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FOXP3 influences recruitment and polarization of macrophages via regulating CCL5 in non-small cell lung cancer

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ABSTRACT: Forkhead box P3 (FOXP3) plays a key role in the malignant progression of non-small cell lung cancer (NSCLC). However, the regulatory effect of FOXP3 on the biological activity of macrophages in NSCLC has not yet been reported. In the present study, we explored the effects of FOXP3 on the polarization and recruitment of macrophages induced by A549 and H1975 cells. RAW264.7 cells were used as macrophages. The transcription and protein levels of FOXP3, chemokine (C-C motif) ligand 5 (CCL5), and other cytokines were evaluated by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Chromatin immunoprecipitation (ChIP) and dual-luciferase reporter assays were performed to confirm the regulatory relationship between FOXP3 and CCL5. Immunofluorescence staining was used to detect the expression of differentiation 206 (CD206) in the macrophages. The capacity of cancer cells to recruit macrophages was evaluated using a macrophage chemotaxis assay. Downregulation of FOXP3 expression inhibits macrophage recruitment by lung cancer cells. Low FOXP3 expression also decreased the expression levels of M2 macrophage markers (CD206, CD163, and interleukin-10 (IL-10)) in macrophages. Inhibition of FOXP3 expression downregulates CCL5 transcription in A549 and H1975 cells. The promotion of macrophage chemotaxis and M2 polarization in lung cancer cells overexpressing FOXP3 was reversed by downregulation of CCL5. Our study revealed that FOXP3 promoted chemotaxis and M2 polarization of macrophages in NSCLC. This effect is caused by the regulation of CCL5 secretion by cancer cells.

KEYWORDS: forkhead box P3, chemokine (C-C motif) ligand 5, non-small cell lung cancer, macrophage recruitment, macrophage polarization

INTRODUCTION

Lung cancer is a leading cause of cancer-related deaths in both men and women [1]. In 2020, lung cancer was expected to account for a higher number of new cases and deaths worldwide [2]. Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer [3]. Despite promising progress in the systematic management of lung cancer, the 5-year survival rate of patients with advanced lung cancer remains low [4]. Hence, providing a more accurate early diagnosis for lung cancer patients and finding effective therapeutic targets has become the most critical scientific problem in clinical research on lung cancer.

In recent years, with the in-depth study of the tumorigenesis mechanism, researchers have found that the growth of tumors depended not only on the tumor cells themselves, but also on the environment they were in, that was, the tumor microenvironment (TME). As a major component of immune infiltration in cancer, tumor-associated macrophages (TAMs) are essential in the TME [5]. TAMs are considered potential indicators of diagnosis and prognosis in patients with NSCLC [6]. In the interaction between cancer cells and TAMs, cancer cells can affect the polarization and recruitment of macrophages through the release of chemokines [7, 8]. Chemokine (C-C motif) ligand 2 (CCL2) and CCL5 have been proved that they could be secreted by NSCLC cells to mediate macrophage infiltration [9]. In colorectal cancer, alternatively activated type 2 (M2) macrophage infiltration can also be promoted by CXCL2 secreted by cancer cells [10]. Given the importance of these chemokines in the regulation of TAMs, it is important to explore the factors that regulate chemokines in lung cancer cells to regulate the infiltration of TAMs.

FOXP3 is a transcription factor that belongs to the forkhead family [11]. Mutations in FOXP3 increase the incidence of autoimmune diseases in humans, especially intestinal diseases [12]. Recent studies have shown that FOXP3 is not only one of the main regulators of Treg cells and related immune diseases but is also an important factor in the development of many cancers [13]. FOXP3 has been identified as a poor prognostic factor in various cancers, including cervical, thyroid, and colorectal cancers. High FOXP3 expression can promote malignant progression of cancer cells [14, 15]. In lung cancer, FOXP3 regulates the biological activity of cancer cells through multiple pathways [16]. FOXP3 has been reported to induce epithelial-mesenchymal transition (EMT) [17] via Wnt/β-catenin or NF-kB signaling pathways in NSCLC [16, 18]. Some

researchers have demonstrated that abnormal levels of FOXP3 ubiquitination could affect the transcription of GINS1 and thus drive the development of NSCLC [19]. FOXP3 expression in lung cancer patients is also positively correlated with macrophage infiltration [20, 21]. However, the role of FOXP3 in the polarization and recruitment of TAMs in NSCLC remains unclear.

To further understand these effects, we explored the regulatory effects of abnormally expressed FOXP3 on polarization and recruitment of macrophages induced by NSCLC. In addition, we proved that FOXP3 promoted M2 polarization and recruitment of macrophages by regulating CCL5 expression in lung cancer. This study revealed the specific role of FOXP3 in mediating the regulation of macrophage recruitment and polarization in NSCLC cells, providing a molecular basis for the study of the effect of cancer-derived FOXP3 on the function of TAMs in lung cancer.

