Crosstalk between lung cancer cells and macrophages contributes to traditional Chinese medicine Feiyanning-induced anti-tumor activities by suppressing M2 macrophage polarization

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New Phenomenon

Crosstalk between lung cancer cells and macrophages contributes to traditional Chinese medicine Feiyanning-induced anti-tumor activities by suppressing M2 macrophage polarization

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Running title: Feiyanning inhibits M2 macrophage polarization

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Lung cancer is one of the leading cancers with high morbidity and fatality worldwide [1]. There are two major types of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for nearly 90% of all lung cancer cases [2]. Although surgery, radiation therapy, chemotherapy, and immunotherapy [3] have been clinically used, the overall survival rate
for lung cancer patients remains at approximately 19%, and the 5-year survival rate is only 5% [4].
Therefore, alternative strategies are urgently needed to improve lung cancer treatment.

Parallel to the Western medicine system, traditional Chinese medicine (TCM) is an important
complementary medicine for cancer treatment in China and other regions of Asia. Feiyanning, a
TCM formula consisting of 11 herbs, has been clinically used for more than 20 years in NSCLC
patients with the benefits of suppressing tumor spread, prolonging patient survival and improving
quality of life [5]. Although clinically effective, the underlying mechanisms of Feiyanning against
lung cancer are not well understood.

Remodeling of the tumor microenvironment (TME) is proposed to be one of the anti-tumor
mechanisms of TCM [6]. Infiltration of the TME by immune and inflammatory cells is involved in
tumor formation and progression. Among tumor-infiltrating cell populations, tumor-associated
macrophages (TAMs) are one of most abundant components and exhibit different phenotypes and
functions in response to various microenvironmental signals derived from tumor and stromal cells
[7]. TAMs can be stimulated to polarize into two phenotypes, classically activated phenotype (M1)
and alternatively activated phenotype (M2) TAMs, which exert different effects on cancer cells. M2
TAMs exhibit pro-tumor effect. Suppression of M2 TAMs is emerging as a promising strategy in
cancer treatment.

Considering the hypothesis that TCM exerts anti-tumor activity through modulation of tumor
microenvironment, we asked whether Feiyanning plays a role in altering the TAM population. Due to
the ethical and technical challenges of obtaining sufficient TAMs from lung cancer patient biopsies,
we carried out in vitro differentiation of human monocyte THP-1 cells to acquire TAMs [8] and
characterize the interaction between A549 cells and TAMs. THP-1 cells were differentiated by PMA
for 24 h and polarized into macrophages, and the THP-1-derived macrophages were further cultured
with A549-conditioned medium for 48 h to generate TAMs (Supplementary Figure S1). To
investigate the interaction of A549 cells and TAMs, lung cancer cells and macrophages were co-
cultured using a cell culture insert with a 0.4-µm porous membrane to separate the top and bottom
chambers in Feiyanning-containing medium (serum free) (Supplementary file). The co-culture in the
absence of Feiyanning was used as the control (Figure 1A).

We first examined the influence of Feiyanning on A549 proliferative activity through a CCK-8
assay and found that the half inhibitory concentration at 48 h (IC_{50}) was 260 µg/mL (Supplementary
Figure S2). The effect of Feiyanning on the migration of A549 cells was then examined via a wound
healing assay (Supplementary file). Figure 1B shows that TAMs promote the migration of lung
cancer cells. Compared with the control (the absence of Feiyanning and macrophages), we observed
that Feiyanning attenuated the TAM-enhanced migration of A549 cells. We further evaluated the
effect of Feiyanning on the invasion of co-cultured A549 cells via Transwell assays. Similar
phenomena were observed as those in the wound healing assay (Figure 1C). Collectively, these
observations indicate that Feiyanning inhibits TAM-mediated migratory and invasive activities of
lung cancer cells.

We next asked whether Feiyanning modulates macrophage polarization for its anti-tumor
activities. To address this question, we incubated macrophages in A549-conditioned medium for 96 h
and examined macrophage markers by flow cytometry. We found no obvious alteration in the
abundance of M1 macrophage marker CD86-positive cells (Figure 2A, B). In contrast, we observed
an increase in the abundance of M2 macrophage marker CD163-positive cells when compared with
the control, indicating that A549 facilitates M2 macrophage polarization (Figure 2A, C). More
importantly, we found that the abundance of CD163-positive cells decreased when Feiyanning was
supplemented in the conditioned medium for 48 or 96 h, indicating that Feiyanning inhibits M2
macrophage polarization in a time-dependent manner (Figure 2A, C). Intriguingly, a very recent
study reported that Feiyanning inhibits M2 macrophage polarization in vivo [9]. These results implied
that Feiyanning could attenuate M2 macrophage polarization triggered by A549-derived conditioned
medium.

