Non-small cell lung cancer (NSCLC) accounts for the majority of all lung cancer cases, which is the leading cause of cancer deaths worldwide. IL-17A, the major effector cytokine derived from Th17 cells, is a key cytokine in tumor pathogenesis and modulates tumor progression. We aimed to identify whether IL-17A derived from Th17 cells promotes the progression of NSCLC. Here we found that the level of Th17 cells was increased in NSCLC and IL-17A was mainly produced by CD4+ cells (Th17 cells) in NSCLC. IL-17A enhanced the migration, invasion and stemness of NSCLC via STAT3/NF-κB/Notch1 signaling. Blockade of this signaling inhibited the migration, invasion and stemness of NSCLC mediated by IL-17A. Th17 cells in NSCLC were closely associated with poor prognosis of NSCLC patients. Our results indicated that Th17 cell-derived IL-17A plays an important role in tumor progression of NSCLC via STAT3/NF-κB/Notch1 signaling. Therefore, therapeutic strategies against this pathway would be valuable to be developed for NSCLC treatment.

**Introduction**

Non-small cell lung cancer (NSCLC) accounts for the majority of all lung cancer cases, which is the leading cause of cancer deaths worldwide. The unfavorable outcome could be at least partially attributed to the poor understanding of NSCLC tumor microenvironment. The complex tumor microenvironment comprises immune cells, fibroblasts, blood and lymphatic vessels, which promotes the proliferation, invasion and metastasis of tumor cells, and contributes to disease progression. T helper (Th) 17 cells are a subtype of Th cell, which have been shown highly pro-inflammatory and to contribute to autoimmune disease. Interleukin (IL)-6 and TGF-β can promote the differentiation of Th17 cells through STAT3. The orphan retinoic acid nuclear receptor (ROR) family transcription factor RORγt is essential for Th17 development and function. IL-17 family is composed of IL-17A-F, among them, IL-17A (also termed IL-17) is the major effector cytokine derived from Th17 cells.

Recently, it has been shown that IL-17A was detected in several tumors. The evidences indicate that tumor development is associated with Th17 cells and IL-17. The adoptive transfer of Th17 cells induced by TGF-β and IL-6 promoted tumor growth in a CD39-dependent manner. IL-17 promotes tumor progression by enhancing angiogenesis in a mouse model of breast cancer. Therefore, we consider that IL-17A is a key cytokine in tumor pathogenesis and modulates tumor progression.

However, the evidence to indicate the protumoral activity of IL-17A in NSCLC is not clear. The aim of this study is to evaluate whether IL-17A derived from Th17 cells promotes the progression of NSCLC. Our results indicate that Th17 cell-derived IL-17A can promote the invasion, migration and cancer stem cell-like properties via STAT3/NF-κB/Notch1 signaling in NSCLC.

**Results**

**The level of Th17 cells was increased in NSCLC**

To determine the relationship between Th17 cells and NSCLC, Th17 cell frequencies in peripheral blood from NSCLC patients and healthy donor were analyzed by flow cytometry (Fig. 1A). CD3+CD4+IL-17A+1FN-γ- cell population was defined as Th17 cells as previously described. The percentage of Th17 cells in peripheral blood from NSCLC patients was significantly higher than that from healthy donor (P<0.001, Fig. 1B). IL-17A mRNA level was...
also significantly higher in peripheral blood from NSCLC patients than that from healthy donor ($P<0.001$, Fig. S1A). Th17 cell frequency in NSCLC tissues was significantly higher than that in non-tumor tissues ($P<0.05$, Fig. 1C). The mRNA level of IL-17A was significantly higher in NSCLC tissues than that in non-tumor tissues ($P<0.05$, Fig. S1B). Moreover, Th17 cell frequency in tumor tissue was increased compared to that in peripheral blood from NSCLC patients ($P<0.01$, Fig. 1D). The relationships between Th17 cell frequency and clinical parameters were shown in Table 1. In addition, we detected the expression of Th17 cells in tumor tissues and non-tumor tissues from NSCLC patients by immunofluorescence. Tumor-infiltrated CD4$^+$ T cells exhibited high-level production of IL-17A (Fig. 1E). Therefore, these results indicate that the level of Th17 cells is increased in NSCLC, and IL-17A is mainly produced by CD4$^+$ cells (Th17 cells) in NSCLC.