MATERIALS AND METHODS

Cell culture and transfection

Mouse monocyte macrophage leukemia cells (RAW264.7), 293T cells (HEK293T), 3 NSCLC cell lines (A549, PC-9, and H1975), and human bronchial epithelial cells (16HBE) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM) (cat. no. 11965092; Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin (cat. no. P4333; Sigma-Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (FBS; cat. no. 16140089; Gibco) was used to culture 16HBE cells, 293T, and RAW264.7 cells. Roswell Park Memorial Institute (RPMI)-1640 medium (cat. no. C11875500BT; Gibco) supplemented with 1% penicillin-streptomycin and 10% FBS was used to culture A549, PC-9, and H1975 cells. The culture conditions for all cells were 37 °C and 5% CO_2. pcDNA3.1-FOXP3 was purchased from RiboBio (Guangzhou, China). Full-length FOXP3 sequence was synthesized and combined with the vector. Control, FOXP3-targeting short hairpin RNAs, and CCL5-targeting short hairpin RNAs (sh-NC, sh-FOXP3, and sh-CCL5, respectively) were purchased from Invitrogen (Waltham, MA, USA) and inserted into the pLKO.1 vector (cat. no. 10878; Addgene, Watertown, MA, USA). For stable overexpression (oe) and sh-cell lines, a lentiviral expression vector was constructed using the ViraPower™ Lentiviral Expression System (cat. no. K4950-00; Invitrogen). The cells were transfected with Lipofectamine 3000 (cat. no. L3000150; Invitrogen) according to the manufacturer's instructions.

Chemotaxis assay of macrophages

The experimental process of chemotactic experiment was based on previous reports [22]. Different groups of A549 and H1975 cells were seeded in a 6-well plate (1×10^6 cells). The cell supernatants were collected. The supernatant of the cells was added to the lower compartment of the Transwell inserts (cat. no. 3422; Corning, Corning City, NY, USA). Macrophages were added to the upper compartment of Transwell inserts at a density of 2×10^5 cells. After incubation for 16 h, macrophages were stained with crystal violet and counted.

Polarization assay of macrophages

Different groups of A549 and H1975 cells were seeded in a 6-well plate (1×10^6 cells). The cell supernatants were collected. Macrophages were seeded at 1×10^6 cells in 6-well plates in a tumor-conditioned medium (medium containing A549 or H1975 cell supernatant) and incubated for 48 h. The expression levels of the M1 (Interleukin-6 (IL-6), C-X-C Motif Chemokine 10 (CXCL10), and CD80) macrophage markers and M2 (CD206, CD163, and CCL22) macrophage markers were determined to study the effects of NSCLC cell supernatant on macrophage polarization.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT- qPCR)

Total RNA was extracted from the cells using TRIzol reagent (cat. no. 15596026, Thermo Fisher Scientific, Waltham, MA, USA). RNA purity (OD260/OD280 nm, 1.8–2.2) was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The RT assay was performed using a PrimeScrip RT reagent kit (cat. no. RR037Q; Takara, Shiga, Japan). Relative mRNA expression levels were determined using SYBR Green Premix Ex Taq (cat. no. RR8A0A; Takara). The reaction protocol was 95 °C for 5 min, 95 °C for 1 min (40 cycles), and 60 °C for 30 s. Primer sequences are listed in Table S1. GAPDH was used as a reference gene. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Chromatin immunoprecipitation (ChIP)-qPCR

Potential FOXP3 binding sites in the CCL5 promoter region were predicted using JASPAR (http://jaspar.genereg.net/). ChIP assays were performed using a ChIP assay kit (cat. no. P2078; Beyotime Biotechnology, Shanghai, China), according to the manufacturer's instructions. A549 and H1975 cells (1×10^6 cells) were cross-linked by adding formaldehyde at a final concentration of 1% for 10 min at 37 °C. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Next, the cells were washed twice in ice-cold PBS containing protease inhibitors, pelleted, and resuspended in 200 µl of SDS lysis buffer (Beyotime Biotechnology). The chromatin was ultra-sonicated to fragments (∼200–500 bp) 10 times with 10 s of ultrasonication at 10 s intervals. Lysates were subsequently incubated with anti-rabbit IgG (cat. no. 2729S; 1:100, Cell Signaling

Technology, Boston, MA, USA) or a ChIP-grade antibody against FOXP3 (cat. no. ab215206; 1:30, rabbit monoclonal, Abcam, Cambridge, UK) at 4 °C overnight and then incubated with Protein A+G Agarose/Salmon Sperm DNA at 4 °C for 3 h. After washing with low salt and high salt buffer, elution, and reverse cross-linking, the DNA was added to EDTA, Tris pH 6.5, and proteinase K at 45 °C for 1 h and then purified for qPCR analysis. The primer sequences used for ChIP-qPCR in the CCL5 promoter region were as follows: CCL5: F: 5′-CTGCCTCAATTTACAGTGTGAGT-3′ and R: 5′-CTCCTTTCCCTCATCCATGGA-3′.

Dual-luciferase reporter assay

To investigate the effect of FOXP3 on the activation of the CCL5 promoter, the construct or truncated promoter region of CCL5 was cloned upstream of the luciferase reporter gene of the pGL3.0 Basic vector (RiboBio). 293T cells (1×10^5 cells) were seeded in a 24-well plate and cultured for 24 h. Cells were then co-transfected with wild-type or mutant luciferase plasmids, pRL-TK plasmid, and FOXP3 shRNA/negative control using Lipofectamine 3000 (cat. no. L3000150; Invitrogen). After transfection for 48 h, luciferase activity was measured using a Dual-Luciferase Reporter Gene Assay Kit (cat. no. RG027; Beyotime Biotechnology). The firefly luciferase activity was normalized to Renilla luciferase activity as a control.