In addition to the direct influence of Feiyanning on M2 macrophage polarization, there is another
possibility that Feiyanning exerts an inhibitory effect on macrophage polarization through A549
cells. To test this hypothesis, we performed transcriptome analysis of Feiyanning-treated A549 cells
that were co-cultured with macrophages through RNA-seq. The raw sequencing data are available in
the EMBL database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-12420.
Compared with co-cultured A549 cells in the absence of Feiyanning, 1,339 differentially expressed
genes (DEGs) were identified in Feiyanning-treated A549 cells (P value < 0.05, |fold change| > 2),
including 594 upregulated genes and 745 downregulated genes (Figure 2D). To understand the
biological relevance of these DEGs, we performed gene ontology (GO) analysis with the DEGs.
Among the top 20 enriched cell component items (Figure 2E), extracellular matrix and extracellular
exosome have been reported to be involved in macrophage polarization [10]. These findings suggest
that Feiyanning potentially modulates the expression program of A549 cells to affect macrophage
polarization.

In conclusion, our findings provide evidence for the crosstalk between macrophages and lung
cancer cells and suggest that Feiyanning potentially contributes to anti-tumor activities by
suppressing M2 macrophage polarization.
Funding
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Conflict of Interest
The authors declare that they have no conflict of interest.

Reference


**Figure legends**

**Figure 1. Feiyanning inhibits the migration and invasion of A549 cells induced by TAMs in vitro**  (A) Schematic diagram of co-culture system in vitro. (B) The effect of Feiyanning on the migration of co-cultured A549 cells was examined through a wound healing assay. Scale bar: 100 μm. (C) The effect of Feiyanning on the invasion of co-cultured A549 cells was examined through Transwell assays. Scale bars: 500 μm for 4× and 100 μm for 10×. Data are expressed as the mean ±SD. ****P < 0.0001, ***P < 0.001, **P < 0.01.

**Figure 2. Feiyanning inhibits M2 macrophage polarization in vitro**  (A-C) TAMs derived from THP-1 cells were incubated in A549-conditioned medium (CM) with or without Feiyanning treatment. These cells were analyzed through flow cytometry with the macrophage M1 marker CD86 (A, B) and the M2 marker CD163 (A, C). (D) Volcano plot showing the DEGs between the two groups with upregulated genes in red and downregulated genes in blue. (E) GO analysis of DEGs with significant changes in expression (|Fold change| > 2). ****P < 0.0001, ***P < 0.001, **P < 0.01.
A

B

C

150x194mm (300 x 300 DPI)
Supplementary Materials

Materials and Methods

Preparation of Feiyanning formula

Feiyanning consists of 11 herb medicines and was obtained from Shanghai chest hospital, Shanghai Jiao Tong University. The components of Feiyanning were shown in Supplementary Table S1. The herb mixture was processed as we previously described [1]. The prepared formula frozen powder was aliquoted into small portions of 15-20 g and diluted into various concentrations for cellular assays. Mass spectrographic fingerprints of the herb formula extract were examined for quality check.

Cell culture and reagents

Human lung adenocarcinoma cell line A549 was purchased from Cell bank of the Chinese Academy of Sciences (Shanghai, China). Immortalized human monocyte cell line THP-1 was kindly provided by Professor Zhifeng Shao’s lab in Shanghai Jiao Tong University. Cells were cultured in RPMI-1640 medium (Servicebio, Wuhan, China) supplemented with 10% FBS (Invitrogen, Carlsbad, USA) and 1% penicillin-streptomycin (Invitrogen) under the condition of 37°C and 5% CO₂. Both cell lines were examined for mycoplasma contamination.

Macrophage polarization

THP-1 cells were differentiated by 100 ng/mL PMA (Sigma-Aldrich, St Louis, USA) for 24 h and polarized into macrophages. To obtain TAMs, THP-1-derived macrophages were cultured with A549-conditioned medium for 48 h as previously described [2]. The effect of Feiyanning on macrophage polarization was examined at 48 h or 96 h after incubation in A549-conditioned medium, respectively. The medium was refreshed every 48 h.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from A549 or TAMs using RNA-easy Isolation Reagent (Vazyme Biotech, Nanjing, China) and reversely transcribed into cDNA using PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer’s protocol. The quantitative PCR reactions were performed using SYBR ® Green Pro Taq HS Premixed qPCR Kit II (Accurate Biotechnology, Changsha, China). The reactions were run on ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA). Relative expression of the examined genes was measured by 2^ΔΔCt calculation. The sequence of the primers in this study were listed in Supplementary Table S2.
**Flow cytometry**

Macrophages were processed into single cell suspension, and incubated with antibodies (PE Mouse Anti-Human *CD86* and FITC Mouse Anti-Human *CD163*, both from BD Biosciences, San Diego USA) for 1 h at room temperature. The cells were washed twice and suspended in 500 μL phosphate-buffered saline (PBS) for analysis. Flow cytometry was performed on FACS Calibur flow cytometer (BD Biosciences) and the results were analyzed by FlowJo software (Treestar, Ashland, USA).

