of Th1 immune response by Shenling Baizhu Powder may effectively enhance tumor microenvironment, as indicated by the high levels of IFN-γ, leading to improved anti-tumor activity. Additionally, we noticed a substantial decline in the manifestation of PD-L1 during the trial. PD-L1 functions as an immune checkpoint protein that interacts with the PD-1 receptor, leading to the suppression of T cell function and facilitating immune evasion. Hence, the intriguing and significant finding of reduced PD-L1 mRNA and protein expression implies that Shenling Baizhu Powder could potentially combat the tumor's immune evasion mechanism. According to the perspective of cell growth and cell death, our findings also demonstrate a significant reduce in the viability of LLC cells and a raise in apoptosis following the administration of Shenling Baizhu Powder. The findings align with previous research on macrophage polarization and immune factor expression, providing further evidence of the notable anti-tumor efficacy of Shenling Baizhu Powder. The Transwell chamber experiment also confirms this perspective, indicating that Shenling Baizhu Powder has a notable impact on diminishing the invasive potential of lung cancer cells. Slowing down the advancement and spread of the tumor has direct clinical importance due to the decrease in invasive capability.

This study effectively illustrates the multi-tier impact of Shenling Baizhu Powder on the regulation of the lung cancer microenvironment, as indicated by the aforementioned findings. Specifically, the medication has demonstrated impressive capability in controlling the characteristics of tumor-related macrophages within the tumor environment, decreasing the manifestation of PD-L1 in cells affected by lung cancer, and enhancing the Th1 immune reaction. Shenling Baizhu Powder has the ability to alter the tumor microenvironment according to macrophages, particularly by facilitating the conversion of M2-type macrophages into M1-type macrophages. Macrophages of the M1 phenotype exhibit potent anti-cancer properties and collaborate with Th1-type immune response to establish a tumor-hostile microenvironment, inhibiting tumor proliferation and metastasis. By altering cytokines and signal pathways, it is possible to achieve this transformation, where the changes in mRNA expression of iNOS and ArgI serve as observable and quantifiable molecular markers. We noticed a significant decrease in the expression of PD-L1, which is an extremely remarkable discovery. Reducing the expression of PD-L1, which acts as an immune checkpoint molecule, can be beneficial in diminishing the immune evasion capability of lung cancer cells. The reason for this alteration could be attributed to the fact that Shenling Baizhu Powder facilitates the conversion from M2 to M1, resulting in the decrease of PD-L1 expression on tumor cell surfaces. The reduction in PD-L1 levels can serve as a possible target for therapy and a molecular indicator for clinical intervention.

CONCLUSION

This study emphasizes the important function of Shenling Baizhu Powder in controlling the polarization of macrophages in the co-culture setup, consequently impacting the growth and infiltration of lung cancer cells. The data from the experiment indicate that the introduction of Shenling Baizhu Powder leads to a conversion of M2-type macrophages into M1-type macrophages that possess

anti-cancer properties. This transformation is evident in the rise of iNOS mRNA levels and the decline of ArgI mRNA levels. The morphological change of macrophages greatly hinders the growth and infiltration of lung tumor cells. The results not only enhance our comprehension of the functioning of Shenling Baizhu Powder, but also offer novel molecular indicators and potential treatment objectives, particularly in the approach to lung cancer treatment, as immunotherapy is progressively gaining prominence.

DECLARATIONS

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IRB approval

This study was approved by The Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, China.

Ethical approval

The study was carried out in compliance with guidelines issued by Ethical Review Board Committee of The Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, China. The official letter would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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