Sijunzi Tang and gefitinib cooperate to inhibit Lung cancer progression by modulating the pre-metastatic niche

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Research

Keywords: Lung cancer, Pre-metastatic niche, Gefitinib, Sijunzi Tang, Immune cells, Cytokines, immunosuppressive

Posted Date: January 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-144090/v1

Abstract

Background:

Although targeted therapies usually trigger great initial responses in patients, the efficacy is transient due to tumor metastasis. The formation of pre-metastatic niche was proposed as the main cause for metastasis, of which the blocking way may be a potential method for inhibiting metastasis. Sijunzi Tang (SJZ), as a complementary drug for targeted therapy, can reduce the recurrence and metastasis of tumors and prolong the survival time of patients. However, how SJZ regulates the formation of pre-metastatic niche to improve the efficacy of targeted therapy remains unclear.

Methods:

Here, we investigated the anti-tumor activity and immunological mechanism of SJZ plus gefitinib based on pre-metastatic niche in Lewis lung carcinoma (LLC) incubated spontaneous metastatic mouse model, using histopathology and immunological methods.

Results:

The results showed that SJZ can improve the effect of gefitinib by inhibiting tumor cell growth, promoting tumor cell apoptosis and preventing metastasis in the lung. Besides, SJZ plus gefitinib could inhibit tumor cell aggregation and the expression of characteristic proteins of Mmp2 and Mmp9 in the lung areas of mice. We also confirmed that SJZ could downregulate the expression levels of c-kit and VEGFR2 on DCs, c-kit on neutrophils, c-kit, VEGFR2 on B lymphocyte in the blood, and c-kit and CXCR1 on monocytes in the lung; Gefitinib could decrease the expression levels of c-kit and VEGFR2 on DCs in the blood and c-kit and CXCR1 on monocytes in the lung, but increase the amount of $c\text{-}kit^+$ monocytes in the blood. SJZ plus gefitinib decrease the proportion of $c\text{-}kit^+$ and $CXCR1^+$ monocytes in the lung. SJZ could regulate pro-metastatic inflammatory responses represented by down-regulating the expression of IL-23、RANTES, GRO-α against that of gefitinib. Moreover, there were significant rises in the expression level of IL-12p70 and IL-15、while declines in the expression level of IL-1β、IL-18、GRO-α in co-treatment group.

Conclusions:

This work identified the immune cells and cytokines in pre-metastatic niche associated with lung cancer affected by gefitinib and SJZ, and further revealed the immunological mechanism of SJZ improving the efficacy of gefitinib.

Background

Worldwide, lung cancer continues to attract special attention because of its high cancer-related morbidity and mortality[1]. Clinical evidence indicates that lung cancer with distant metastases is usually accompanied with poor prognosis[2]. It was reported that 85% of lung cancer patients are diagnosed as

non-small cell lung cancer (NSCLC), who may probably be in the terminal stage of cancer[1]. In recent years, gefitinib, as the first-line drug of Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor (EGFR-TKI) greatly improved survival rate of patients for multiple types of cancer including NSCLC compared with chemotherapy[3]. Although many EGFR-mutated NSCLC patients benefit from EGFR-TKI therapy, whether gefitinib or osimertinib, tumor recurrence and metastasis are inevitable due to the prevalence of loss of drug sensitivity[4]. Increasing evidences show that Traditional Chinese medicine(TCM) has the characteristics of overall systematicity and multiple targets, which can improve the sensitivity of anti-tumor drugs and inhibit tumor metastasis [5–7]. However, the underlying mechanism of how TCM improves the sensitivity of EGFR-TKI remains unclear.

Lately, a concept was proposed that metastasis is related to the formation of pre-metastatic niche which is driven by cooperation between tumors and their distant metastatic organs[8]. In this process, the primary tumor can regulate distant organs by secreting substances including inflammatory cytokines, adhesion molecules, chemokines and exosomes which may cause inflammation and immunosuppressiv[9, 10]. For instance, Human Granulocyte Colony Stimulating Factor (G-CSF) secreted by tumor tissue will mobilize peripheral $CD11b^{+}Gr1^{+}$cells into the lungs, as a result, force the formation of pre-metastatic niche in the lung[11]. Tumor-derived factors represented by inflammatory cytokines factors play an important role of in the initiate step of the formation of pre-metastatic niches (PMNs)[9]. The conception of PMNs has shifted our attention from cancer cell killing strategy to niche component regulation in the prevention of metastasis. XIAOPI formula treatment remarkably could reduce the populations of hematopoietic stem and progenitor cells (HSPCs) in the bone marrow and Myeloid-derived suppressor cells(MDSCs) in the lung tissues and reduce CXCL1 expression in a dose-dependent manner[12].Sijunzi Tang (SJZ) is a typical Chinese medicine formula derived from ‘Prescriptions People's Welfare Pharmacy’ which consists of four herbs including Panax ginseng C.A.Mey.,Atractylodes macrocephala Koidz., Poria cocos (Schw.)Wolf and Nardostachys jatamansi DC.. As reported, it has been used as the adjunct drug which can enhance the efficacy of anti-tumor drugs including of EGFR-TKI, inhibit tumor recurrence and metastasis and prolong the survival of patients. Our previous study showed that gefitinib can increase the expression of cytokines represented by IL-6 and IL-1α in serum and tumor tissue, which may aggravate the inflammation in pre-metastatic niche[13]. The results suggested that the decreased efficacy of gefitinib may associated with the up-regulation of some inflammatory cytokines. Jia and colleagues found that there was either no significant change in antitumor effector cells and an increasing secretion of IL-10 and CCL2 in serum after a period of EGFR-TKI treatment[14], which can exactly approve our point of view. However, the mechanistic details of the signaling network and the relevance to pulmonary physiology are poorly understood. Herein, to further investigate the efficacy of gefitinib and SJZ on LLC model in vivo and its immunological mechanism, we evaluated the situation of tumor growth and apoptosis. Further, we set out to identify the influence of gefitinib and SJZ on pro-metastatic inflammatory responses and pre-metastatic niche formation.

Materials And Methods

Reagents and antibodies

Rabbit monoclonal antibodies against MMP9, MMP2 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

The mouse lewis lung cancer cells were obtained from Guang' anmen Hospital China academy of Chinese medical sciences and then cultured in DMEM medium, which is supplemented with 10 % fetal bovine serum, 1×10^5 u·L^{-1} penicillin, and 100 mg·L^{-1} streptomycin (Gibco, USA). The cell line was maintained in a humidified atmosphere at 37 °C and 5 % CO2.