Immunofluorescence assay

Macrophages were fixed in 4% paraformaldehyde (cat. no. P6148; Sigma-Aldrich) for 20 min and 0.5% Triton X-100 (cat. no. 93443; Sigma-Aldrich) at room temperature for 15 min and incubated with the primary antibodies, CD206 (cat. no. ab300621; 1:50, rabbit monoclonal; Abcam) at 4 °C overnight. The samples were then cleaned and treated with Alexa Fluor-647-conjugated anti-rabbit IgG antibody (cat. no. ab150079; 1:200, Abcam) for 1 h. After staining with 4,6-diamino-2-phenyl indole (DAPI, cat. no. D9542; Sigma-Aldrich), the samples were visualized under a fluorescence microscope (cat. no. BX53; Olympus, Tokyo, Japan).

Enzyme linked immunosorbent assay (ELISA)

ELISA Kit of IL-10 was obtained from R&D Systems (cat. no. M1000B; Minneapolis, MN, USA), and an ELISA Kit of TNF-α was obtained from Abcam (cat. no. ab208348) according to the manufacturer's instructions. Briefly, the cell culture supernatant was collected, centrifuged at 1,000*g* at 4 °C for 30 min, and stored at −80 °C until use. Samples to be tested were thawed on ice. Standard wells were set up, and 50 µl of pre-replaced standards at different concentrations were added to each well. Subsequently, 50 µl of each sample was added to each well. Next, 50 µl of horseradish was added to each well and incubated for 1 h at 37 °C. The liquid was discarded, and 300 µl of the cleaning solution was added. After 2 min, the cleaning solution was discarded, and the microwell plate was patted dry on absorbent paper to remove any residual liquid. The microwell plate was rinsed 5 times, and 50 µl of the substrate was added to each well and incubated for 15 min. Finally, 50 µl of stop solution was added to terminate the reaction, and the absorbance of each well was measured at 450 nm using a spectrophotometer (cat. no. Multiscan MK3 (Thermo Fisher Scientific).

Statistical analysis

The experimental data are presented as mean ± standard deviation (SD). Data obtained from these experiments were analyzed using GraphPad Prism software (version 8.0). The unpaired Student's *t*-test was used for two-group comparisons. Statistical analyses involving multiple group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$.

RESULTS

Downregulation of FOXP3 inhibited the recruitment of macrophages by NSCLC cells

We first observed the expression of FOXP3 in 3 NSCLC cell lines (A549, H1975, and PC-9) and 16HBE cells. FOXP3 expression in NSCLC cell lines (A549, H1975, and PC-9) was significantly higher than that in 16HBE cells with higher expression observed in the A549 and H1975 cell lines (Fig. 1A). Hence, we constructed A549 and H1975 cell lines with low FOXP3 expression levels. The results of RT-qPCR showed that the expression of FOXP3 was inhibited in the sh-FOXP3 group (Fig. 1B). Next, we placed each tumor-conditioned medium in the lower chamber of the Transwell chamber and performed chemotactic experiments. Compared with the tumor-conditioned medium of the control group, macrophages did not produce better chemotactic ability than the tumor-conditioned medium of the sh-NC-CM group (Fig. 1C). Concurrently, the conditioned medium of the sh-FOXP3-CM group showed the lowest recruitment capacity for macrophages (Fig. 1C). We showed that reducing FOXP3 expression in lung cancer cells may inhibit the chemotaxis of macrophages to cancer cells.

Downregulation of FOXP3 inhibited M2 macrophage polarization by NSCLC cells

We first treated macrophages with different groups of A549 and H1975 conditioned media. Furthermore, we determined the changes in macrophage polarization by detecting differences in the expression of M1 and M2 macrophage markers in different groups of macrophages. The results of RT-qPCR showed that only CD80 expression of M1 macrophage