**IL-17A promotes the migration and invasion of NSCLC cells**

Whether IL-17A derived from Th17 cells has the effect on NSCLC progression, the migration and invasion assays were firstly performed *in vitro*. After the treatment of rhIL-17A *in vitro*, the migration activity of A549 and H460 cells was significantly increased compared to control ($P<0.05$, Fig. 2A and B). Moreover, the invasion activity of A549 and H460 cells with the treatment of rhIL-17A was also increased compared to untreated group *in vitro* ($P<0.05$, Fig. 2C and D). Therefore, these results indicate that the level of Th17 cells is increased in NSCLC, and IL-17A is mainly produced by CD4$^+$ cells (Th17 cells) in NSCLC.
Effect of IL-17A on epithelial-mesenchymal transition (EMT) and angiogenesis of NSCLC was evaluated in vitro. We detected the expression of N-cadherin (mesenchymal-related gene) in A549 and H460 cells with or without rhIL-17A treatment by western blotting and qPCR. The results showed that the expression of N-cadherin was significantly higher in cells with rhIL-17A treatment than that in untreated groups (P<0.05, Fig. 2E and F). Snail and vimentin, two other EMT-related genes, were investigated by western blotting and qPCR as well. The expressions of snail and vimentin were significantly higher in cells with rhIL-17A treatment than that in untreated groups (P<0.05, Fig. S2A-C). Next, the effect of IL-17A on angiogenesis of NSCLC was evaluated. First, the expression of IL-17 receptor on tumor tissues and endothelium of blood vessels was detected. The results showed that IL-17 receptor expression in NSCLC tissues was significantly higher than that in non-tumor tissues (P<0.05, Fig. S2D). Similarly, the expression of IL-17 receptor in primary endothelium from NSCLC tissues was significantly higher than that from non-tumor tissues (P<0.05, Fig. S2E). Then we found tube formation was enhanced with rhIL-17A treatment than that in untreated groups (P<0.05, Fig. S2F). Further, the relationship between

| Characteristics                | Number | P value |
|-------------------------------|--------|---------|
| Age at diagnosis              |        |         |
| <65 years old                 | 30     | 0.8662  |
| ≥65 years old                 | 20     |         |
| Gender                        |        |         |
| Male                          | 35     |         |
| Female                        | 15     | 0.9409  |
| Histological type             |        |         |
| Adenocarcinoma                | 25     | 0.2449  |
| Squamous carcinoma            | 20     |         |
| Large cell carcinoma          | 5      |         |
| Tumor grade                   |        |         |
| Good                          | 8      |         |
| Moderate                      | 21     |         |
| Poor                          | 21     | 0.0203  |
| TNM stage                     |        |         |
| I                             | 18     |         |
| II                            | 14     |         |
| III                           | 11     | 0.0340  |
| IV                            | 7      |         |
| Lymph node metastasis         |        |         |
| Positive                      | 28     |         |
| Negative                      | 22     | 0.0110  |
| Smoking habit                 |        |         |
| Yes                           | 21     | 0.9686  |
| No                            | 29     |         |

**Table 1. Correlations between Th17 expression and clinicopathological parameters**

**Figure 2.** IL-17A promoted the migration and invasion of NSCLC cells in vitro. The migration activities of A549 (A) and H460 (B) cells, and the invasion activities of A549 (C) and H460 (D) cells before and after treatment of rhIL-17A was analyzed using transwell assay. One representative analysis is shown. Data are presented as a histogram. (E) The expression of N-cadherin in A549 and H460 cells before and after treatment of rhIL-17A was analyzed using western blotting. (F) The relative expression of N-cadherin in A549 and H460 cells before and after treatment with rhIL-17A was analyzed using qPCR. (G) The expressions of IL-17A and CD31 in tumor tissues from NSCLC patients were detected by immunofluorescence. * indicates P < 0.05. Scale bar represents 50 μm.
IL-17A and CD31, which is the biomarker of angiogenesis, was investigated. We found that IL-17A expression was associated with CD31 (P<0.05, Fig. S2G). Lastly, the expression of CD31 was investigated in NSCLC tissues by immunofluorescence. CD31 was highly or lowly expressed in NSCLC tissues in correspondence with high or low expression of IL-17A (Fig. 2G). The results suggest that IL-17A promotes the migration and invasion of NSCLC cells.