Preparation and quality control of Sijunzi Tang

SJZ was extracted from a mixture of 4 herbs including Panax ginseng C.A.Mey., Atractylodes macrocephala Koidz., Poria cocos (Schw.) Wolf and Nardostachys jatamansi DC. by refluxing extraction method. Its quality control was applied by detecting content, heavy metals, pesticide residues and aflatoxin according to Pharmacopoeia of the People's Republic of China. The detailed preparation and quality control method has been previously reported[15].

Animals and Tumor model

Animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, with the approval of animal ethics committee which belongs to Chinese Academy of Medical Sciences Cancer Hospital (Beijing, China). Age-and sex-matched C57BL/6J mice (male, 6–8 weeks old, 16–20 g) were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rodents used in the study were housed under standardized light- and temperature-controlled conditions with free access to food and water. To establish spontaneous lung metastatic models, 1×10^6 LLC cells were injected subcutaneously in the shaved right lateral axilla of mice.

Treatment regime

84 mice were randomly divided into four groups with 21 mice in each group at each time point. The four groups of mice were administered the following dosing regimen once a day by intragastric administration (a) saline solution; (b) gefitinib (50 mg·kg^{-1}); (c) SJZ (25.74g·kg^{-1}); (d) gefitinib + SJZ. Gefitinib was suspended in 0.5% CMC-NA solution. Mice were sacrificed separately on 7, 14, 21 days.

Flow cytometry assay

Mouse peripheral blood was collected in sodium Heparin blood collection tube. Erythrocyte cells were harvested, washed, and resuspended in 100 μL PBS solution at a density of 1×10^6 cells. Mouse lung were also collected, shredded and digested with collagenase. The density of lung cell suspension is also 1×10^6 cells. All the cells were labeled by BD Pharmingen™ APC-Cy™7 Rat Anti-Mouse CD45 antibody (557659, BD Biosciences, San Jose, CA, USA). For neutrophils and monocytes analysis, BD Horizon™ BV711 Rat Anti-CD11b (563168, BD Biosciences, San Jose, CA, USA), PE-Cy™7 Rat Anti-Mouse Ly-6G (560601, BD

Biosciences, San Jose, CA, USA) and BV605 Rat Anti-Mouse Ly-6C (563011, BD Biosciences, San Jose, CA, USA) were used. For detection of dendritic cells, Ms CD11c BUV395 HL3(563011, BD Biosciences, San Jose, CA, USA) and BV650 rat anti-mouse I-A/I-E (743873, BD Biosciences, San Jose, CA, USA) were used. For identification of Lymphocytes, lung cells and peripheral blood cells were incubated with BUV496 Hamster Anti-Mouse CD3e (612955, BD Biosciences, San Jose, CA, USA) and BUV737 Rat Anti-Mouse CD19 (564296, BD Biosciences, San Jose, CA, USA). Besides, the surface receptors of c-Kit, CXCR1, CCR2 and VEGFR2 on immune cells were also identified by PE anti-mouse CD117 (c-Kit) Antibody (105807, BD Biosciences, San Jose, CA, USA), PE Rat Anti-Mouse CD181 (CXCR1) (566383, BD Biosciences, San Jose, CA, USA), Alexa Fluor® 647 Mouse anti-Human CD192 (CCR2) (561744, BD Biosciences, San Jose, CA, USA) and BV421 Rat Anti-Mouse FLK-1 Clone Avas 12α1 (RUO) (562941, BD Biosciences, San Jose, CA, USA). After incubation, cells were washed with PBS and subjected into the FACS Canto™ II flow cytometer (BD Biosciences, San Jose, CA, USA).

HE staining

H&E staining was performed as described[16]. Lungs were inflated with 10% formalin and embedded in paraffin. Subsequently, 5-μm sections were cut for hematoxylin/eosin staining.

TUNEL staining

Cell apoptosis in tumor tissues were detected using the TUNEL method. The assay was carried out with Roche TUNEL assay kit according to the manufacturer's protocols. The tumor tissues of mice were first immersed in 10% neutral formalin. After dehydration with graded ethanol (70%-100%) and degrease disposing with xylene, the paraffin embedded tumor tissues were made for further use. Sections were deparaffinized, re-hydrated and incubated with 20 mg/ml proteinase K for 20 min at room temperature. The TUNEL reaction mixture was prepared as follows: 50 μL TdT enzyme, 450μL Biotin-dUTP. The slides were incubated in the mixture for 60 min at 37 °C and then incubated in the 50 μl converter-POD for 30 min at 37 °C. And DAB solution was applied to the sections for 10 min at 15–25℃. Images of the cells were captured using a microscope.

Immunohistochemistry

5 μm sections of the paraffin embedded femur were incubated at 60℃ for 24 h and then followed by defatting in xylene and hydrating with graded ethanol (100%-70%). After successively incubating with antigen retrieval solution and 3% H_2O_2 for 30 min, the slides were rinsed and incubated with the primary antibody (MMP-2, MMP9) overnight at 4℃. For the negative controls, the primary antibodies were replaced by non-immunized goat serum. The next day, the slides were rinsed and incubated with the second antibody for a period of time followed by DAB and haematoxylin staining, respectively. Quantification of positive cells was examined in at least five random fields from each section.

Cytokine measurement

Mouse testing samples were obtained by centrifugation from tumor tissue homogenate at 10000 rpm for 15 min. After protein quantification to 10 $pg{\cdot}mL^{-1}$, the samples were stored in -80℃ environment.

Cytokines (ENA-78, Eotaxin, G-CSF, GM-CSF, GROα, IFN-γ, IFNα, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-15/IL-15R, IL-17A, IL-18, IL-22, IL-23, IL-27, IL-28, IL-31, IP-10, LIF, MCP-1, M-CSF, MCP-3, MIP-1α, MIP-1β, MIP-2, RANTAS, TNF-α) in tumor issue were tested by ProcartaPlex™ Mouse Cytokine&Chemokine Panel.

Statistical analysis

Numerical data are presented as mean ± SD. All data analyses were performed by Graph Pad Prism (version8.0, Graph Pad Software, Inc.). Between-group analyses were conducted using one-way ANOVA and unpaired t-Test.

