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Interleukin-7 promotes lung-resident CD14$^+$ monocytes activity in patients with lung squamous carcinoma

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ABSTRACT

Interleukin (IL)-7 enhances cytokines secretion by CD14$^+$ monocytes, and induces recruitment of monocytes to endothelium. As an important regulator to different types of immune cells, the role of IL-7 in modulation of CD14$^+$ monocytes is still not fully elucidated. Thus, the aim of current study was to investigate the immunoregulatory activity of IL-7 to peripheral and lung-resident CD14$^+$ monocytes in lung squamous carcinoma patients. Thirty-seven lung squamous carcinoma patients and eighteen healthy individuals were enrolled. CD14$^+$ monocytes and CD4$^+$ T cells were purified from both peripheral bloods and bronchoalveolar lavage fluids (BALF). IL-7 expression in plasma and BALF was measured by ELISA, and CD127 expression in peripheral and lung-resident CD14$^+$ monocytes was investigated by real-time PCR and flow cytometry, respectively. Cellular proliferation, cytokine production, and molecules in IL-7 signaling pathway was assessed in CD14$^+$ monocytes in response to IL-7 stimulation. IL-7-induced CD14$^+$ monocytes activity to CD4$^+$ T cells was also assessed in direct and indirect contact co-culture system. There were no remarkable differences of plasma IL-7 concentration or CD127 level between healthy individuals and lung squamous carcinoma patients. However, IL-7 expression was significantly reduced in BALF from tumor site in squamous carcinoma patients, especially in stage III and IV. IL-7 stimulation not only promoted proliferation, cytokines secretion, and STAT-5 phosphorylation in lung-resident CD14$^+$ monocytes, but also enhanced CD14$^+$ monocytes-induced Th1 and T follicular helper cells activation, which presented as elevated interferon-γ and IL-21 secretion by CD4$^+$ T cells. This process required direct cell-to-cell contact, and was dependent on IL-6 secretion. The current data revealed a potential immunopromotive property of IL-7 to lung-resident CD14$^+$ monocytes in lung squamous carcinoma.

1. Introduction

Non-small cell lung cancer (NSCLC), which mainly comprises squamous carcinoma, adenocarcinoma, and large-cell lung cancer, accounts for approximate 85% of all lung cancers. Current therapies for NSCLC include surgery, chemotherapy, radiotherapy, and molecularly targeted therapy to immune check point inhibitors [antibodies against programmed death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)], which provide promising therapeutic management for lung cancer patients even in advanced stages [1–3]. However, lung cancer remains the most common cause of cancer-related deaths both worldwide and in China due to migration, invasion, recrudescence, and acquired resistance to anti-cancer drugs [4]. Thus, there remains needs for better understanding the pathogenesis and for development of new therapeutic achievements of lung cancer.

Interleukin (IL)-7, which is mainly secreted by stromal cells, plays a central role in the adaptive immune system by promoting lymphocytes development and maintaining T cell homeostasis and survival [5,6]. IL-7 influences generation and maintenance of T follicular helper (Tfh) cells in both normal and pathological condition [7,8]. Moreover, IL-7 signaling pathway is also involved in various aspects of innate lymphoid cells developmental and functional programs, indicating an immunoregulatory activity of IL-7 to innate immune response [9,10]. IL-7 enhances cytokines secretion by CD14$^+$ monocytes in tumor patients [11,12], and induces recruitment of monocytes/marcophage to endothelium [13]. Blood monocytes in combination with IL-7/IL-15 maintain human CD4$^+$ memory cells with mixed helper/regulatory function in autoimmune disorders [14]. However, modulatory activity of IL-7 to CD14$^+$ monocytes is not completely understood.

The immune capacity of cancer patients is suppressed, which is characterized by lower immune cell counts, exhausted/dysfunctional phenotypes, and increased expression of immunosuppressive cytokines

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[15,16]. Due to the potential immunotherapeutic strategy of IL-7/IL-7 receptor α (CD127) signaling pathway, we hypothesized that IL-7 administration also induced $CD14^+$ monocytes function in lung squamous carcinoma patients. To test this possibility, $CD14^+$ monocytes purified from peripheral bloods and lung residence from patients with lung squamous carcinoma were stimulated with recombinant human IL-7. Cellular proliferation, cytokine production, and key important molecules in IL-7 signaling within $CD14^+$ monocytes were investigated. The regulatory role of IL-7-induced $CD14^+$ monocytes activity to $CD4^+$ T cells was also assessed in lung squamous carcinoma.

2. Materials and methods

2.1. Subjects

The study protocol was approved by Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University, and written consent was obtained from each enrolled subjects. A total of thirty-seven patients with pathologically diagnosed lung squamous carcinoma were enrolled in the present study. All patients were hospitalized in Department of Thoracic Surgery of The First Affiliated Hospital of Xi'an Jiaotong University between July 2017 and April 2018. No patients were afflicted with pneumonia, chronic obstructive pulmonary disease, autoimmune disorders (including asthma), chronic viral hepatitis, or human immunodeficiency virus (HIV) infection. Moreover, patients received surgery, radiotherapy, chemotherapy, or molecular targeted therapies before sampling were excluded from the study. The tumor-node-metastasis (TNM) stage was evaluated following the American Joint Committee on Cancer/Union for International Cancer Control TNM classification (7th ed.). For healthy controls, eighteen individuals with matched sex ratio and age were also enrolled. The clinical characteristics of all enrolled subjects were shown in Table 1.

2.2. Isolation of plasma and peripheral blood mononuclear cells (PBMCs)

20 mL EDTA-anticoagulant peripheral bloods were obtained from each enrolled subjects. Plasma was collected by direct centrifugation at 300 ×*g* for 10 min at room temperature. PBMCs were isolated using Ficoll-Hypaque (Sigma, St. Louis, MO, USA) density gradient centrifugation, and then were stored in liquid nitrogen in fetal bovine serum (FBS) supplemented with 10% DMSO.

2.3. Preparation of bronchoalveolar lavage fluid (BALF)

BALF was prepared as previously described [17,18]. Briefly, local anesthesia was performed by injection of 2 mL 5% lidocaine in lung section through biopsy hole. The top of bronchofiberoscope closely wedged into opening of subsegmental bronchus. 50 mL sterilized saline was rapidly injected, and were immediately recovered under 100 mm Hg negative pressure with recover rate between 40% and 60%. This process was repeated four times. The collected BALF was filtered with double sterilized gauze and the total amounts were recorded. BALF was then centrifuged at 1200 ×*g* for 10 min at 4 °C. The supernatants were harvested and stored at −70 °C for subsequent experiments, while cellular precipitations were washed twice and cultured in DMEM supplemented with 10% FBS, L-Glutamine (10 g/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37 °C under 5% CO_2 condition.