The STAT3/NF-κB/Notch1 signaling was critical for IL-17A-induced migration and invasion in NSCLC cells

Recent study showed that IL-17A could promote the transition from chronic pancreatitis to pancreatic cancer through stimulating STAT3 activation.\(^8\) It is shown that tumorigenesis capacity was mediated by NF-κB signaling in ovarian cancer.\(^9\) It is also demonstrated that IL-17 could induce Notch1 activation in oligodendrocyte progenitor cells that enhanced proliferation and inflammatory gene expression.\(^20\) Furthermore, Notch1 signaling pathway is associated with cancer stem cell (CSC)-like properties in tumors.\(^21\) To determine whether the STAT3/NF-κB/Notch1 signaling is involved in IL-17A-induced migration and invasion in NSCLC, the expression of phospho-STAT3, phospho-p65 and cleavage-Notch1 in A549 and H460 cells treated with rhIL-17A was investigated by western blotting. The results showed that rhIL-17A increased phospho-STAT3, phospho-p65 and cleavage-Notch1 in A549 and H460 cells (Fig. 3A, Fig. S3A-C). Then, we investigated whether STAT3/NF-κB/Notch1 depletion would affect IL-17A-mediated tumor progression in NSCLC. rhIL-17A enhanced the migration and invasion activities in A549 and H460 cells treated with or without DMSO, but could not do so in A549 and H460 cells treated with STAT3, or NF-κB, or Notch1 inhibitors, respectively (Fig. 3B-G). The result was confirmed with STAT3, or NF-κB, or Notch1 knockdown, respectively (Fig. S3D-H). In addition, IL-17A-mediated high level of N-cadherin expression in NSCLC cells was blocked in A549 and H460 cells treated with signaling inhibitors compared to cells treated with or without DMSO (Fig. 3H). All of these results demonstrate that STAT3/NF-κB/Notch1 signaling is critical for IL-17A-induced migration and invasion in NSCLC cells.

IL-17A promoted the CSC-like properties of NSCLC cells

Stemness is an important characteristic of tumor progression. To determine the effect of IL-17A on the stemness of NSCLC, sphere formation assay was firstly investigated in vitro. The sphere formation was enhanced in A549 and H460 cells treated with rhIL-17A compared to untreated cells (P<0.01, Fig. 4A and B). In addition, the expression of CSC-related gene Oct4 in A549 and H460 cells treated with rhIL-17A was significantly higher than that in untreated cells (Fig. 4C). Nanog and Sox2, two other CSC-related genes, were significantly higher with rhIL-17A treated than that in untreated cells (Fig. S4A-C). After the treatment with docetaxel, the apoptosis of A549 and H460 cells was significantly increased compared to control (<0.05, Fig. 4D and E). Moreover, the apoptosis of A549 and H460 cells with the treatment of docetaxel was blocked by rhIL-17A in vitro (Fig. 4D and E), indicating that IL-17A induces the resistance of NSCLC cells.

To further evaluate whether IL-17A is required for CSC-like properties in NSCLC, stable IL-17A expression in H460 cells was established using IL-17A-expressing plasmid (P<0.01, Fig. 4F and G). The results showed that tumor volumes in IL-17A-expressing H460 cell group were significantly higher than those in control group (P<0.05, Fig. 4H, I and K), and the tumor weights in IL-17A-expressing H460 cell group were increased as well (P<0.05, Fig. 4I), suggesting that IL-17A enhanced tumor growth in NSCLC. Collectively, IL-17A promotes the CSC-like properties in NSCLC cells.