Results

SJZ plus gefitinib enhance the anti -tumor activity

The LLC spontaneous model was generated to investigate the anti-tumor activity after treatment by gefitinib and SJZ. The mice were sacrificed separately on 7, 14, 21 days. Based on the previous study, gefitinib and SJZ was administered via intragastric administration (i.g.) route at the dose of 50 $mg \cdot kg^{-1}$ and 25.74 $g \cdot kg^{-1}$, respectively[15]. As shown in Fig. 1, the tumor growth curve indicated that gefitinib combined with SJZ can significantly reduce the tumor volume compared with the model group. The average volume of tumors in co-treatment group increased at a significantly slower rate than the average volume of tumors in the model group at 14,21days($P < 0.001$). All of three treatment groups significantly decreased the tumor weight compared with that of the model group, and the co-treatment group behave more obvious at 14、21days($P < 0.01$ or $P < 0.001$) (Fig. 1D, E). Furthermore, the co-treatment group did not show obvious side effects on weight loss in body weight and internal organs (lung, liver, kidney, and heart) during the experiment (Fig. 1B, C). These results demonstrated that SJZ could enhance the anti-tumor activity of gefitinib without obvious toxic side effects on internal organs.

In addition, the TUNEL assay was conducted to evaluated the cell death following treatment with gefitinib, SJZ or both. The result showed that the amounts of TUNEL-positive cells in the gefitinib or SJZ-treated cells were significantly higher compared with that in the model group. By contrast, the addition of SJZ further promoted the apoptotic effect of gefitinib, leading to a markedly higher amounts of apoptotic cells in the gefitinib plus SJZ group (Fig. 1F). Thus, it can be concluded that SJZ was able to enhance the apoptotic effect of gefitinib on the LLC cells.

SJZ and gefitinib treatment prevents lung pre-metastatic niches formation and metastasis

The pre-metastatic niche plays a critical role in the metastatic process as it prepares the ‘fertile soil’ in distinct organs for tumor metastasis[8]. In order to confirm the anti-metastasis effects of SJZ and gefitinib in the spontaneous metastatic mouse model, the formation of micro-metastases was examined by HE staining. The results showed that a large number of tumor cells aggregated and surrounded the

small bronchioles, consequently forming the micro-metastases in the lung in model group. There was lower aggregation in lung metastasis in the gefitinib or SJZ group than in the model. However, there was no tumor cell aggregation in the lung areas of mice after co-treatment with gefitinib and SJZ (Fig. 2A). Based on the result of H&E staining, we found that a more severe level of micro-metastasis in the model group. Furthermore, the expression of characteristic proteins of Mmp2 and Mmp9, which are reported to promote tumor cell invasion, migration and colonization in the metastatic niche, were also assessed by IHC analysis. We found that the treated group by gefitinib or SJZ can effectively downregulate the expression of Mmp2, Mmp9 after administration than model group. Besides,the gefitinib plus SJZ group showed a significantly lower expression of Mmp2, Mmp9 after administration than single treatment. Thus, SJZ may prevent the formation of the pre-metastatic niche and improve the therapeutic efficacy of gefitinib. Therefore, the combination of SJZ and gefitinib can effectively inhibit the lung pre-metastatic niche formation.

The effect of SJZ and gefitinib on immune cells in the pre-metastatic niche

Several lines of evidence have shown that BMDCs are recruited to metastatic organ sites to form a metastatic niche. It has been shown that neutrophils may facilitate cancer lung metastasis. According to the recent published researches, $CD11b^+$ myeloid cells were reported to remarkably expanded in the pre-metastatic niche, among which $CD45^+CD11b^+$ $Ly6G^+$ $Ly6C^{int}$ neutrophils were the most dominant[17]. Therefore, we analyzed the cellular composition of the lung and blood in mice after tumor inoculation and drug administration. The fluorescence-activated cell sorting (FACS) analysis revealed that $CD45^+$ $CD11b^+$ $Ly6G^+$ $Ly6C^{int}$ neutrophils and $CD11c^+$ Ia/e^+ dendritic cells were remarkably decreased in the treatment group in the pre-metastatic niche,while $Ly6G^-Ly6C^+$ monocytes, lymphocytes and $F4/80^+$ macrophages showed no difference (Fig. 3B). These data indicate that neutrophils and DCs may be key biomarkers in pre-metastatic niche that gefitinib and SJZ focus on, although other myeloid cells may be also involved. The above findings indicated that gefitinib and SJZ could inhibit myeloid cells accumulation especially for neutrophils and DCs in target organs, since myeloid cells were reported to modulate by chemokines or cytokines relying on the chemokine receptors or proto-oncogene on their surface, it is necessary to explore the changes of receptors expression in response to gefitinib and SJZ treatment. $c\text{-}kit^+$, $CCR2^+$, $CXCR1^+$ and $VEGFR1^+$ myeloid cells are the main BMDCs gathered in the pre-metastatic niche [17, 18]. As showed in Fig. 4, there is a significantly change in some receptor positive myeloid cell following the treatment. Obviously, compared with the model group, gefitinib or SJZ treatment can downregulate the expression of c-kit and VEGFR2 on DCs in the blood (Fig. 4A). Besides, the $c\text{-}kit^+$ neutrophils and $c\text{-}kit^+$, $VEGFR2^+$ B lymphocytes in the blood was significantly reduced after SJZ treatment compared with the model group (Fig. 4B and 4D). Surprisingly, we found that gefitinib can elevated the $c\text{-}kit^+$ monocytes in the blood (Fig. 4C). We also tested the receptors expression on the immune cells in the lung of mice which is considered as the metastatic niche (Fig. 4E-4H). The result showed that gefitinib or SJZ treatment apparently can't affect the receptors expression of neutrophils and dendritic cells. However, gefitinib and co-treatment can effectively decrease the proportion of $c\text{-}kit^+$ and

$CXCR1^+$ monocytes (Fig. 4G). In addition to this, the expression of CXCR1 of B lymphocytes significantly reduced after gefitinib treatment. Therefore, gefitinib and SJZ treatment have different effects on immune cells in the pre-metastatic. The regulation on immune cells in the pre-metastatic by SJZ might be the major reason for improving the efficacy of gefitinib.