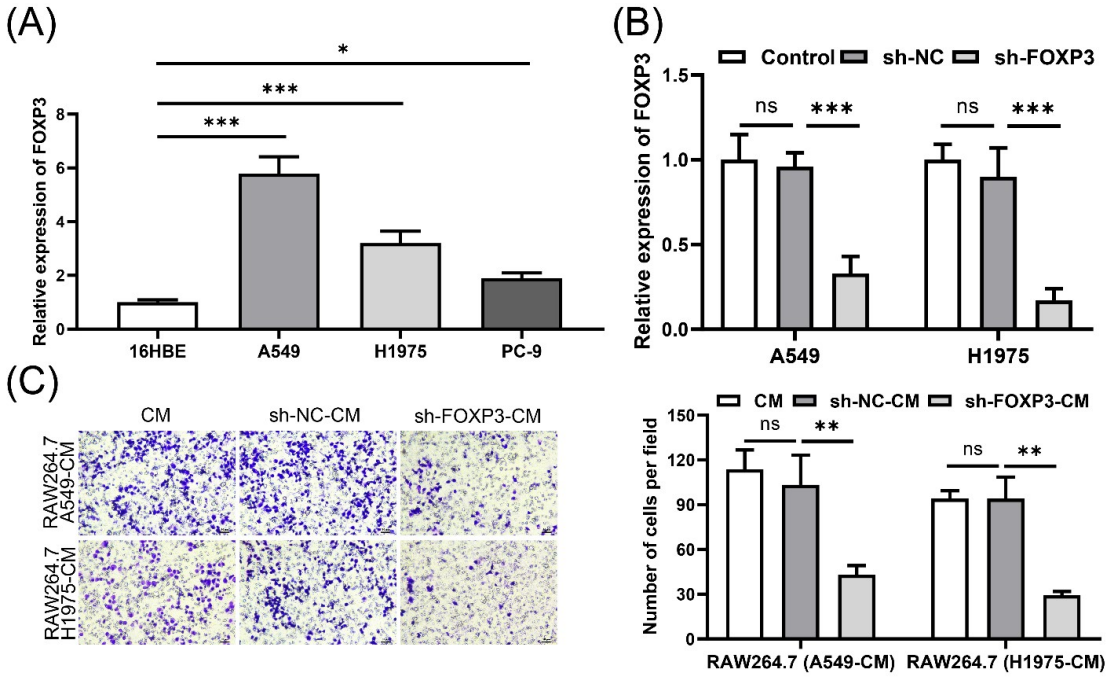

Fig. 1 Downregulation of FOXP3 inhibiting the recruitment of macrophages by NSCLC cells. (A) Expression of FOXP3 in human bronchial epithelial cells (16HBE) and NSCLC cell lines determined by RT-qPCR. (B) FOXP3 transcription levels in A549 or H1975 cells of different treatment groups estimated via RT-qPCR. (C) The effect of FOXP3 on the recruitment of macrophages by A549 and H1975 cancer cells of different treatment groups measured by chemotaxis assay of macrophages. ns, no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

markers (IL-6, TNF-α, and CD80) was changed in macrophages treated with A549 cell-conditioned medium (Fig. 2A). For macrophage M2 polarization in A549 cell-conditioned medium, the expression of M2 macrophage markers (CD206, CD163, and IL-10) decreased significantly after FOXP3 expression was inhibited (Fig. 2B). Similarly, the inhibition of FOXP3 expression in H1975 conditioned medium had no effect on the transcription level of M1 macrophage markers but significantly reduced the expression of M2 macrophage markers (Fig. 2B). The results of the ELISA also showed that downregulating the expression of FOXP3 in A549 and H1975 cells did not affect the level of TNF-α in macrophages but significantly reduced the expression level of IL-10 (Fig. 2C,D). All the above results showed that the inhibition of FOXP3 expression only downregulated the expression of M2 macrophage markers. Therefore, we further detected the expression of the M2 macrophage marker protein CD206 in the macrophages of each group by immunofluorescence staining. The results also showed that the expression of CD206 in the macrophages of the A549/H1975-sh-FOXP3-CM group was significantly downregulated (Fig. 2E,F). Hence, the regulatory effect of FOXP3 on TAMs is mainly mediated by M2 macrophages.

CCL5 is one of the factors indicating that FOXP3 regulates macrophage infiltration

We further explored the effects of FOXP3 inhibition on chemokine expression in A549 and H1975 cells. We examined differences in the expression of 3 chemokines, CCL2, CCL5, and CXCL2, in different groups of cancer cells. At the transcriptional level, the inhibition of FOXP3 expression in A549 and H1975 cells only downregulated CCL5 expression (Fig. 3A,B). ELISA results also showed that inhibiting FOXP3 expression in cancer cell lines reduced the level of CCL5 protein secreted by cancer cells (Fig. 3C,D). We investigated whether FOXP3 directly regulated CCL5 transcription and predicted FOXP3 binding sites in the CCL5 promoter region using JASPAR website. Furthermore, combined with previous reports, we regarded the −483 to −432 bp (ATAAATA) site as the binding site of FOXP3 and CCL5 promoter region for follow-up studies [23]. ChIP-qPCR confirmed that FOXP3 was recruited to the promoter region of CCL5 (Fig. 3E,F). The results of the dual-luciferase reporter assay showed that downregulation of FOXP3 decreased the wild-type (WT) CCL5 reporter activity. However, the luciferase activity of mutant (MUT) CCL5 was not affected by FOXP3 knockdown (Fig. 3G). Taken together, these data sug-