Table 1
Clinical characteristics of enrolled subjects.

	Healthy individual	Lung squamous carcinoma
Case (n)	18	37
Gender (male/female)	13/5	27/10
Age (years)	50.1 ± 9.7	51.6 ± 14.4
Smoking history (n)	4	31
Stage (I/II/III/IV)	Not available	9/10/11/7

2.4. Purification of $CD14^+$ monocytes and $CD4^+$ T cells

$CD14^+$ monocytes and $CD4^+$ T cells purified using human CD14 Microbeads (Miltenyi, Bergisch Gladbach, Germany) and human $CD4^+$ T-Cell Isolation Kit (Miltenyi) according to instructions of manufacturer, respectively. Briefly, PBMCs or cells from BALF were centrifuged at 300 ×*g* for 10 min, and were resuspended by separation buffer mixed with CD14 Microbeads for a 15-min incubation at 4 °C in dark. Cell were then washed, and loaded into LD column (Miltenyi). The column was washed by separation buffer. Cells adhered in LD column, which were identified as $CD14^+$ monocytes, were collected and cultured in DMEM supplemented with 10% FBS. Cells in elutriant were also collected by centrifugation at 300 ×*g* for 10 min. Enriched cells were resuspended by separation buffer mixed with $CD4^+$ biotin-antibody cocktail microbeads for a 10-min incubation at 4 °C in dark. Cell were then washed, and loaded into LD column. Cells which flow through LD column were identified as $CD4^+$ T cells. The purity of $CD14^+$ monocytes and $CD4^+$ T cells was > 95% by flow cytometry determination.

2.5. Cell culture and stimulation

The sorted cells were cultured under three different conditions. (*i*) $CD4^+$ T cells or $CD14^+$ monocytes culture alone: 10^5 $CD4^+$ T cells or 10^5 of $CD14^+$ monocytes were added into 24-well plate. (*ii*) Direct cell-to-cell contact co-culture: 5×10^4 of $CD4^+$ T cells and 5×10^4 of $CD14^+$ monocytes were mixed and added into 24-well plate. (*iii*) Indirect cell-to-cell contact co-culture: 5×10^4 of $CD14^+$ monocytes were resuspended in 200 μL complete culture medium and were added into upper chamber of Transwell plate (Corning, Corning, NY, USA), while 5×10^4 of $CD4^+$ T cells were resuspended in 500 μL complete culture medium and were added into lower chamber. The chambers were separated by a semipermeable membrane with 0.04 μm pore size, which allowed circulation of medium only but not passage of cultured cells. Cells were stimulated with anti-CD3 antibody (eBioscience, San Diego, CA, USA) for 24 h. In certain experiments, $CD14^+$ monocytes were pretreated with recombinant human IL-7 (R&D Systems, Minneapolis, MN, USA; final concentration: 10 ng/mL) and or anti-IL-6 neutralizing antibody (InvivoGen, San Diego, CA, USA; final concentration 20 ng/mL) for 6 h or 48 h. Three independent experiments were performed for each subjects.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Expressions of cytokines and chemokines in plasma and cultured supernatants were measured using commercial ELISA kits (eBioscience) according to instructions of manufacturer.

2.7. Real-time PCR

Total RNA was purified using RNeasy Mini kit (Qiagen, Hilden, Germany) according to instruction of manufacturer. First strand cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa, Beijing, China) with random hexamers and Oligo(dT). Real-time PCR was performed using TB Premix Ex *Taq* (TaKaRa) according to instruction of manufacturer. Relative gene expression was semi-quantified by $2^{-\Delta\Delta CT}$ method using Applied Biosystems 7500 System Sequence Detection Software (Applied Biosystems, Foster, CA, USA). The primer sequences were cited from previous study [19].

2.8. Cellular proliferation assay

Cellular proliferation was measured by Cell Counting Kit-8 (CCK-8, Alexis Biochemicals, San Diego, CA, USA) according to instructions of

manufacturer.

2.9. Western blot

Cells were lysed in 50 μL lysis buffer by incubation on ice for 15 min. Cell lysis were harvested by centrifugation at 12,000 ×*g* for 1 min after incubation at 95 °C for 10 min. Proteins were separated on SDS-PAGE by eletrotransferring onto PVDF membrane. The membrane was soaked in blocking solution (PBS containing 5% non-fat milk and 0.05% Tween 20) for 2 h, and incubated overnight in the presence of antibodies targeting suppressor of cytokine signaling 3 (SOCS3), total signal transducer and activator of transcription-5 (STAT-5), phosphorylated STAT-5, and β-actin. All antibodies were purchased from Abcam (Cambridge, MA, USA), and were diluted in blocking solution (1: 1000 dilution) before incubation. Horseradish peroxidase-conjugated antibody IgG (Abcam, 1: 2000 dilution) was added to the membrane for additional 2-hour incubation. Antigen-antibody complexes were observed using enhanced chemiluminescence (Western Blotting Luminol Reagent, Cell Signaling Technology, Danvers, MA, USA).

2.10. Flow cytometry

PBMCs or cells from BALF were stained with anti-CD14-FITC (eBioscience) and anti-CD127-PE (eBioscience). In certain experiments, co-cultured cells were washed after stimulation, and then were cultured with PMA (50 ng/mL) and ionomycin (1 μg/mL), with brefeldin A (BFA, 10 μg/mL) for 5 h for intracellular staining of cytokine production. Cells were then stained with anti-CD4-PerCP Cy5.5 (eBioscience) along with anti-interferon-γ (IFN-γ)-APC (eBioscience; for intracellular staining) and anti-IL-21-PE (eBioscience; for intracellular staining). Data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and were analyzed using FlowJo software version 8.6.2 (Tree Star Inc., Ashland, OR, USA).

2.11. Statistical analyses

All data were analyzed using SPSS Version 21.0 software for Windows (SPSS, Chicago, IL, USA). Shapiro-Wilk test was used for normal distribution analysis. Data following normal distribution were presented as mean ± standard deviation, and differences were determined by Student's *t*-test, paired *t*-test, or One-Way ANOVA. Data following skewed distribution were presented as median [Q1, Q3], and differences were determined by Mann-Whitney test, Wilcoxon matched pairs test, or Kruskal-Wallis test. All tests were two tails, and P values < 0.05 were indicated as significant differences.