The effect of SJZ and gefitinib on cytokines secretion in the pre-metastatic niche

The tumor microenvironment plays a vital role in the initiation and progression of many cancers[19]. Primary tumor-secreted soluble molecules contribute to preparing distant sites for the pre-metastatic niche formation, thereby promoting metastasis and even determining metastatic organotropism[9]. Therefore,these soluble molecules is also considered as niche-promoting molecules. In our study, the secretion of cytokine levels in tumor issues were measured using Luminex xMAP technology. The expression differences of the cytokines levels in each treatment group were analyzed during the pre-metastatic niche formation. We identified the 9 main inflammatory cytokines (IL-12p70, IL-1β, TNF-α, IL-18, IL-23, GRO-α, IP -10, RANTES, IL-15) as the mediator of primary tumor after drug treatment. As shown in the Fig. 5, compared with model group, the expression levels of IL-23 and RANTES increased after gefitinib treatment ($P < 0.05$), while the expression level of TNF-α decreased($P < 0.05$). Besides, compared with the model group, the expression levels of TNF-α, IL-18, IL-23, GRO-α, IP -10, RANTES showed a downward trend in the SJZ group, while the expression levels of GRO-α and IP-10 decreased remarkably($P < 0.05$). Compared with the model group, co-treatment with gefitinib and SJZ could increase the activity of IL-12p70 and IL-15 and decrease the activity of IL-1β、IL-18、GRO-α. Specially, compared with the model group, gefitinib can increase the levels of IL-23 and RANTES, while SJZ could decrease them and the co-treatment group couldn't change them. However, compared with the gefitinib group, the levels of IL-23 and RANTES in the co-treatment group indicated a downward trend. Thus, in the pre-metastatic niche, the increased levels of inflammatory cytokines caused by gefitinib might be one of the important reasons for metastasis in the later stage of the tumor. Additionally, SJZ improved the efficacy of gefitinib by regulate the expression of immune cytokines.

Discussion

In this study, the inhibition rate and growth of tumors and the weight changes of mice were observed to evaluate the anti-tumor effects of gefitinib and SJZ. Compared with gefitinib or SJZ administration alone, the co-treatment has a better control of tumor growth and promoting tumor apoptosis. It has been proposed that the presence of apoptotic tumors enhances antigen-presenting ability in dendritic cells as well as T-cell response[20]. These results indicated that SJZ can significantly improve the effect of gefitinib in tumor-bearing mice.

In 2005, Kaplan *et al.* put forward the concept of 'pre-metastatic niche'[8]. It refers to the adaptive transition in the metastatic organ before tumor metastasis, so as to create a 'fertile soil' suitable for tumor cell colonization[9]. In other words, the primary tumor can secrete tumor-derived factors such as

chemokines, inflammatory factors or exosomes which can mobilize immune cells from bone marrow[21]. As for lung metastasis, tumor cells can move along blood vessels or lymphatics to the lung after the matrix prepared[22]. Of note, we found that there were no apparent lung metastatic lesions after LLC inoculation in 2th weeks in all groups, but HE staining showed that gefitinib and SJZ could inhibit the process of transferring of tumor cells to the lung, which means the co-treatment may block the formation of pre-metastatic niche in the lung. The formation of PMNs can be basically divided into three stages: 1) Tumor tissues secrete soluble cytokines or components into the circulatory system; 2) Immunosuppressive cells in bone marrow are activated and transferred in located site. 3) Cell matrix were captured in distant organ. In other words, the PMNs development is an overall process involving multiple components, cells and organs, which means there would be more possibilities improving clinical outcomes of patients by attempting more effective targeting strategies.

Recognition of primary tumor-derived signals to initiate subsequent immune responses in distinct organs is a critical but poorly defined process in pre-metastatic niche formation. It was proved that chemokine receptors, which are mainly expressed on the surface of immune cells such as neutrophils and monocytes, can promote tumor metastasis, mediate inflammation and play an important role in regulating the recruitment of immune cells[23]. CXCR1 and CCR2 have been receiving particular interest on their roles in cancer metastasis[24, 25]. The VEGF signaling pathway is associated with cellular proliferation and migration and endothelial cell permeability[26]. Vascular endothelial growth factor receptor 1 positive ($VEGFR1^+$) BMDC clusters are recruited to the pre-metastatic sites before the arrival of LLC cells in the spontaneous metastasis model[27].In our study, we found that gefitinib and SJZ single treatment can significantly downregulate the $VEGFR2^+$ DC in blood of mouse, but they have no effect on the other proteins on immune cells. So, VEGFR2 protein may not be the dominant targets in PMNs modulated by gefitinib and SJZ. In the metastasis model, $c\text{-}kit^+$ neutrophils also expanded systemically, as tumors grew larger[18]. We surprisingly discovered that gefitinib can elevated the $c\text{-}kit^+$ monocytes in the blood. Besides, the gefitinib and SJZ single treatment can downregulate the expression of c-kit on DCs in blood of mice Besides, the $c\text{-}kit^+$ neutrophils and $c\text{-}kit^+$ B lymphocytes of was significantly reduced after SJZ single treatment compared with the model group in blood of mice. Notably, co-treatment can effectively decrease the proportion of $c\text{-}kit^+$ and $CXCR1^+$ monocytes. Generally, c-kit and CXCR1 seem the potential targets that gefitinib and SJZ can do the joint regulation.

As the initiate step, tumor-secreted cytokines play an important role in lung metastasis. In a word,PMNs formation is a complex procedure that involves multiple cytokines[12]. Our results showed that increased levels of RANTES and IL-23 were observed after gefitinib treatment. While SJZ could down-regulate the expression levels of RANTES and IL-23. RANTES, known as chemokine C-C motif ligand 5 (CCL5)has also been detected in ovarian cancer [28], prostate cancer[29],pancreatic cancer[30], and melanoma[31] and breast cancer[32]. RANTES produced by cells in the tumor microenvironment has been considered as an important contributor to metastatic disease[32]. It was proved that CCL5-deficiency inhibited tumor growth and metastasis of colorectal cancer cells by increasing the infiltration of $CD8^+$ T cells into central tumor