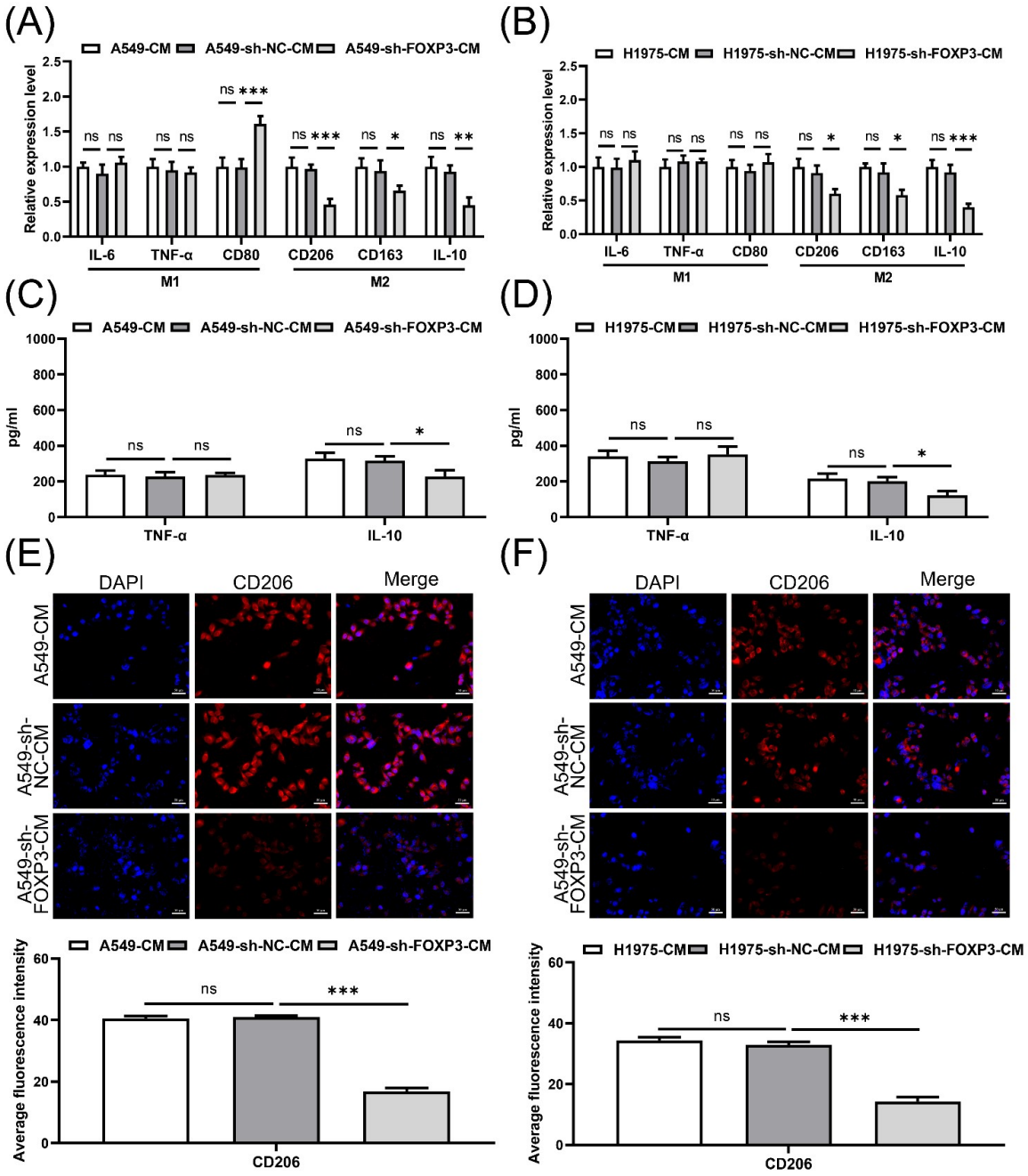

Fig. 2 Downregulation of FOXP3 inhibiting M2 macrophage polarization by NSCLC cells. (A–B) The transcription levels of M1 macrophage markers (IL-6, TNF-α, and CD80) and M2 macrophage markers (CD206, CD163, and IL-10) in macrophages treated with A549 (A) or H1975 (B) medium of different treatment groups detected by RT-qPCR. (C–D) The protein levels of TNF-α and IL-10 in macrophages treated with A549 (C) or H1975 (D) medium of different treatment groups detected by ELISA. (E–F) The expression levels of CD206 in macrophages treated with A549 (E) or H1975 (F) medium of different treatment groups detected by immunofluorescence staining. ns, no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

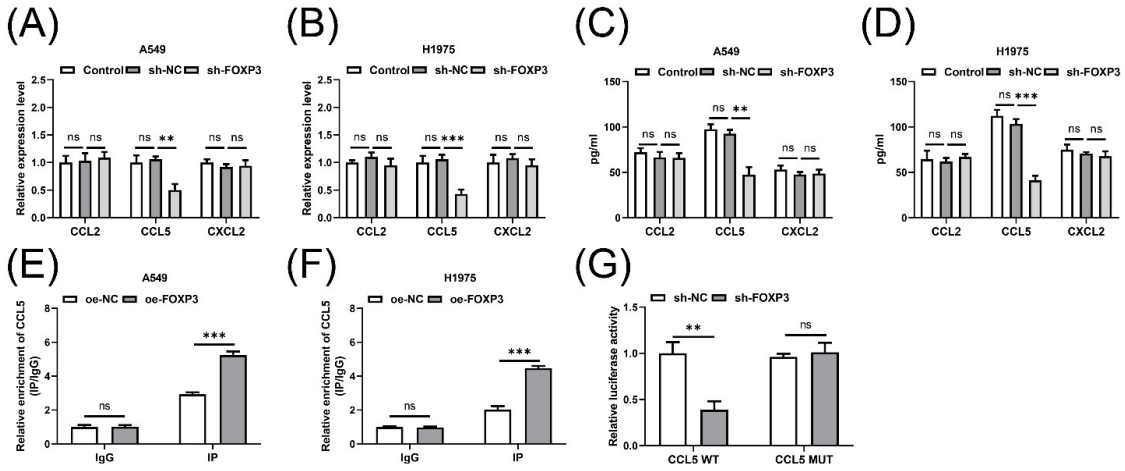

Fig. 3 Downregulation of FOXP3 decreasing CCL5 expression in NSCLC cells. (A–B) The transcription levels of CCL2, CCL5, and CXCL2 in A549 cells (A) or H1975 cells (B) of different treatment groups detected by RT-qPCR. (C–D) The protein levels of CCL2, CCL5, and CXCL2 in A549 cells (C) or H1975 cells (D) of different treatment groups detected by ELISA. (E–F) ChIP-qPCR analysis of FOXP3 binding on CCL5 promoter in A549 cells (E) or H1975 cells (F). (G) Dual-luciferase reporter assay for detecting the activity of wild-type (WT) or mutant (MUT) CCL5 promoters in 293T cells which were transfected with sh-FOXP3 or sh-NC. ns, no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

gest that FOXP3 can promote the secretion of CCL5 by activating CCL5 transcription in NSCLC cells.