**Co-culture assay**

The lung cancer cells and macrophages were co-cultured using a cell culture insert (Corning, Steuben County, USA) with a 0.4-μm porous membrane to separate the top and bottom chambers. THP-1 monocytes were differentiated as described above. After differentiation, macrophages (1 × 10⁵ cells) were washed three times with PBS and placed in 6-well plates where A549 cells (5 × 10⁵ cells/well) were preplated. A549 cells and macrophages were cultured in the Feiyanning-containing medium (serum free). The co-culture in the absence of Feiyanning was used as the control.

**Cell viability assay**

Cell viability assay was conducted by the Cell Counting Kit-8 (CCK-8; Sangon, Shanghai, China). A549 cells were seeded in 96-well plates (3.5 × 10^3 cells/well) and cultured for 24 h. These lung cancer cells were then incubated in the medium containing various concentrations of Feiyanning (0, 75, 150, 225, 300, 400, 500, 750, and 1000 μg/mL) for 24, 48 and 72 h, respectively. After Feiyanning treatment, 10 μL of CCK-8 solution was added into each well and incubated for 3 h. The absorbance was measured at 450 nm with a spectrophotometric plate reader (Omega Bio-Tek, Norcross, USA).

**Wound healing assay**

Wound-healing assay was performed as we reported previously [3]. A549 cells were seeded in 6-well plates. Upon reaching confluency, cells were serum-starved for 24 h, and then the cell layer was scratched with a sterile plastic tip and washed twice with PBS and cultured in RPMI-1640 (serum free) at 37°C in a humidified incubator with 5% CO₂. At different time points, photo images of the plates were taken under a microscope.

**Transwell migration and invasion assays**
Transwell assays were performed as we described previously [4]. In brief, cell migration assay was performed in 24-well Transwells (8 μm pore size; Corning) without Matrigel, and cell invasion assay was performed in 24-well Transwells pre-coated with Matrigel. In each well about 4×10^4 A549 cells were plated in top chamber in serum-free medium, while 750 μL of 20% FBS-containing medium was placed in the bottom chamber. After 24 h of incubation, cells adhering to the bottom surface were fixed with 4% paraformaldehyde and stained by 0.1% crystal violet for visualization.

**RNA-seq assay**

Total RNA was extracted using RNA-easy Isolation Reagent (Vazyme Biotech, Nanjing, China), and the purified mRNA was used to generate the strand-specific RNA-seq libraries with NEBNext Ultra Directional RNA Library Prep Kit (NEB, Ipswich, USA). In brief, the fragmented mRNA fractions with the size of 300 - 500 bp were reversely transcribed into cDNA, and further synthesized into double-strand DNA. The resulting double-strand DNA fragments were purified with AMPure beads (Beckman Coulter, Pasadena, USA) and ligated with deep-sequencing adapters. The RNA-seq libraries were sequenced with HiSeq X (Illumina, San Diego, USA). The raw sequencing data are available in the EMBL database (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-12420.

**Bioinformatics analysis**

Bioinformatics analysis was basically performed as we described previously [1]. The raw sequencing reads were mapped to the human genome (hg19) using TopHat v2.1.1 [5]. Differentially expressed gene analysis was carried out by comparing genes in Feiyanning-containing co-cultured A549 cells with those in co-cultured A549 in the absence of Feiyanning using cuffdiff v2.2.1. Cufflinks v2.2.1 [5] was used to quantify their expression by fragments per kilobase of transcript per million reads. Gene ontology (GO) analysis was performed using Gene Ontology Resource (http://geneontology.org/). DAVID was used for KEGG pathway analysis (https://david.ncifcrf.gov/tools.jsp).

**Statistical analysis**

All experiments were conducted at least twice independently. Statistical analysis was performed with GraphPad Prism 8.0 software (La Jolla, USA) and the results were represented in the form of Mean ± standard deviation (SD). The one-way ANOVA and unpaired t test were used to examine statistical
significance of differences between groups. Differences were considered to be significant if \( P \) value was less than 0.05.

**Supplementary Figure S1. Establishment of human tumor-associated macrophages in vitro**

(A) Representative images of THP-1 and the differentiated macrophages. Scale bar: 100 \( \mu \)m. (B) The expression of pan-macrophage markers \( CD11b \) and \( CD68 \) were evaluated by RT-PCR. All results were normalized by the genes expression in THP-1 cells. (C,D) Flow cytometry indicated the presence of M1 phenotype marker \( CD86 \) (PE Mouse Anti-Human \( CD86 \)) and M2 marker \( CD163 \) (FITC Mouse Anti-Human \( CD163 \)) on the surface of TAMs. Data are expressed as the mean±SD. ****\( P < 0.0001 \), ***\( P < 0.001 \), **\( P < 0.01 \).
Supplementary Figure S2. Feiyanning inhibits the proliferation of A549 cells in vitro  

(A,B) The cell viability results after 24, 48 and 72h of treatment with different concentrations (75, 150, 225, 300, 400, 500, 750, 1000 µg/mL) of Feiyanning. Data are expressed as the mean±SD.

Supplementary Table S1. Components of Traditional Chinese medicine formula Feiyanning

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<td>Corium bufonis</td>
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Supplementary Table S2. Sequences of primers used for RT-qPCR

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References