area. Besides, CCL5 can also modulate the differentiation of MDSCs to promote tumor progression in luminal and triple-negative breast cancer[33]. Interleukin (IL)-23 is a pro-inflammatory cytokine which consists of IL-12 p40 and IL-23 p19 subunits secreted by macrophages and dendritic cells, inducing autoimmunity by T-cell-mediated inflammation through impacting T helper 17 (Th17) cell response[34]. IL-23-mediated responses were indicated to be crucial in promoting tumor progression[35]. SHENG *et al.* found that blocking the function of IL-23 inhibited the proliferative activity and induced the apoptotic activity of tumor cells in MCF-7 cells[36]. IL-23 is associated with the metastasis of cancer, as IL-23 promoted the metastasis of hepatocellular carcinoma via matrix metalloproteinase 9 (Mmp9)[37, 38]. It was found that in the transgenic mice with $EGFR^{L858R}$ driven mutation, gefitinib and osimertinib treatment can significantly improve the level of expression of IL-10 in mice serum and CCL-2 in tumor tissue[14].The over secretion of IL-6 by tumor cells may be responsible for the decreased efficacy of tyrosine inhibitors such as icotinib and erlotinib, further resulting in the drug resistance[39]. Previously, we confirmed that gefitinib treatment induced higher level of inflammatory cytokines such as IL-1α which may cause inflammation in the pre-metastatic niche[13]. Therefore, the increased expression levels of IL-23 and RANTES might be associated with the formation of pre-metastatic niche and SJZ could inhibit it. IL-12p70, as well as IL-15, was a common immune promoting factors[40, 41]，while IL-1α, IL-1β，IL-18 and GRO-α were important cytokines involved in inflammatory processes which are considered as the facilitators in cancer metastasis[42–45]. We found that there were significant rises in the expression level of IL-12p70 and IL-15，while declines in the expression level of IL-1β，IL-18，GRO-α in co-treatment group. It seems that SJZ treatment can maintain a relative balance in the tumor tissues, which means there were increased expression in immune promoting factors and decreased expression in immunosuppressive factors. Generally, we found that although SJZ treatment and co-treatment may cause different cytokines changes. Moreover, only the expression level of GRO-α showed a collaborative downward trend significantly. Studies have shown that GRO-α can mediate the activation of apoptosis signaling pathways through interacting with CXCR2 and increase the ability of tumor cell invasion and migration[46]. So if GRO-α could be the key target of controlling the initiate step for the formation of PMNs in tumor tissues that gefitinib and SJZ focus on has not been defined. Anyway, this study enlightened us that SJZ can modulate the tumor cell secretion to a state of balance which is favorable for improving anti-tumor effect of gefitinib.

It is recognized that the process of tumor metastasis not only depends on the behavior of tumor cells themselves, but also depends on the adaptive change of extracellular matrix in the distant organ[47]. To rule out if gefitinib and SJZ can interfere the pre-metastatic niche with extracellular matrix, we performed IHC analysis of MMP2 and MM9 on lung tissues which are involved in the degradation of extracellular matrix (ECM). The results showed that gefitinib and SJZ could reduce the expression of these two proteins, and down-regulation of them were more obvious in co-treatment group. Recent studies reveal that the expression of matrix metalloproteinases (MMPs) is extremely high in lung tumors compared with non-malignant lung tissue. Recekamp *et al.* proved that the expression level of MMP-9 in serum of non-

small cell lung cancer patients was closely related to the drug sensitivity of when receiving erlotinib treatment [48].

Conclusions

In conclusion, these results implied that SJZ could improve the effect of gefitinib by targeting the tumor-derived cytokines as well as regulating matrix remodeling related proteins, therefore, resulting in the suppression of PMNs formation. Targeting the pre-metastatic niche is a potential approach to prevent metastasis.

Abbreviations

SJZ: Sijunzi Tang

LLC: Lewis lung carcinoma

NSCLC: non-small cell lung cancer

EGFR-TKI: Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor

TCM: Traditional Chinese medicine

G-CSF: Granulocyte Colony Stimulating Factor

PMNs: pre-metastatic niches

MDSCs: Myeloid-derived suppressor cells

VEGFR1+: Vascular endothelial growth factor receptor 1 positive

Th17:T helper 17

MMPs: matrix metalloproteinases

ECM:extracellular matrix

Declarations

Acknowledgements

None.

Funding

This research was supported by grants from the CAMS Innovation Fund for Medical Sciences (CIFMS) (grant no. 2016-I2M-1-001).

Author information

Guohui Li designed the research. Mingyu Zhang carried out the experiments and performed data analysis. Chunyu Li and Chen wei participated part of the experiments. Mingyu Zhang and Chunyu Li wrote the manuscript. Ruisheng Li and Xingjie Li revised the manuscript. All of the authors have read and approved the final manuscript.

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Contributions

Guohui Li designed the research. Mingyu Zhang carried out the experiments and performed data analysis. Chunyu Li and Chen wei participated part of the experiments. Mingyu Zhang and Chunyu Li wrote the manuscript. Ruisheng Li and Xingjie Li revised the manuscript. All of the authors have read and approved the final manuscript.