FOXP3/CCL5 axis promoted the recruitment of macrophages by NSCLC cells

We further investigated whether the chemotactic effect of FOXP3 on macrophages is mediated by CCL5. We first constructed FOXP3 overexpression and CCL5 stable underexpression cell lines for the A549 and H1975 cells. RT-qPCR results showed that the transcription level of FOXP3 in the oe-FOXP3 group was significantly higher (Fig. 4A). The expression of CCL5 was also significantly inhibited in cell lines with CCL5 knocked out (Fig. 4B). We further inhibited CCL5 expression by overexpressing FOXP3 in the cancer cell lines. Both transcriptional and protein levels showed that FOXP3 overexpression promoted CCL5 expression in A549 and H1975 cells (Fig. 4C,D). However, silencing of CCL5 in cancer cells reversed the upregulation of CCL5 expression by FOXP3 (Fig. 4C,D). After successfully constructing cancer cell lines with abnormal FOXP3 and CCL5 expressions, we performed macrophage chemotaxis experiments using the supernatants of the cancer cells from each group. The results showed that the supernatant of cancer cells overexpressing FOXP3 recruited more macrophages (Fig. 4E). However, the enhancement of macrophage chemotactic levels by FOXP3 was significantly downregulated after the inhibition of CCL5 (Fig. 4E). These results confirmed that the expression of CCL5 is regulated by FOXP3 and thus affects the recruitment of macrophages by lung cancer cells.

FOXP3/CCL5 axis promoted M2 macrophage polarization by NSCLC cells

We further treated macrophages with the supernatant of cancer cells in each group. The transcription levels of 3 markers of M2 macrophages (CD206, CD163, and IL-10) were significantly increased after treatment of macrophages with tumor-conditioned medium overexpressing FOXP3 (Fig. 5A,B). Meanwhile, macrophages treated with conditioned media that inhibited CCL5 expression downregulated the expression of these 3 marker genes (Fig. 5A,B). At the protein level, overexpression of FOXP3 also promoted the expression of IL-10, while silencing of CCL5 reversed this trend (Fig. 5C,D). Finally, we used an immunofluorescence assay to detect the expression of CD206 in the macrophages of each group. The results also proved that overexpression of FOXP3 promoted the influence of cancer cells on macrophage M2 protein expression, while CCL5 showed the opposite effect (Fig. 5E,F). Experimental results show that CCL5 can inhibit the promotion of FOXP3 to M2 macrophage polarization.

DISCUSSION

In previous studies, it was believed that FOXP3 mainly acts on T cells and their immune escape [24]. Recent studies have shown that FOXP3 plays a key role in regulating immune cell infiltration in the tumor microenvironment. In breast cancer, FOXP3 regulates the expression of multiple chemokines, thus affecting the chemotactic level of T cells to cancer cells [25]. In lung cancer, researchers have found that several immune-related pathways are closely related to abnor-

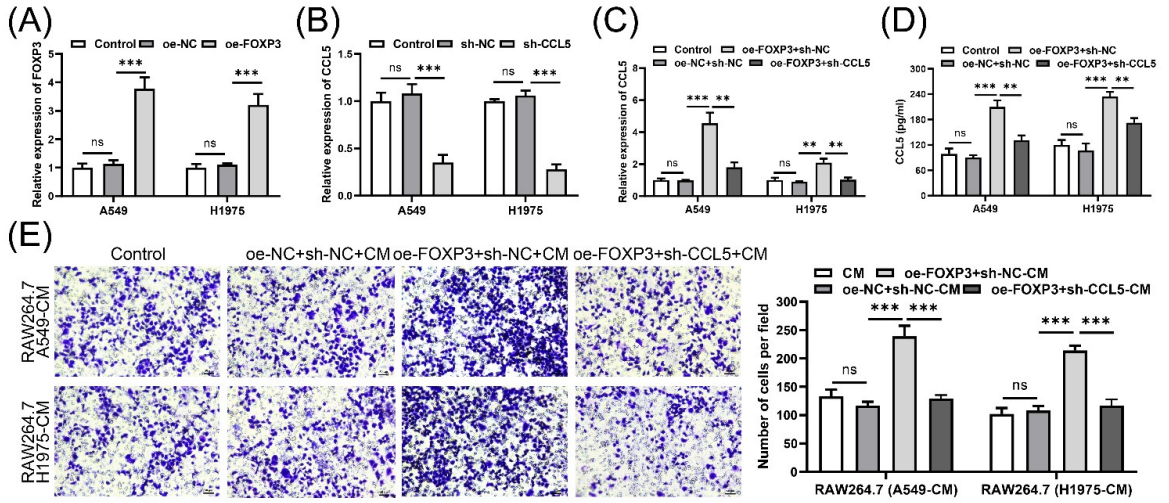

Fig. 4 FOXP3/CCL5 axis promoting the recruitment of macrophages by NSCLC cells. (A) The transcription levels of FOXP3 in A549 cells and H1975 cells of different treatment groups detected by RT-qPCR. (B) The transcription levels of CCL5 in A549 cells and H1975 cells of different treatment groups detected by RT-qPCR. (C) The transcription levels of CCL5 in A549 cells and H1975 cells of different treatment groups detected by RT-qPCR. (D) The protein levels of CCL5 in A549 cells and H1975 cells of different treatment groups detected by ELISA. (E) The effect of FOXP3/CCL5 axis on the recruitment of macrophages by A549 and H1975 cancer cells of different treatment groups measured by chemotaxis assay of macrophages. ns, no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

mal FOXP3 expression [26]. In this study, we explored the role of FOXP3 in mediating the recruitment and polarization of macrophages by cancer cells. Our results suggest that FOXP3 promotes the chemotactic level of TAMs and macrophage M2 polarization.