3. Results

3.1. IL-7 expression was down-regulated in BALF from tumor sites in patients with lung squamous carcinoma

Plasma samples were obtained from all enrolled subjects, while BALF samples were obtained from both tumor and nontumor site from all enrolled lung squamous carcinoma patients. IL-7 concentration was measured in plasma and BALF by ELISA. Plasma IL-7 expression was comparable between healthy individuals (2602 ± 784.9 pg/mL) and lung squamous carcinoma patients (2621 ± 700.8 pg/mL; Student's *t*-test, $P = 0.931$, Fig. 1A). There was also no significant difference of plasma IL-7 concentration among patients in different disease stages (One-Way ANOVA, $P = 0.263$, Fig. 1B). Moreover, IL-7 expression was notably down-regulated in BALF from tumor site (75.73[57.37, 117.9] pg/mL) than from nontumor site (122.8[82.22, 201.1]pg/mL; Mann-Whitney test, $P = 0.0022$, Fig. 1C). Although there was no remarkable difference of BALF IL-7 in tumor site among patients in different stages (Kruskal-Wallis test, $P = 0.243$), Il-7 expression in BALF from tumor site was reduced in stage III (Mann-Whitney test, $P = 0.018$, Fig. 1D) and stage IV (Mann-Whitney test, $P = 0.0079$, Fig. 1D), not in stage I (Mann-Whitney test, $P = 0.947$, Fig. 1D) or stage II (Mann-Whitney test, $P = 0.317$, Fig. 1D).

3.2. CD127 expression was comparable in $CD14^+$ monocytes between healthy individuals and patients with lung squamous carcinoma

$CD14^+$ monocytes were purified from peripheral bloods of all enrolled subjects and from BALF of all enrolled patients with lung squamous carcinoma. CD127 mRNA relative expression was semi-quantified by real-time PCR in 10^4 of $CD14^+$ monocytes. There were no significant differences of CD127 mRNA expression in peripheral $CD14^+$ monocytes between healthy controls and patients with lung squamous carcinoma (Student's *t*-test, $P = 0.288$, Fig. 2A), or among patients in different stages (One-Way ANOVA, $P = 0.279$, Fig. 2B). Furthermore, CD127 mRNA relative level was also comparable in lung-resident $CD14^+$ monocytes from lung squamous carcinoma patients between nontumor site and tumor site (Student's *t*-test, $P = 0.517$, Fig. 2C), or among different stages (One-Way ANOVA, $P = 0.654$, Fig. 2D). PBMCs from ten healthy individuals and eleven lung squamous carcinoma patients, and cells from BALF of eleven lung squamous carcinoma patients were stained with CD14 and CD127. The mean fluorescence intensity (MFI) value corresponding to CD127 on $CD14^+$ monocytes were assessed. The typical flow histogram analysis of membrane CD127 on $CD14^+$ monocytes were shown in Fig. 2E. There was no remarkable difference of membrane CD127 expression on either peripheral $CD14^+$ monocytes between healthy control and squamous carcinoma patients (143.5 ± 38.25 *vs.* 152.4 ± 51.78; Student's *t*-test, $P = 0.660$, Fig. 2F). Membrane CD127 expression on lung-resident $CD14^+$ monocytes also did not reveal significant difference between non-tumor site and tumor site (110.2 ± 35.64 *vs.* 105.8 ± 24.55; Student's *t*-test, $P = 0.751$, Fig. 2G).

3.3. IL-7 promoted proliferation and cytokine production of $CD14^+$ monocytes from patients with lung squamous carcinoma

We selected $CD14^+$ monocytes from both nontumor site and tumor site in eleven patients with lung squamous carcinoma (three in stage I, four in stage II, and four in stage III). 10^5 of purified $CD14^+$ monocytes were stimulated with recombinant human IL-7 for 48 h. Cells and supernatants were harvested for proliferation and ELISA assay. IL-7 stimulation significantly promoted cellular proliferation, which presented by increased cultured cell counts in both nontumor site ($[1.52 \pm 0.21] \times 10^5$ *vs.* $[1.28 \pm 0.15] \times 10^5$; paired *t*-test, $P = 0.0006$, Fig. 3A) and tumor site ($[1.56 \pm 0.24] \times 10^5$ *vs.* $[1.25 \pm 0.22] \times 10^5$; paired *t*-test, $P = 0.0009$, Fig. 3A). A global analysis of cytokines/chemokines released by monocytes was investigated in cultured supernatants. IL-1β and IL-6 concentration in the supernatants was significantly lower in the supernatants of cultured $CD14^+$ monocytes from tumor site than from nontumor site (Student's *t*-tests, $P = 0.0008$ and $P = 0.025$, respectively, Fig. 3B and C). However, IL-8 and tumor necrosis factor-α (TNF-α) level in the supernatants of $CD14^+$ monocytes was comparable between nontumor and tumor site (Mann-Whitney test, $P = 0.870$, Fig. 3D; Student's *t*-test, $P = 0.609$, Fig. 3E). IL-7 stimulation robustly enhanced all tested cytokines production by cultured $CD14^+$ monocytes (all $P < 0.05$, paired *t*-tests or Wilcoxon matched pairs tests, Fig. 3B–E). Moreover, levels of IL-12, soluble tumor necrosis factor related apoptosis-inducing ligand (sTRAIL), CCL2, CCL4, and CCL5 were also reduced in the supernatants of monocytes from tumor site, and revealed similar trends in response to IL-7 stimulation (Fig. S1). Important molecules in IL-7 signaling pathway, including SOCS3, total STAT-5, phosphorylated STAT-5 (pSTAT-5) were also assessed by Western blot. IL-7 stimulation significantly induced SOCS3 expression and STAT-5 phosphorylation (Fig. 3F). However, total STAT-5 expression did not change remarkably