Blockade of STAT3/NF-κB/Notch1 signaling inhibited NSCLC cell stemness promoted by IL-17A

To examine if the STAT3/NF-κB/Notch1 signaling is required for IL-17A-induced CSC-like properties in NSCLC cells, the blockade of this signaling in A549 and H460 cells was investigated. The results showed that the enhancing activity of sphere forming in A549 and H460 cells induced by IL-17A was reversed by the treatment of STAT3, or NF-κB, or Notch1 inhibitors, respectively (Fig. 5A, B and C). The result was also confirmed with STAT3, or NF-κB, or Notch1 knockdown, respectively (Fig. S5A-C). After the treatment with STAT3, or NF-κB, or Notch1 inhibitor, Oct4 expression in A549 and H460 cells treated with rhIL-17A was decreased (Fig. 5D). In addition, IL-17A-mediated resistance in NSCLC cells was blocked in A549 and H460 cells treated with signaling inhibitors compared to cells treated with DMSO (Fig. 5E and F). Taken together, STAT3/NF-κB/Notch1 signaling is critical for IL-17A-induced CSC-like properties in NSCLC cells, and blockade of this signaling inhibits the stemness of NSCLC cells mediated by IL-17A.

Th17 cells were associated with poor prognosis in NSCLC patients

Th17 cells were reported associated with a poor clinical outcome in glioblastoma recently.\(^22\) So the relationship between Th17 frequency and clinical parameters (TNM stage, tumor grade and lymph node metastasis), and the impact of Th17 cells on therapeutic effect and survival were evaluated in NSCLC patients. The level of Th17 frequency in peripheral blood was increased in patients with II/III TNM stage (P<0.05, Fig. 6A), poor tumor grade (P<0.05, Fig. 6B), and positive lymph node metastasis (P<0.05, Fig. 6C). After chemotherapy treatment (cisplatin+ docetaxel), the percentage of Th17 cells was significantly increased in patients with progressive disease (PD) (P<0.05, Fig. 6D), decreased in patients with partial responses (PR) (P<0.01, Fig. 6E), and no significant difference in patients with stable disease (SD) (P<0.05, Fig. 6F). Upon IL-17A expression from immunohistochemistry results, patients were grouped as “high” or “low” using the respective median (2.8) as a cut-off point (Fig. 6G). NSCLC patients with dense infiltration of Th17 cells had a worse overall survival (OS) (P<0.05, Fig. 6H). Taken together, Th17 cells in NSCLC are closely associated with poor prognosis in NSCLC patients, and could be
Figure 3. The STAT3/NF-κB/Notch1 signaling was critical for IL-17A-induced migration and invasion in NSCLC cells. (A) The activation of STAT3, NF-κB and Notch1 in A549 and H460 cells treated with rhIL-17A was analyzed using western blotting. (B) The migration activities of A549 and H460 cells treated with or without rhIL-17A and STAT3, or NF-κB, or Notch1 inhibitor were assessed by transwell assay. One representative analysis is shown. The data from A549 (C) and H460 (D) cells are presented as histogram. (E) The invasion activities of A549 and H460 cells treated with or without rhIL-17A and these molecular inhibitors were assessed by transwell assay. One representative analysis is shown. The data from A549 (F) and H460 (G) cells are presented as a histogram. (H) The expression of N-cadherin in A549 and H460 cells treated with or without rhIL-17A and STAT3, or NF-κB, or Notch1 inhibitor was analyzed using western blotting. * indicates \( P < 0.05 \). Scale bar represents 50 µm.
served as a potential biomarker and target for prognosis and treatment of NSCLC.

Discussion

The link between inflammation and tumorigenesis is well known, studies have implicated many inflammatory components, such as IL-6, as a key player in tumor development, growth, and metastasis.\(^{23}\) Recently, it is shown that the numbers of Th17 cells and expression of IL-17A are increased in several tumors.\(^{24,25}\) The combination of IL-6 and TGF-β induces IL-17-producing cells (Th17). Moreover, IL-6 can use TGF-\(\beta\) produced by thymus-derived natural regulatory T cells (nTregs) to convert them to Th17 cells. However, IL-2 and TGF-\(\beta\) induce naive T cells to become forkhead/winged helix transcription factor (Foxp3) positive regulatory cells (iTregs), which are resistant to Th17 conversion by IL-6.\(^{9}\) Although numerous studies have been reported that IL-17A was involved in inducing and mediating inflammatory responses,\(^{26,27}\) the role of IL-17A in cancer initiation, growth, and metastasis was very controversial.\(^{28-30}\) Therefore, we consider that IL-17A is an important cytokine in tumor progression. In this study, we aimed to evaluate the role of IL-17A derived from Th17 cells in tumor progression of NSCLC.