As an important factor in the interaction between cancer cells and TAMs, changes in the recruitment of macrophages by cancer cells can profoundly affect the malignant progression of tumors. In NSCLC, the recruitment of macrophages by tumor cells is also regulated by multiple factors. High NOX4 expression in NSCLC cells promotes recruitment by activating ROS/PI3K signaling [27]. Lung cancer cells have also been found to regulate glycolysis and infiltration of macrophages by releasing exosomes [28]. Promoting VEGF-C expression in A549 and H441 cells was also found to promote migration of RAW264.7 cells [29]. Although FOXP3 is expressed in both cancer cells and TAMs, studies on the regulation of macrophage chemotaxis by FOXP3 have not been reported [30]. In this study, we revealed that the recruitment of macrophages using tumor-conditioned media that inhibited FOXP3 expression significantly inhibited macrophage chemotactic ability.

In the TME, M1 macrophages usually inhibit the activity of cancer cells, whereas M2 macrophages play a role in promoting tumor progression [31]. Therefore, it is important to explore targets that regulate M2 macrophage polarization in NSCLC for the development of novel immunotherapies. We proved that treatment of macrophages with tumor-conditioned media that inhibited FOXP3 expression did not change the expression of the M1 macrophage polarization factor but significantly inhibited the M2 polarization of macrophages. These results suggest that the polarization of FOXP3 to macrophages is targeted at the M2 type rather than the M1 type. The regulation of M2 macrophage polarization by lung cancer cells has been confirmed in several studies. Some researchers found that A549 cells with OCT4 overexpression were closely related to M2 TAM polarization [32]. Lung cancer cells were also found to inhibit M2 macrophage polarization by releasing exosomal microRNA (miR)-770 [33]. For FOXP3, its high expression in Hodgkin lymphoma in Argentina is thought to promote M2-type polarization of macrophages [34]. In nasopharyngeal carcinoma tissues, FOXP3 expression has also been shown to be positively correlated with M2-type macrophage infiltration [35]. This study further demonstrated that FOXP3 significantly promotes the polarization of M2 macrophages during the malignant progression of NSCLC.

The secretion of chemokines has been considered one of the important ways in which cancer cells regulate the chemotaxis and polarization of TAMs [36]. Among them, CCL2, CCL5, and CXCL2 are important chemokines in the regulation of immune cell infiltration in the TME. In mouse breast tumor models, CCL2, CCL5, and CXCL2 were found to be highly expressed and were associated with T lymphocyte infiltration [37]. In studies of the immunotherapy response of melanoma cells, abnormal levels of CCL2,CCL5, and