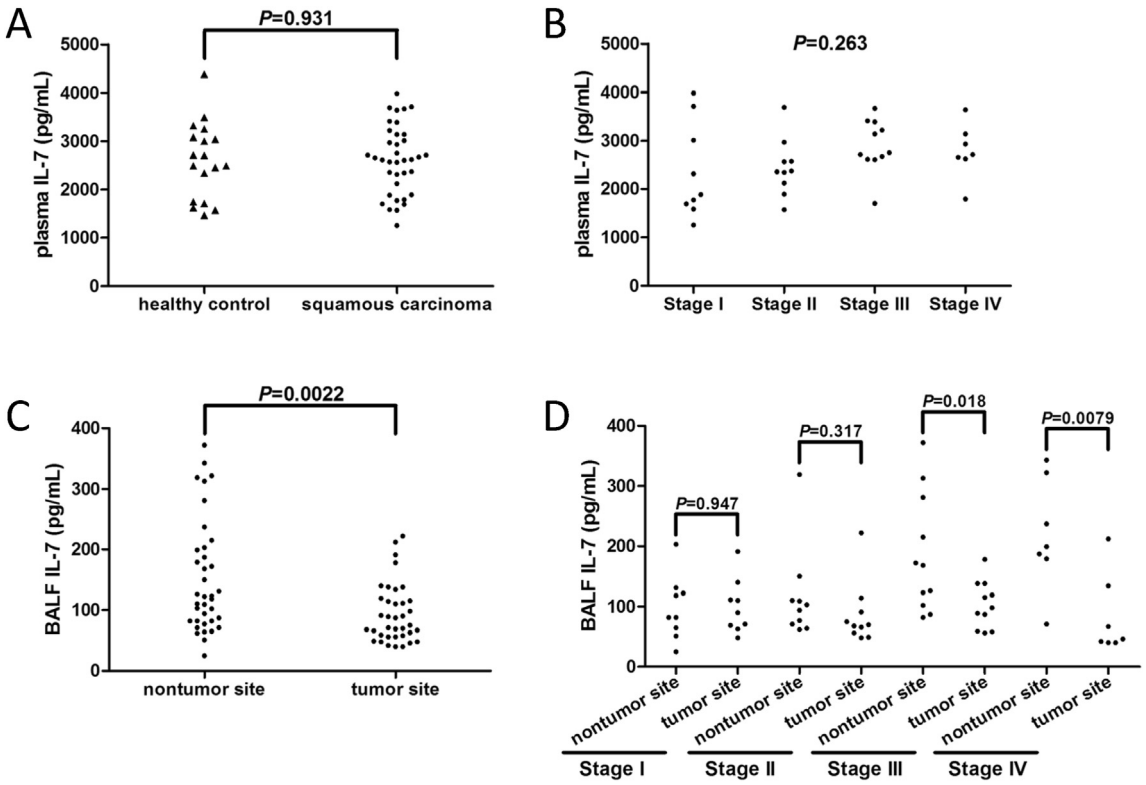

Fig. 1. IL-7 expression in plasma and BALF in healthy controls and patients with lung squamous carcinoma. IL-7 expression was measured by ELISA. (A) IL-7 expression in plasma in healthy individuals (n = 18) and lung squamous carcinoma patients (n = 37). (B) Plasma IL-7 expression in lung squamous carcinoma patients of different stages (stage I, n = 9; stage II, n = 10; stage III, n = 11, stage IV, n = 7). (C) IL-7 expression in BALF from nontumor site and tumor site in lung squamous carcinoma patients (n = 37). (D) IL-7 expression in BALF from tumor site and nontumor site was compared in lung squamous carcinoma patients of different stages (stage I, n = 9; stage II, n = 10; stage III, n = 11, stage IV, n = 7). Individual level of each enrolled subject was shown.

in response to IL-7 treatment (Fig. 3F).

3.4. IL-7 enhanced CD14$^+$ monocytes-induced activation of CD4$^+$ T cells, which required direct cell-to-cell contact, in patients with lung squamous carcinoma

We selected CD4$^+$ T cells and CD14$^+$ monocytes from tumor site in fifteen patients with lung squamous carcinoma (four in stage I, four in stage II, five in stage III, and two in stage IV). CD14$^+$ monocytes were pretreated with recombinant human IL-7 for 6 h, and then washed twice to remove exogenous IL-7. 5×10^4 of stimulated CD14$^+$ monocytes were then cultured with autogenic 5×10^4 of CD4$^+$ T cells in the presence of anti-CD3 antibody in both direct contact and indirect contact manner. Cells were harvested 24 h post co-culture for flow cytometry analysis. The representative flow plots of IFN-γ and IL-21 secretion within CD4$^+$ T cells were shown in Fig. 4A. The percentage of IFN-γ$^+$ within CD4$^+$ cells in direct contact co-culture system (4.69 ± 0.70%) was significantly higher than in indirect contact co-culture system (2.84 ± 0.76%; Student's *t*-tests, $P < 0.0001$, Fig. 4B) and CD4$^+$ cells cultured alone (2.75 ± 0.99%; Student's *t*-tests, $P < 0.0001$, Fig. 4B). However, there was no remarkable difference of IFN-γ$^+$ cells frequency within CD4$^+$ T cells between indirect contact system and CD4$^+$ T cells cultured alone (Student's *t*-tests, $P = 0.782$, Fig. 4B). IL-7 pretreatment to CD14$^+$ monocytes induced higher IFN-γ production in CD4$^+$ T cells in direct contact system (5.58 ± 0.98%; paired *t*-test, $P < 0.0001$, Fig. 3B), but not in indirect contact system (3.05 ± 0.69%; paired *t*-test, $P = 0.075$, Fig. 3B). IL-21 production in CD4$^+$ T cells revealed similar trends with IFN-γ. The percentage of IL-21$^+$ within CD4$^+$ T cells in direct contact co-culture system (4.82 ± 1.09%) was notably higher than in indirect contact system (3.04 ± 0.38%; Student's *t*-tests, $P < 0.0001$, Fig. 4C) and CD4$^+$ cultured alone (2.98 ± 1.21%; Student's *t*-tests, $P = 0.0003$, Fig. 4C), however, the difference of IL-21$^+$CD4$^+$ cells percentage did not indicate statistical significance between indirect contact co-culture and CD4$^+$ T cells alone (Student's *t*-tests, $P = 0.870$, Fig. 4C). IL-7 pretreatment to CD14$^+$ monocytes also enhanced IL-21 secretion by CD4$^+$ T cells in direct contact co-culture system (5.68 ± 1.16%; paired *t*-test, $P = 0.0031$, Fig. 4C), but not in indirect contact system (3.14 ± 0.50%; paired *t*-test, $P = 0.099$, Fig. 4C). IL-6 production in the cultured supernatants was also measured by ELISA. IL-6 expression was significantly elevated in both direct and indirect contact co-culture system (266.1 ± 54.80 pg/mL and 200.1 ± 51.07 pg/mL) than in CD4$^+$ T cells cultured alone (103.4 ± 24.46 pg/mL; Student's *t*-tests, $P < 0.0001$, Fig. 4D). IL-7 pretreatment to CD14$^+$ monocytes robustly increased IL-6 production in both direct contact (339.7 ± 81.37 pg/mL; paired *t*-test, $P = 0.0001$, Fig. 3D) and indirect contact system (262.0 ± 53.11 pg/mL; paired *t*-test, $P = 0.0004$, Fig. 3D).