In this study, the percentage of Th17 cells in peripheral blood from NSCLC patients was significantly higher than that from healthy donors, and Th17 cells in NSCLC were closely associated with the therapeutic effect and prognosis of NSCLC patients. Other studies have also proven that Th17 cells and IL-17A were correlated with worse prognosis in cancer patients. Vizio et al. found that increased expression of IL-17 predicted a poor response to chemotherapy and adverse prognosis in pancreatic carcinoma.\(^{31}\) Yamada et al. reported that Th17 cell abundance and IL-17 expression were also elevated in gastric
cancer and indicated a poor prognosis. In addition, high level of IL-17 expression and tumor-infiltrating Th17 cells were associated with worse prognosis in patients with invasive ductal carcinoma, colorectal and lung cancer patients. Abundant evidences have indicated that tumor development is closely correlated with IL-17. IL-17 could promote angiogenesis in tumor, mainly by stimulating surrounding fibroblasts and endothelial cells to produce proangiogenic factors such as VEGF. Furthermore, IL-17-transfected HCC cells significantly promoted angiogenesis, neutrophil recruitment and tumor growth in vivo. Recently, Treg enrichment in cancer patients correlated with the increase of IL-17A expression which induced tumor invasiveness. In addition, IL-17 promoted mammary tumor progression by changing tumor cell behavior and eliciting tumorigenic neutrophils recruitment. IL-17 contributed to ovarian cancer malignancy through promoting CD133+ CSCs self-renewal. In this study, we found that IL-17A promoted the migration and invasion of NSCLC by transwell assays, enhanced N-cadherin expression in NSCLC cells and CD31 expression in xenograft tumors. Moreover, our data showed that rhIL-17A enhanced the sphere formation of NSCLC cells, the expression of Oct4 and resistance in NSCLC cells, and tumor growth in NSCLC, indicating that IL-17A promotes the CSC-like properties in NSCLC cells. Taken together, IL-17A promotes the migration, invasion and stemness to induce tumor progression in NSCLC.

The signaling mediated IL-17A-induced tumor progression is reported by several studies. Celine et al. found that IL-17 functions through the novel REG3b/JAK2/STAT3 signaling pathway to promote the transition from chronic pancreatitis to pancreatic cancer. The effect of IL-17 on tumor progression was operated through AKT signaling activation, which resulted in IL-6 production. Then, IL-6 activated JAK2/STAT3 signaling and subsequently up-regulated IL-8, MMP2, and VEGF expression. According to the results from other studies, IL-17 also could promote tumor growth through stimulating production of IL-6 and STAT3 activation. In addition, IL-17 not only promoted sphere formation of ovarian in vitro, but also enhanced the tumorigenesis in vivo, by p38 MAPK and NF-κB activation. Meanwhile, IL-17 could induce Notch1 activation in oligodendrocyte progenitor cells that enhanced proliferation and inflammatory gene expression. Pardoll et al. showed that maintenance of NF-κB activity in tumors requires Stat3. Pfeffer et al. showed that STAT3 and p65 regulated Notch1 expression in adherent CSCs by directly binding to the Notch1 promoter. In our study, we found that IL-17 significantly induced STAT3 and NF-κB phosphorylation and Notch1 cleavage in NSCLC cells. These results indicated that STAT3, NF-κB and Notch1 activation play an important role in NSCLC progression.