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Ethics declarations

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

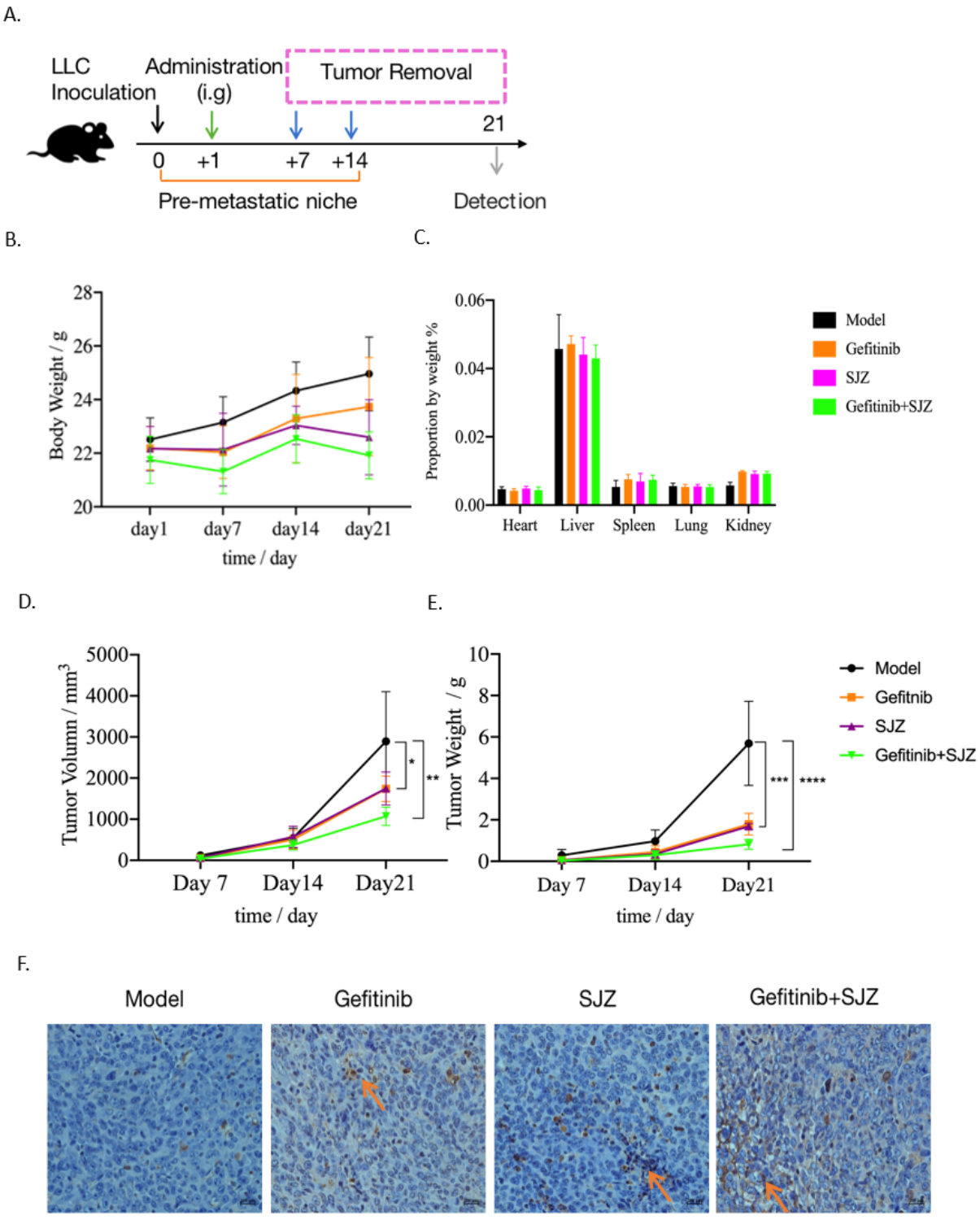

Figure 1

Anti-tumor activity of gefitinib and SJZ Tang in LLC mouse model. (A) Schematic illustration for the spontaneous metastatic models in this study. (B) The changes in body weight of mice (C)Tumor weight and (D) volume of mice was measured on 7, 12 and 21 days after gefitinib and SJZ treatment. The

weight(E) and coefficient (F) of main organ (Heart, Liver, Spleen, Lung, Kidney). (G) Representative images of cell apoptosis in tumor tissue.Scale bar, 200μm.

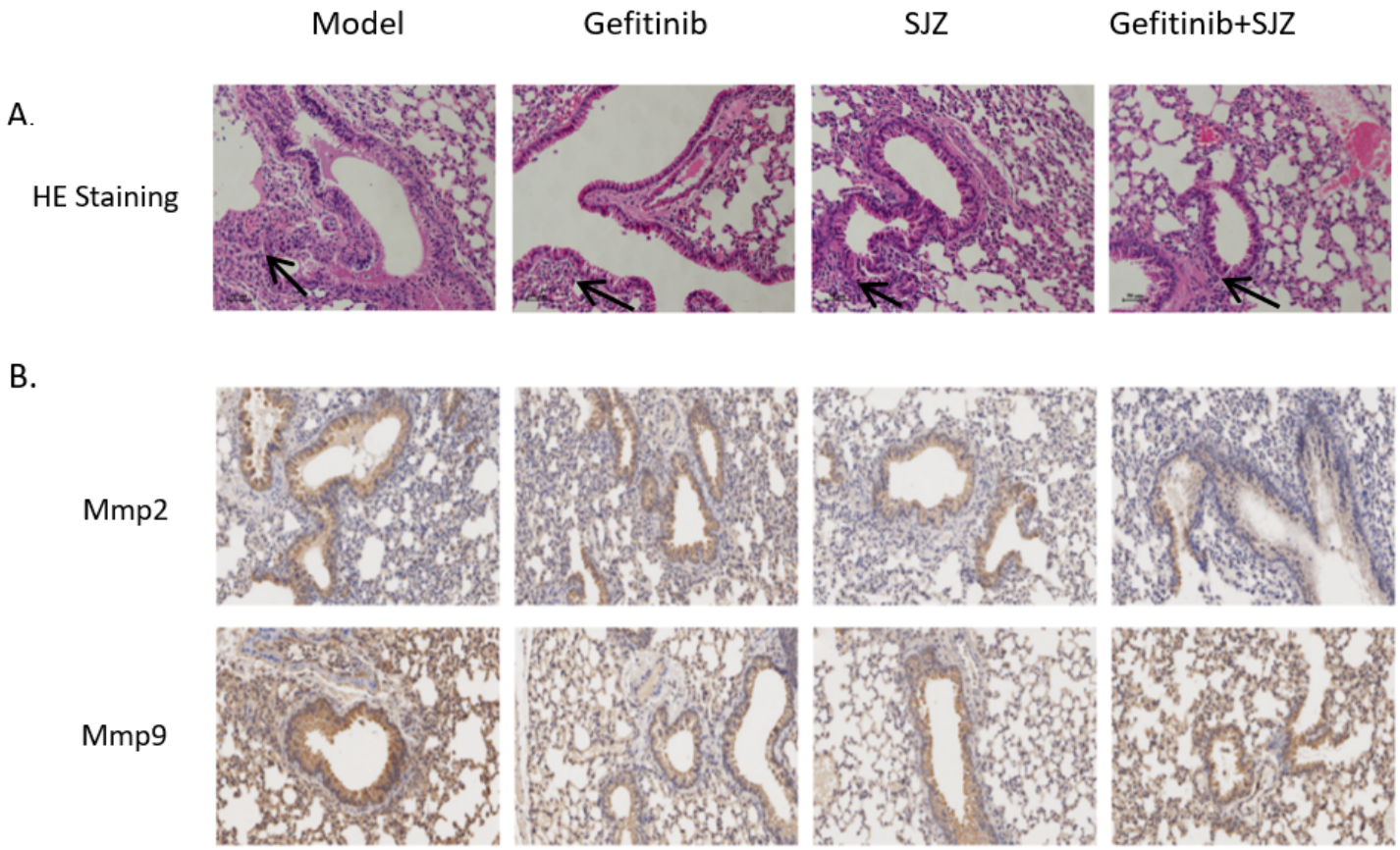

Figure 2

Gefitinib and Sijunzi Tang inhibit the formation of PMNs. (A) Representative images of lung sections stained of tumor-bearing mice after implantation. scale bars, 50μm. (B) Representative images of Mmp2 and Mmp9 staining in tumor tissues.