3.5. The enhancement of IL-7 stimulation to CD14$^+$ monocytes-induced CD4$^+$ T cells activation was dependent on IL-6 secretion

CD4$^+$ T cells and CD14$^+$ monocytes from tumor site were selected from ten patients with lung squamous carcinoma (three in stage II, three in stage III, and four in stage IV). CD14$^+$ monocytes were pretreated with recombinant human IL-7 for 6 h, and then washed twice to remove exogenous IL-7. 5×10^4 of stimulated CD14$^+$ monocytes were then cultured with autoglous 5×10^4 of CD4$^+$ T cells in the presence of anti-CD3 antibody and anti-IL-6 neutralizing antibody in direct contact manner. Administration of anti-IL-6 neutralizing antibody did not affect IFN-γ (4.64 ± 1.09% *vs.* 4.37 ± 0.69%; paired *t*-test, $P = 0.521$, Fig. 5A) or IL-21 (4.18 ± 0.92% *vs.* 4.08 ± 1.16%; paired *t*-test, $P = 0.830$, Fig. 5B) production by CD4$^+$ T cells. However, anti-IL-6

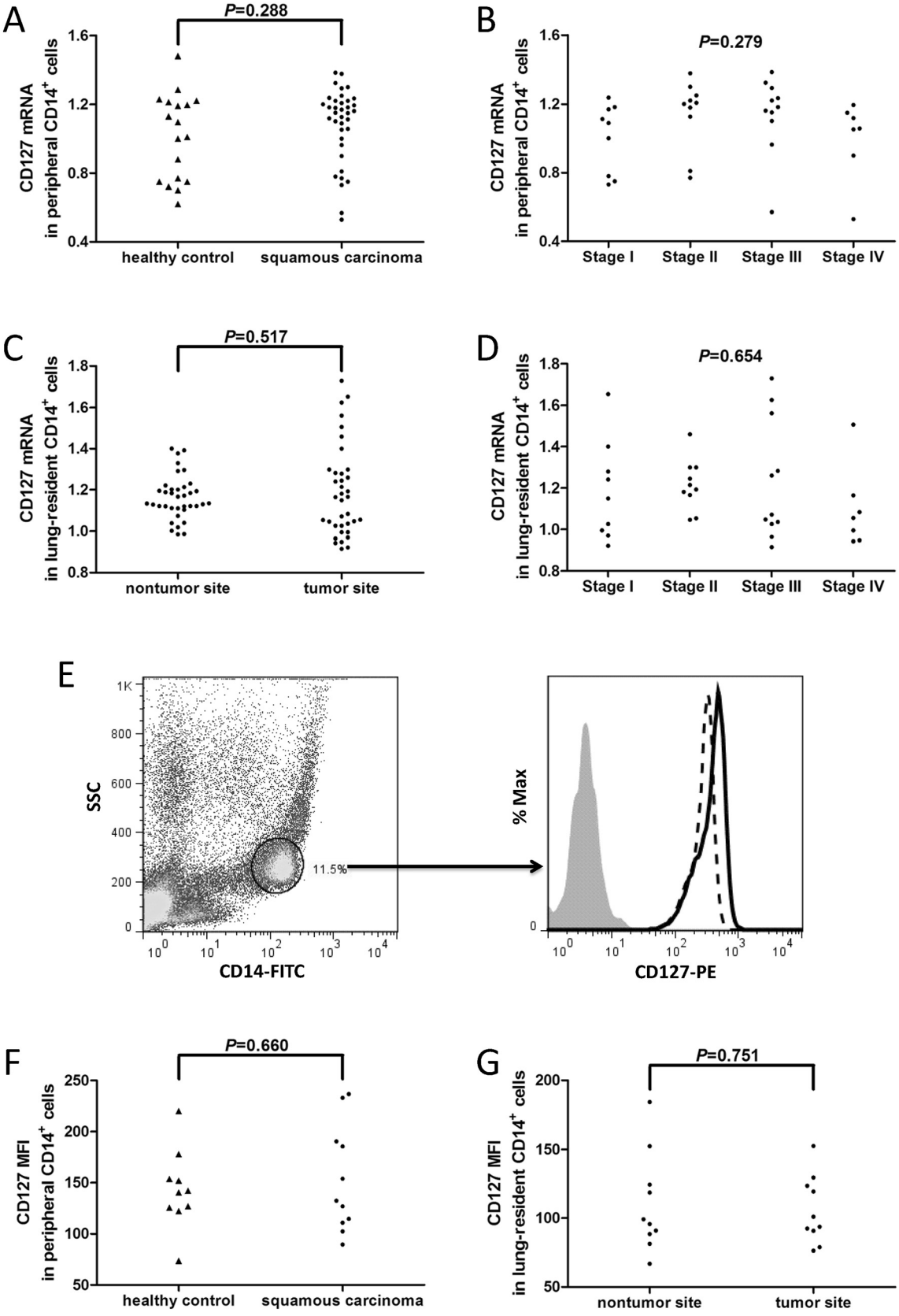

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Fig. 2. CD127 expression in peripheral and lung-resident $CD14^+$ monocytes from healthy individuals and patients with lung squamous carcinoma. $CD14^+$ monocytes were purified from all enrolled subjects, and CD127 mRNA expression within $CD14^+$ monocytes was semi-quantified by real-time PCR. (A) CD127 mRNA expression in peripheral $CD14^+$ monocytes in healthy individuals (n = 18) and lung squamous carcinoma patients (n = 37). (B) CD127 mRNA expression in peripheral $CD14^+$ monocytes in lung squamous carcinoma patients of different stages (stage I, n = 9; stage II, n = 10; stage III, n = 11, stage IV, n = 7). (C) CD127 mRNA expression in lung-resident $CD14^+$ monocytes from nontumor site and tumor site in lung squamous carcinoma patients (n = 37). (D) CD127 mRNA expression in lung-resident $CD14^+$ monocytes from tumor site in lung squamous carcinoma patients of different stages (stage I, n = 9; stage II, n = 10; stage III, n = 11, stage IV, n = 7). PBMCs from ten healthy individuals and eleven lung squamous carcinoma patients, and cells from BALF of eleven lung squamous carcinoma patients were stained with CD14 and CD127 to analyze CD127 expression on $CD14^+$ monocytes by flow cytometry. (E) The typical flow histogram analysis of CD127 expression on peripheral $CD14^+$ monocytes. The grey line represented isotype control, the black line the healthy control, the black dot line the squamous carcinoma patient. (F) Membrane CD127 expression on peripheral $CD14^+$ monocytes in healthy individuals (n = 10) and lung squamous carcinoma patients (n = 11). (G) Membrane CD127 expression in lung-resident $CD14^+$ monocytes from nontumor site and tumor site in lung squamous carcinoma patients (n = 11). Individual level of each enrolled subject was shown.