Figure 5. Blockade of STAT3/NF-κB/Notch1 signaling inhibited NSCLC stemness promoted by IL-17A. (A) After the treatment with STAT3 or NF-κB or Notch1 inhibitor, the sphere forming of A549 and H460 cells induced by rh-IL-17A was blocked. One representative photomicrograph is shown. The results from A549 (B) and H460 (C) cells were shown as histogram. (D) The expression of Oct4 in A549 and H460 cells treated with or without rh-IL-17A and these molecular inhibitors were assessed by western blotting. The apoptosis of A549 (E) and H460 (F) cells treated with or without rh-IL-17A and these molecular inhibitors were assessed by transwell assay. * indicates P < 0.05. Scale bar represents 100 μm.
Our study supports the principle that IL-17A/STAT3/NF-κB/Notch1 may participate in NSCLC progression. Thus, targeting IL-17A/STAT3/NF-κB/Notch1-mediated signaling pathway in tumors may provide a novel approach to controlling tumor growth. There are several approaches available for blocking this signaling. It is shown that IL-17 blockade significantly reduced the tumor volume and inhibited tumor growth in TC-1 tumor model.33 Tumor growth was inhibited in IL-17−/− mice in another B16 melanoma model.42 IL-17A-neutralizing or IL-17RA blocking antibodies (secukinumab, ixekizumab, and brodalumab), already proven effective in autoimmune disorders such as psoriasis, psoriatic arthritis, and ankylosing spondylitis,45 could be used as potential neo-adjuvants in the treatment of early, non-metastatic colorectal cancer.47 Furthermore, agents targeted STAT3/NF-κB/Notch1 signaling for cancer treatment are used in preclinical and clinical trials. Niclosamide, an inhibitor of STAT3, has the potential to target the IL-6-STAT3-AR pathway to inhibit migration and invasion in advanced prostate cancer.46 1-O-acetylbritannilactone, a Chinese traditional medicine, has been performed to have the activity of anticancer with decreased NF-κB expression.47 A clinical phase I trial was to investigate that enoticumab, a fully human IgG1 monoclonal antibody that binds human Delta-like Ligand 4 and disrupts Notch-mediated signaling, was tolerated, and the antitumor activity included two partial responses and 16 patients with stable disease.48 Similarly, our data showed that blockade of STAT3/NF-κB/Notch1 signaling inhibited the CSC-like properties in NSCLC cells, indicating that STAT3/NF-κB/Notch1 signaling could be served as the potential therapeutic targets of NSCLC.

In summary, high level of IL-17A derived from Th17 cells in NSCLC enhanced the migration, invasion and stemness of NSCLC cells via STAT3/NF-κB/Notch1 signaling, which could be blocked by these signaling molecular inhibitors. Therefore, therapeutic strategies that target this pathway could represent an effective method for NSCLC treatment.

Materials and methods

Patients and samples

From February 2014 to March 2015, 110 patients with NSCLC from The First Affiliated Hospital of Zhengzhou University were enrolled. These patients were subjected to routine laboratory diagnosis, and the samples were analyzed using conventional cytology. In addition, 41 NSCLC patients were enrolled from May 2014 to June 2015 and administrated with cisplatin (75 mg/m²) plus docetaxel (30 mg/m² on day 1, 8) every three weeks for 4 cycles. Samples from all these patients’ peripheral blood were obtained. Samples from 21 healthy donors and 9 non-tumor patients were used as control. All patients gave the written informed consent. The whole consent procedure was in accordance with the standards defined by Institutional Review Boards of The First Affiliated Hospital of Zhengzhou University (approval number 2013-LW-1201).

Flow cytometry analysis

Fresh human peripheral blood mononuclear cells (PBMCs) were isolated using ficoll-hypaque (huajing Biology) density gradient centrifugation, and stimulated with 1 mg/mL phorbol-12-myristate-13-acetate (PMA, Sigma, #P1585) and 1 mg/mL ionomycin (Sigma, #I9657) in the presence of Brefeldin-A (BFA, BioLegend, #420601) for 5 h. Cells were stained with
anti-human CD3 (BD, #555332), CD4 (BD, #550631), and then intracellular staining for IFN-γ (BD, #557643) and IL-17A (BD, #580437) was performed as described. Cells were analyzed using flow cytometry (BD, FACS CantoII, USA).

**Immunofluorescence**

The protocol of immunofluorescence is described elsewhere. Mouse anti-CD4 (Abcam, #ab846), rabbit anti-IL-17A (PeproTech, #500-P07) and mouse anti-CD31 (R&D, #BBA7) were used as the primary antibodies. The secondary antibodies of Alexa Fluor 555-conjugated goat anti mouse IgG (Life technology, #A21422), Dylight 488-conjugated donkey anti-rabbit IgG (Thermo Fisher, #A21206), and Dylight 555-conjugated goat anti-rabbit IgG (Thermo Fisher, #A21430) were used in immunofluorescence. The nuclear was stained with 40,6-diamidino-2-phenylindole (DAPI, Solarbio, #C0065). Images were analyzed using a fluorescent microscope (200×, Leica, DMI8, Germany).