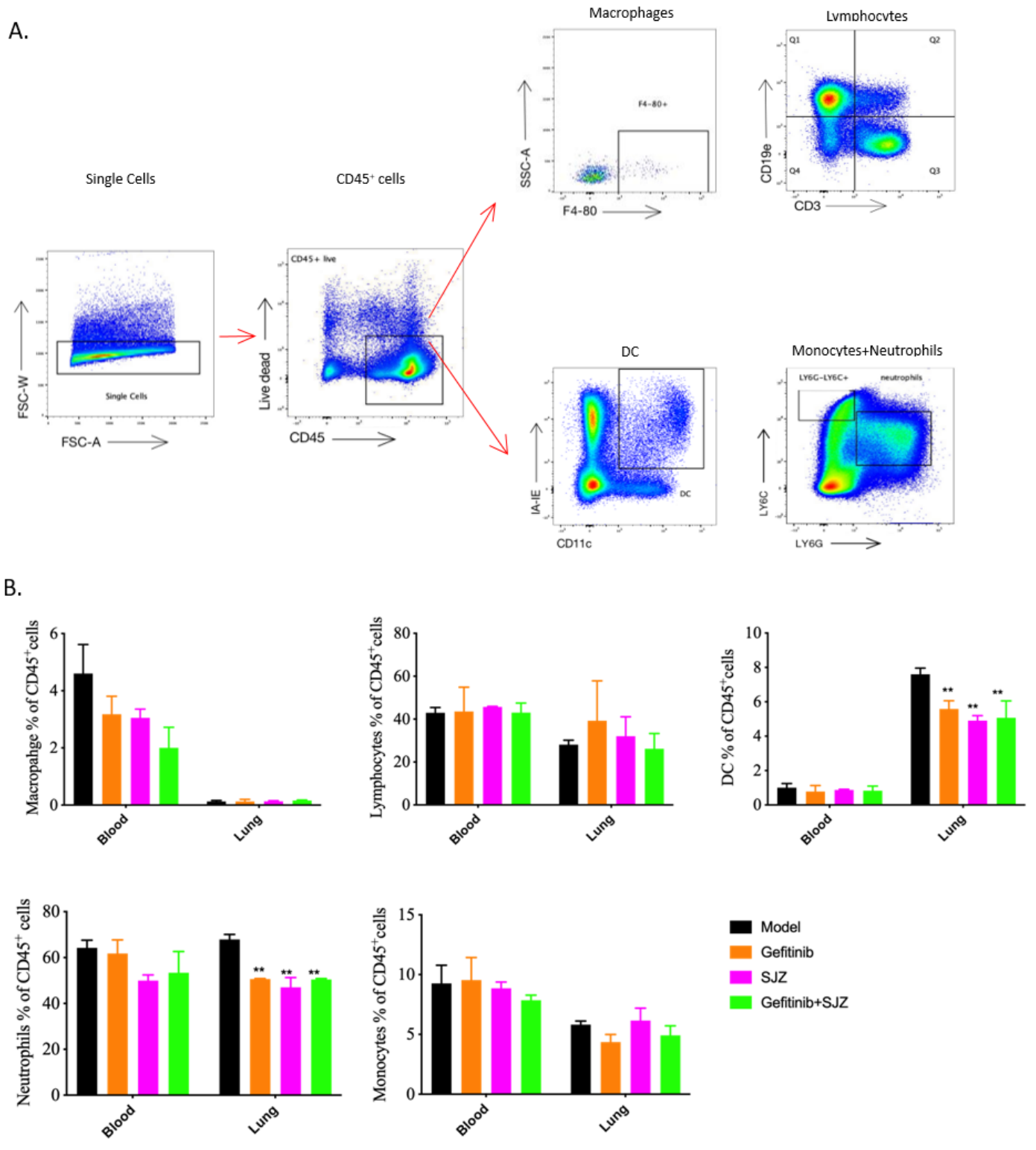

Figure 3

Different immune cells were detected by flow cytometry in C57 mice after LLC tumor inoculation after 14days. (A) Schematic diagram of sketch pattern of flow chart. (B) The proportions of macrophages, lymphocytes, dendritic cells, neutrophils and monocytes in the blood and lung.Results were presented as mean ± SEM. N = 2–3 per group. To compare differences between model group and treatment, unpaired student t test was used. *P < 0.05, **P < 0.01, ***P < 0.001.