neutralizing antibody reduced IL-7-induced $CD14^+$ monocytes activation to $CD4^+$ T cells, which presented as decreased percentages of IFN-γ^+CD4^+ cells (4.44 ± 1.26% *vs.* 5.79 ± 1.18%; paired *t*-test, $P = 0.024$, Fig. 5B) and IL-21^+CD4^+ cells (4.25 ± 0.93% *vs.* 5.62 ± 1.48%; paired *t*-test, $P = 0.023$, Fig. 5B).

4. Discussion

In the present study, plasma IL-7 expression and CD127 mRNA level in peripheral $CD14^+$ monocytes was comparable between healthy individual and lung squamous carcinoma patients. Lung-resident IL-7 revealed reduced level in tumor site, especially in stage III and IV. Administration of recombinant human IL-7 *in vitro* promoted the activity of lung-resident purified $CD14^+$ monocytes, which presented as increased cellular proliferation, elevated proinflammatory cytokines production, STAT-5 phosphorylation. IL-7 stimulation also enhanced $CD14^+$ monocytes-induced $CD4^+$ T cells activation, while this process required direct cell-to-cell contact, and was dependent on IL-6 secretion. The current results indicated a pivotal immunoactive property and a potential therapeutic strategy of IL-7 in lung squamous carcinoma.

IL-7 is a member of common γ-chain family of cytokines [20], which is known to be essential in promoting immunity during chronic infection and cancer [21,22]. Serum IL-7 concentration was significantly increased in colorectal cancers, and this elevation was associated with lymph node involvement and right-sided tumor location [23,24]. Systemic IL-7 elevation correlated with skin involvement of breast cancer in Iranian patients [25]. Elevated IL-7 expression has also reported in prostate cancer tissues, which was closely associated with poor prognosis [26,27]. In contrast, reduced serum IL-7 level was observed in patients with chronic hepatitis C [28] and HCC [29]. We found that peripheral IL-7 expression was comparable between healthy controls and lung squamous carcinoma patients, however, lung-resident IL-7 level was robustly down-regulated in tumor site. This was consistent with a current study by Zhang et al., which indicated a reduction of IL-7 expression in BALF in NSCLC patients [30]. However, higher IL-7 level was found in bone-aggressive NSCLC cells in both mice model and human histological biopsies [31]. The controversy on IL-7 production in lung cancer might be partly due to the diverse source of IL-7. Tumor cells, cells of non-hepatopoietic origin, dendritic cells, and even monocytes/macrophages could express and produce IL-7 [32], which made it difficult to confirm the exact origin of lung-resident IL-7. Moreover, human tumor lesions showed aberrant IL-7 variant expression profile, and six different isoforms could be detected in lung tissue [32]. Commercial antibodies targeting human IL-7 might bind to different IL-7 variants or motifs for detection, leading to distinct results by different groups. Further experiments might be needed to investigate differentiated expression of common region of IL-7 mRNA within different variants in organs and tumor tissues. Furthermore, it was ideal for comparison of BALF IL-7 from healthy controls with BALF from lung squamous carcinoma patients, however, this is not ethical.

Although arguments still remained as to the direct pro-tumor or anti-tumor activity to lung cancer cells [30,33,34], it has been well accepted that IL-7 was an important regulator to different types of immune cells. Tumor-infiltrating myeloid-derived suppressor cells dysregulated B cell responses in lung cancer through impaired IL-7 and STAT5 [35]. IL-7 did not impact natural killer (NK) cells proliferation, but induced elevation of IFN-γ-secreting $CD56^{bright}$ NK cells and enhancement of cytotoxicity in $CD56^{dim}$ NK cells from multiple sclerosis patients [36]. However, IL-7 promotes CXCR3 ligand-dependent T cell antitumor reactivity in lung cancer [37]. IL-7 treatment promoted cytotoxic $CD8^+$ T cells activity in chronic hepatitis and HCC, and these process was independent on CD127 expression on $CD8^+$ T cells [28,29], However, few studies focused on the direct regulatory activity of IL-7 to $CD14^+$ monocytes. IL-7 administration to chronic HIV infected patients *in vivo* reduced plasma level of soluble CD14, which indicative of systemic inflammation [38]. IL-7 gene variant lacking exon 5 (IL-7δ5)-encoding protein stimulation resulted in a proinflammatory phenotype of $CD14^+$ monocytes and increased expression of genes involved in lipid metabolism [39]. In this study, membrane CD127 protein as well as total CD127 mRNA level (including membrane and soluble CD127) in $CD14^+$ monocytes was comparable between health and cancer in both peripheral and lung-resident issues, suggesting $CD14^+$ monocytes responsiveness to IL-7 was dispensable of IL-7 receptor α chain level. This was similar to the comparable CD127 expression in liver-resident $CD8^+$ T cells [28,29], in which IL-7 also revealed immunoactive property. Furthermore, IL-7 stimulation *in vitro* enhanced lung-resident $CD14^+$ monocytes functionality (including proliferation, proinflammatory cytokines production, STAT5 phosphorylation, and direct regulation to $CD4^+$ T cells activation. This was consistent with previous study by Standiford et al., which showed an elevation of IL-8-secreting $CD14^+$ monocytes in response to IL-7 stimulation [11]. The current results indicated that $CD14^+$ monocytes in tumor site were more suppressive rather than proinflammatory, and IL-7 might restore $CD14^+$ monocytes function in lung squamous carcinoma patients.