**Migration and invasion assay**

A transwell system (Corning, #3422) was used to analyze cell migration and invasion. A549 or H460 cells (1×10⁴ cells/mL) were harvested and suspended in serum-free RPMI-1640. The suspension was seeded onto the upper chamber of the transwell system. Recombinant human interleukin-17A (rhIL-17A) was suspended in serum-free RPMI-1640 at the concentration of 100 ng/mL in the bottom wells. For blocking STAT3/NF-κB/Notch1 pathway with inhibitors, STAT3 inhibitor (Niclosamide, Sigma, #N3510), NF-κB inhibitor (PDTMC, MCE, #HY-18738) and Notch1 inhibitor (LY411575, MCE, #HY-50752) were added respectively in the bottom 30 minutes before rhIL-17A was added. For blocking STAT3/NF-κB/Notch1 pathway with siRNAs, A549 and H460 cells were transfected with siRNAs 24 hours before rhIL-17A was added. A549 cells were seeded onto Matrigel matrix chambers (Corning, #354248) following the manufacturer’s instructions in invasion assay. The cells were incubated with 5% CO₂ for 48 h at 37°C, then stained with crystal violet and counted under a microscope (100×). Triplicate in-dependent experiments were performed.

**Western blotting**

Quantified protein lysates were measured with a Protein BCA Assay Kit (Thermo Fisher, #23228) according to the manufacturer’s instructions. The proteins in lysates were resolved on SDS-PAGE gels, transferred onto PVDF membranes (Millipore, #ISEEQ00010), and immunoblotted with anti-human N-cadherin (Cell signaling Technology, #13116), snail (Cell signaling Technology, #8387), vimentin (Cell signaling Technology, #5741), p-STAT3 (Cell signaling Technology, #9145), p-p65 (Cell signaling Technology, #3033), cleaved-Notch1 (Cell signaling Technology, #4147), Oct4 (Cell signaling Technology, #2750), Nanog (Cell signaling Technology, #4903), Sox2 (Cell signaling Technology, #3579), IL-17A (PeproTech, #500-P07) and anti-human β-actin antibody (ProteinTech, #20536-1-AP-50).

**RNA extraction and quantitative real-time polymerase chain reaction (qPCR)**

For detecting IL-17A mRNA level in peripheral blood, total RNA was extracted from PBMC of healthy donors and NSCLC patients by TRIzol reagent (Takara, #9109) according to the manufacturer’s instructions. For detection of IL-17A and IL-17R mRNA in tissues, total RNA was extracted from tumor tissues and non-tumor tissues of NSCLC patients. Total RNA was extracted from rhIL-17A-treated A549 and H460 cells to investigated EMT and CSC-related genes. The first-strand cDNA was synthesized from 1 μg of total RNA using Prime Script™ RT reagent kit (Takara, #RR047A). qPCR was performed using SYBR Premix Ex Taq II (Takara, #RR820A) and assessed by Agilent Mx3005P. The abundance of mRNA for each gene of interest was normalized to GAPDH. Details of the primer sequences used for qPCR are listed in Table S1.

**Cell sorting**

To obtain CD31⁺ and CD31⁻ endothelial cells, Fresh tumor and non-tumor tissues were cut into small pieces and digested in RPMI 1640 (Gibco, USA) supplemented with 0.25% trypsin (Gibco), 0.002% DNaseI (Gibco), and 20% fetal bovine serum (FBS, Gibco) at 37°C for 20 min. Dissociated cells were filtered through a 40 μm mesh. Also, CD31 MicroBeads (Miltenyi, #130-091-935) were used to enrich for endothelial cells through magnetic cell separation as per manufacturer’s protocol. Total RNA was extracted from CD31⁺ and CD31⁻ endothelial cells and IL-17R mRNA expression was detected by qPCR.