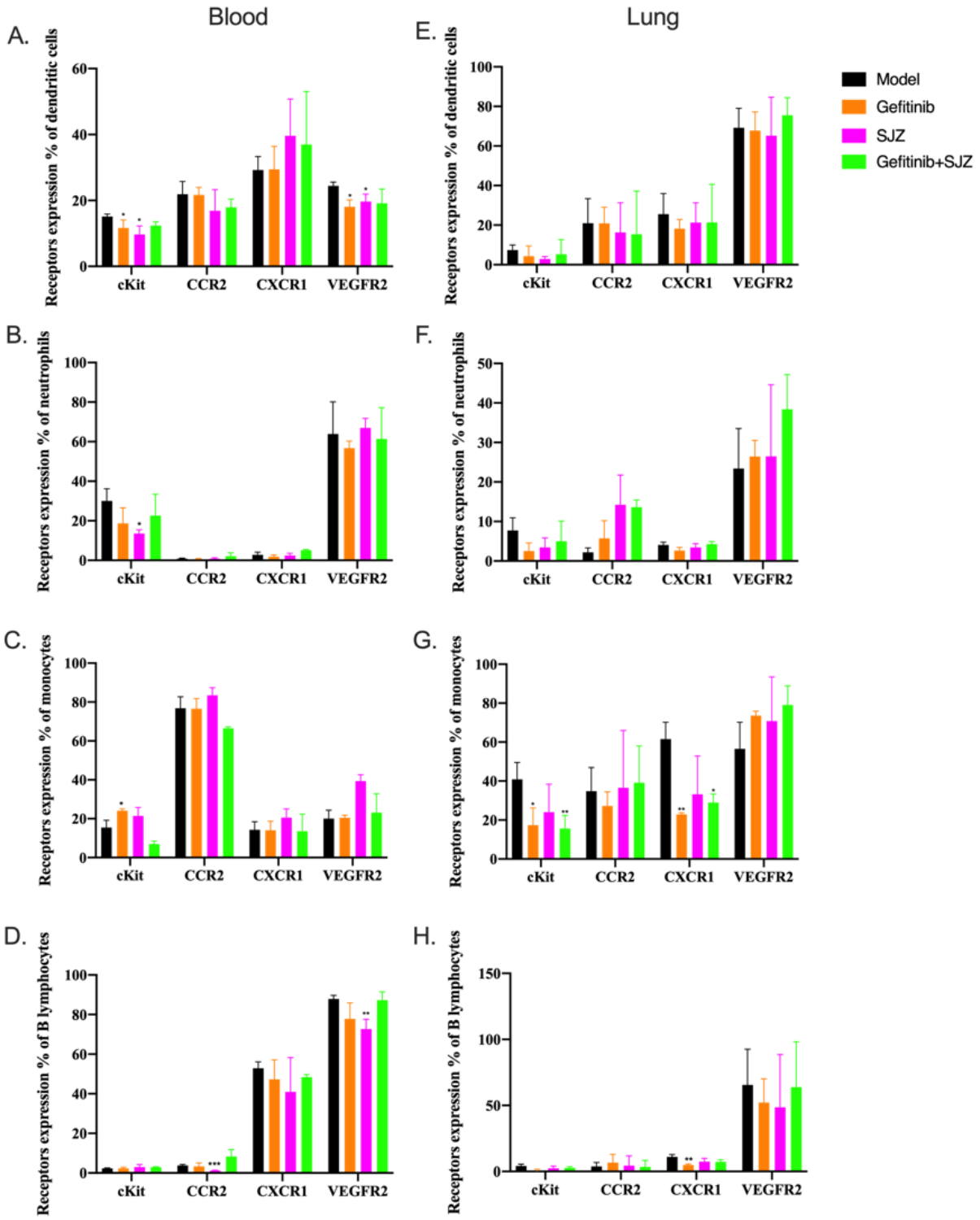

Figure 4

Fluorescence intensity of ckit, CCR2 , CXCR1 and CXCR4 expression on (A,E) dendritic cells, (B,F) neutrophils , (C,G) monocytes and (D,H) B lymphocytes in blood and lung detected by flow cytometry in C57 mice after LLC tumor inoculation after 14days. Results were presented as mean ± SEM. N = 2-3 per group. To compare differences between model group and treatment, unpaired student t test was used. *P < 0.05, **P < 0.01, ***P < 0.001.

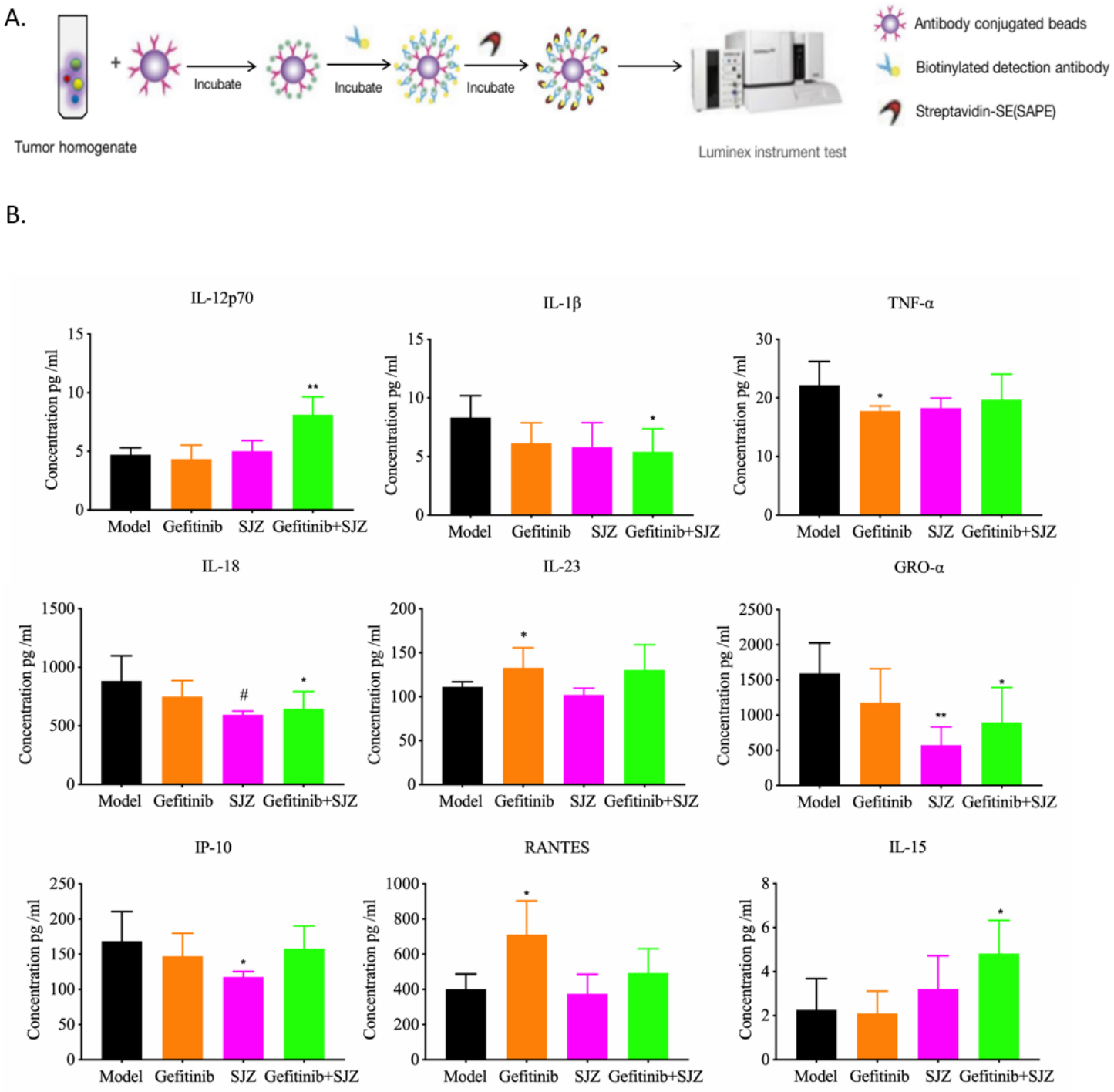

Figure 5

Gefitinib and Sijunzi Tang treatment changed tumor-derived inflammatory cytokines secretion. A.Luminex xMAP assay process. Briefly, 3mg of tumor tissue was dealt with PBS solution into tumor homogenate, then added up with beads, antibody and SAPE step by step. The complex was detected by Luminex test system. B.The levels of IL-12p70, IL-1β, TNF-α, IL-18, IL-23, GRO-α、IP-10, RANTES, IL-15, IL-1α and IL-6 concentration in tumor tissues from different treatment groups were measured by Luminex xMAP assay.

Results were presented as mean ± SEM. N = 5–6 per group. To compare differences between model group and treatment, unpaired student t test was used. *P < 0.05, **P < 0.01, ***P < 0.001.