Activated monocytes induced $CD4^+$ T cells response in rheumatoid arthritis patients, and this process was independent of cytokines stimulation, but required cell-to-cell contact [40]. We found that the presence of activated $CD14^+$ monocytes could not induce Th1 and Tfh phenotype differentiation without direct contact to $CD4^+$ T cells. The only condition under direct contact of $CD14^+$ monocytes and $CD4^+$ T cells effectively induced Th1 and Tfh differentiation, suggesting an important cell-to-cell contact in guiding differentiation of Th1 and Tfh cells from $CD4^+$ T cells in lung squamous carcinoma patients. The current results were consistent with the study by Yang et al., which indicated a direct cell contact manner of $CD14^+$ monocytes to Th17 differentiation in rheumatoid arthritis patients [41]. Moreover, IL-7 directly regulated Tfh cells and $CD8^+$ T cells function in a HCV-specific manner, which was dependent on IL-6 production [8,28]. We found that IL-7-induced $CD14^+$ monocytes presented promising regulatory activity to $CD4^+$ T cells in a cell-to-cell contact manner. This process was also dependent on IL-6 secretion because neutralization of IL-6 suppressed IL-7-mediated $CD14^+$ monocytes regulation to $CD4^+$ T cells.

In summary, lung squamous carcinoma might down-regulate IL-7 production in lung-residence, leading to dysfunction of $CD14^+$ monocytes. Recombinant IL-7 stimulation restored lung-resident $CD14^+$

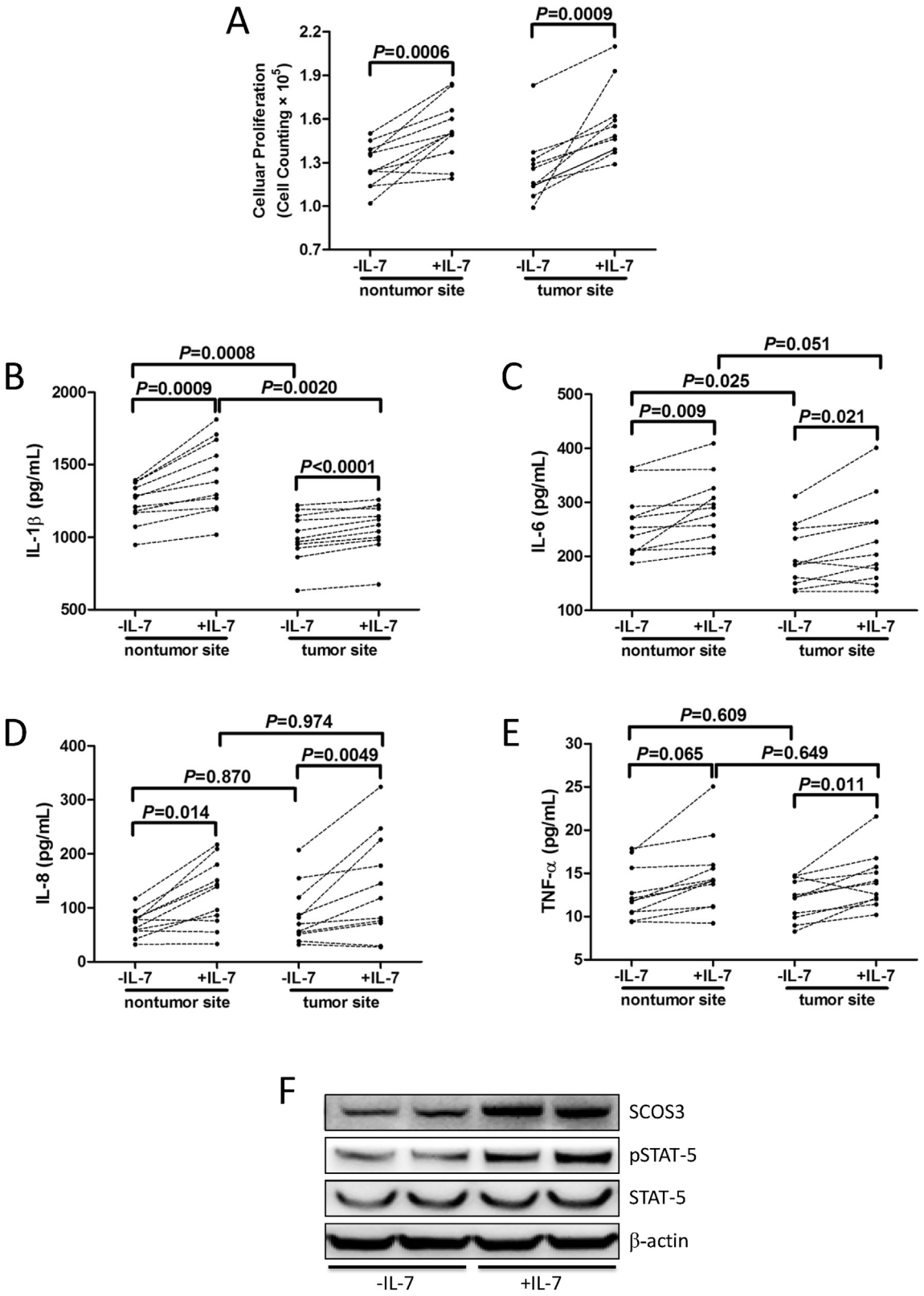

Fig. 3. Effect of recombinant human IL-7 stimulation to $CD14^+$ monocytes from patients with lung squamous carcinoma. 10^5 of $CD14^+$ monocytes from eleven patients with lung squamous carcinoma (three in stage I, four in stage II, and four in stage III) were stimulated with recombinant human IL-7 for 48 h. (A) Cellular proliferation was assessed by CCK-8 in both $CD14^+$ monocytes from nontumor and tumor sites in the presence or absence of IL-7 stimulation. (B) IL-1β, (C) IL-6, (D) IL-8, and (E) TNF-α expression in the cultured supernatants were measured by ELISA in the presence or absence of IL-7 stimulation. Individual level of each enrolled subject was shown. (F) Expressions of SOCS3, total STAT5, phosphorylated STAT5, and β-actin in $CD14^+$ monocytes in the presence or absence of IL-7 stimulation were assessed by Western blot.