**Tube-forming assay**

Ice-cold growth factor-reduced basement membrane matrix (Matrigel; Corning, #354230) was added at 64 ul per well to precooled 96-well plates and allowed to polymerize at 37°C for 30 minutes. Human umbilical vein endothelial cells (HUVEC, 3×10⁴ cells/well) were seeded in 100 ul of EGM-2 MV basal medium with or without 100 ng/mL IL-17A were plated onto the gel surface and incubated at 37°C for 5 h. Cell rearrangement and tube formation were visualized by microscopy (200×, Leica, DMI8, Germany).

**Sphere formation assay**

A549 or H460 cells were suspended in DMEM/F12 medium (Gibco, #11330032) with 4 μg/mL heparin (Solarbio, #H8270), B27 (1.50, Gibco, #17504044), 20 ng/mL EGF (PeproTech, #AF-100-15-100), 20 ng/mL bFGF (PeproTech, #AF-100-18B) in 24-well ultra-low cluster plates (Corning, #3473). The cells were treated with or without rhIL-17A (PeproTech, #200-17). For blocking STAT3/NF-κB/Notch1 pathway with inhibitors, STAT3 inhibitor, NF-κB inhibitor and Notch1 inhibitor were added respectively 30 minutes before rhIL-17A was added. For blocking STAT3/NF-κB/Notch1 pathway with siRNAs, A549 and H460 cells were transfected with siRNAs 24 hours before rhIL-17A was added.
added. After culturing for 7 days, the numbers of spheres were counted.

**Chemotherapy resistance assay**

$10^5$ A549 or H460 cells were treated with 100 ng/mL of rhIL-17A for 0.5 h. Then docetaxel (Sangon Biotech, #A606561) was added to the wells at a final concentration of 1 μg/mL for 24 h. Cells untreated with docetaxel and rhIL-17A were used as control group. In the assay of inhibitors’ treatment, A549 or H460 cells were treated with rhIL-17A and inhibitors for 0.5 h, then 1μg/mL docetaxel was added. Lastly, the apoptosis percentage was analyzed by ow cytometry.

**Transfected cell line**

To evaluate the effect of IL-17A on tumor growth, H460 cells were stably transfected with IL-17A-expressing vector (Sangon Biotech) to induce the overexpression of IL-17A in H460 cells. The control vector was used to generate the control H460 cells. To evaluate the signaling of IL-17A-induced tumor progression in NSCLC, A549 cells and H460 were transfected with vectors containing an STAT3-specific siRNA to knock down STAT3 expression, NF-κB-specific siRNA to knock down NF-κB expression, Notch1-specific siRNA to knockdown Notch1 expression, respectively. A549 and H460 cells were also transfected with a vector containing a scramble siRNA as a control. The sequences of the siRNAs used are as follows. STAT3: 5'-UGGCCCAUG- GAAUCAGCUACAGCA-3', NF-κB: 5'-GAGAGGAGCACAGA UACCAACAGA-3' and Notch1: 5'-GACGAUGUGCCAGGA AACACUGCA-3'.

**In vivo xenograft experiments**

Animal protocols were approved by the Review Board of the First Affiliated Hospital of Zhengzhou University. Severe combined immunodeficient mice (female, 4–6-week old) were purchased from the Chinese Academy of Medical Sciences. Mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions. For xenograft experiments, $4 \times 10^6$ H460 cells transfected with IL-17-expressing or control vector were resuspended in 1 ml PBS and injected into each mice subcutaneously. By visual observation and palpation, engrafted mice were inspected every 2 days for tumors. At the 21 days after cell transplantation, mice were sacrificed by cervical dislocation and tumors were isolated for analysis.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded sections were provided by the Department of Pathology of our hospital, and confirmed by histopathological results. The protocol of immunohistochemistry is described elsewhere. Rabbit anti-IL-17A (PeproTech, #500–P07) was used as primary antibody. Three fields of images per sample were taken. Non-immune rabbit IgG at the same dilution was used as negative control. Photos were recorded under a microscope (200×, Leica, DMi8, Germany).

**Statistical analysis**

All the data from quantitative assays were expressed as mean ± standard deviation by GraphPad Prism 5 software. Statistical analyses were performed using t-test or one-way analysis of variance. Kaplan-Meier curves in combination with log-rank tests were used to compare the survival of patients in different groups. The difference was considered statistically significant when $P < 0.05$.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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