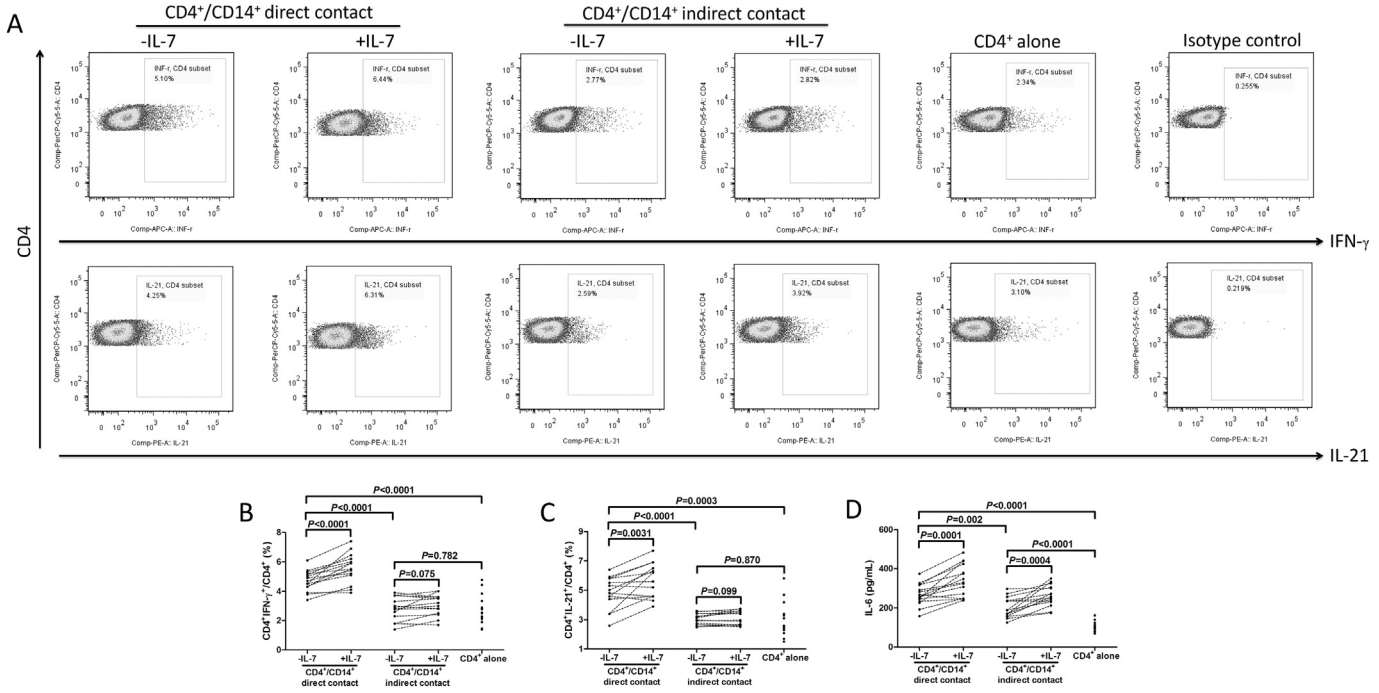

Fig. 4. Effect of recombinant human IL-7 to CD14$^+$ monocytes-induced activation of CD4$^+$ T cells in patients with lung squamous carcinoma. CD4$^+$ T cells and CD14$^+$ monocytes from tumor site were selected from fifteen patients with lung squamous carcinoma (four in stage I, four in stage II, five in stage III, and two in stage IV). CD14$^+$ monocytes were pretreated with recombinant human IL-7 for 6 h, and then washed twice to remove exogenous IL-7. 5×10^4 of stimulated CD14$^+$ monocytes were then cultured with autoglous 5×10^4 of CD4$^+$ T cells for 24 h in the presence of anti-CD3 antibody in both direct contact and indirect contact manner. (A) The representative flow plots of IFN-γ and IL-21 secretion within CD4$^+$ T cells in the presence or absence of IL-7 stimulation. (B) IFN-γ and (C) IL-21 secretion by CD4$^+$ T cells was measured by flow cytometry. (D) IL-6 expression in the cultured supernatants was investigated by ELISA. Individual level of each enrolled subject was shown.

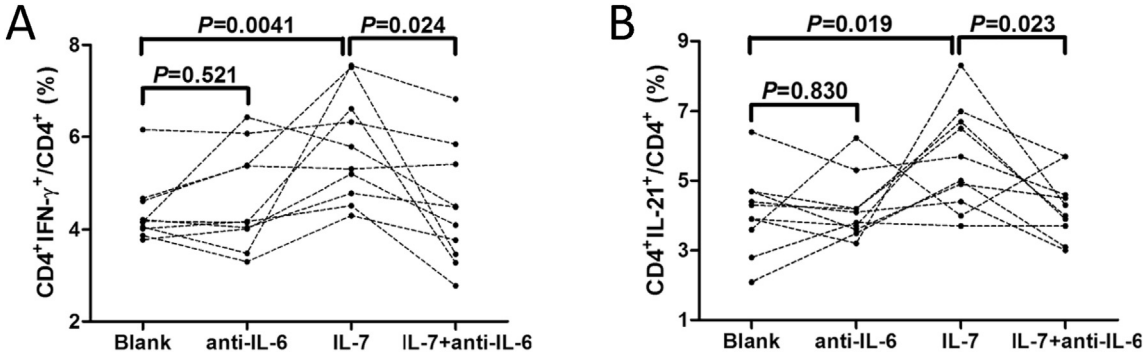

Fig. 5. Effect of anti-IL-6 neutralizing antibody to IL-7 enhanced CD14$^+$ monocytes-induced activation of CD4$^+$ T cells in patients with lung squamous carcinoma. CD4$^+$ T cells and CD14$^+$ monocytes from tumor site were selected from ten patients with lung squamous carcinoma (three in stage II, three in stage III, and four in stage IV). CD14$^+$ monocytes were pretreated with recombinant human IL-7 for 6 h, and then washed twice to remove exogenous IL-7. 5×10^4 of stimulated CD14$^+$ monocytes were then cultured with autoglous 5×10^4 of CD4$^+$ T cells in the presence of anti-CD3 antibody and anti-IL-6 neutralizing antibody in direct contact manner. (A) IFN-γ and (B) IL-21 secretion by CD4$^+$ T cells was measured by flow cytometry. Individual level of each enrolled subject was shown.

monocytes functionality, however, the regulatory activity of CD14$^+$ monocytes to CD4$^+$ T cells required direct cell-to-cell contact and was dependent on IL-6 secretion. This critical function of IL-7 signaling pathway in CD14$^+$ monocytes expanded our insight and might be useful in approaching novel therapeutic strategies for lung squamous carcinoma.

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Disclosure statement

No competing financial interests exist.

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