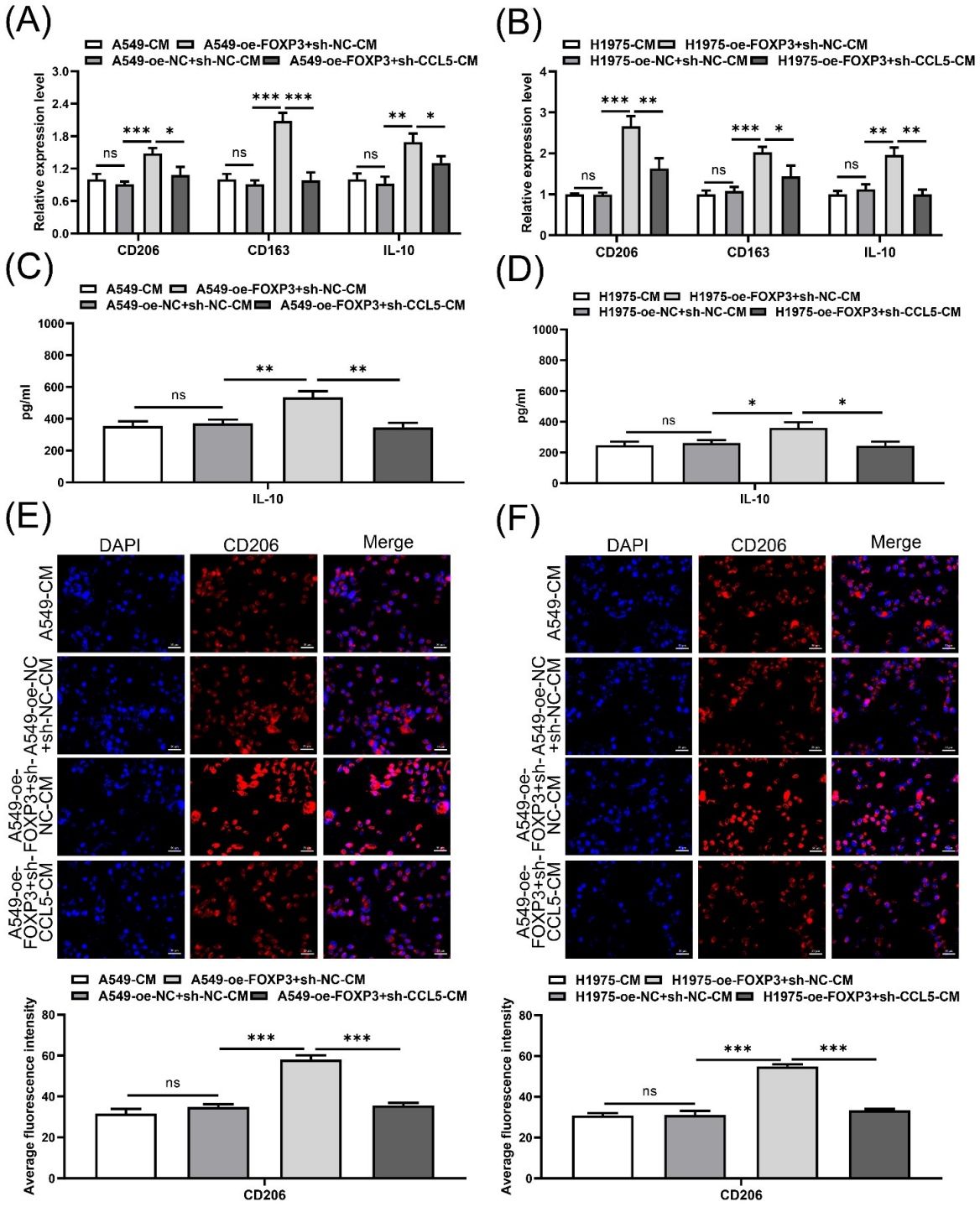

Fig. 5 FOXP3/CCL5 axis promoting M2 macrophage polarization by NSCLC cells. (A–B) The transcription levels of M2 macrophage markers (CD206, CD163, and IL-10) in macrophages treated with A549 (A) or H1975 (B) medium of different treatment groups detected by RT-qPCR. (C–D) The protein levels of IL-10 in macrophages treated with A549 (C) or H1975 (D) medium of different treatment groups detected by ELISA. (E–F) The expression levels of CD206 in macrophages treated with A549 (E) or H1975 (F) medium of different treatment groups detected by immunofluorescence staining. ns, no significance, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

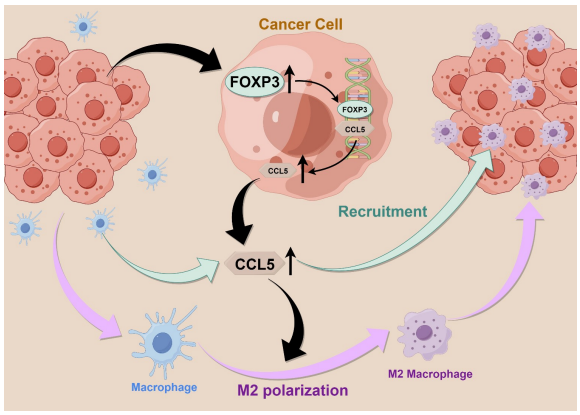

Fig. 6 Schematic illustrating the proposed molecular mechanism by which FXOP3 promotes recruitment of macrophages and macrophage M2 polarization via promoting the expression of CCL5 in NSCLC cells.

CXCL2 have also been shown to be correlated with T cells and M1 macrophages [38]. Xia et al [22] evaluated the role of EZH2 in the regulation of CCL2, CCL5, and CXCL2 chemokines in lung cancer cells. It was found that EZH2 could increase the secretion of CCL5 to promote macrophage infiltration. Considering the role of these 3 chemokines in the regulation of TEM and TAM activities, this study also explored the regulatory role of FOXP3 in these 3 chemokines. We demonstrated that the upregulation of FOXP3 expression induced lung cancer cells to secrete more CCL5. FOXP3 also mediates the regulation of macrophage chemotaxis and M2 polarization through the upregulation of CCL5. Some researchers have found that upregulation of CCL5 in NSCLC tissues is consistent with the high infiltration levels of macrophages [39]. IL-6 has also been shown to mediate macrophage recruitment by upregulating CCL5 [9]. The results of this study further demonstrate that CCL5 plays an important role in the regulation of TAM activity in cancer cells.

The present study had certain limitations that should be acknowledged. First, the regulation of FOXP3 on macrophage activity may be produced through various pathways and chemokines. This study focused on only one chemokine, CCL5, and more studies are needed to determine other regulatory pathways of FOXP3. What's more, in this study, only some M1 or M2 macrophage markers were selected to verify the effect of FOXP3/CCL5 axis on macrophage polarization. More markers and experiments need to be performed in the future to verify the changes in macrophage polarization. Finally, although we demonstrated that FOXP3 regulation of macrophages is mediated by CCL5, the specific molecular mechanism of CCL5 regulation of macrophage activity was not demonstrated in this study. Therefore, further research is needed to address this issue.

Collectively, FOXP3 has been shown to promote the recruitment of macrophages by lung cancer cells and macrophage M2 polarization. FOXP3 regulates the activity by promoting the secretion of CCL5 by cancer cells (Fig. 6). These results provide a new perspective for exploring the molecular mechanisms of lung cancer cell interactions with TAMs. Our study also suggested that FOXP3 is a potential target for inhibiting M2 macrophage infiltration in NSCLC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.021.

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Appendix A. Supplementary data

Table S1 Primer sequence information used in the study.

Gene	Forward (5′–3′)	Reverse (5′–3′)
FOXP3	AAGAGAGAGGTCTGCGGCTT	GACTGACAGAAAAGGATCAGCC
IL-6	TTCGGTCCAGTTGCCTTCTC	GAGGTGAGTGGCTGTCTGTG
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
CD80	TCTCAGAAGTGGAGTCTTACCCT	GATTGGAGGGTGTTCCTGGG
CD206	CCAAACGCCTTCATTTGCCA	ACCTTCCTTGCACCCTGATG
CD163	CCGGGAGATGAATTCTTGCCT	GGTATCTTAAAGGCTCACTGGGT
IL-10	AGGGCACCCAGTCTGAGAAC	TCTTCACTCTGCTGAAGGCAT
CCL2	AGCAGCAAGTGTCCCAAAGA	TTGGGTTTGCTTGTCCAGGT
CCL5	TACACCAGTGGCAAGTGCTC	CTTGTTCAGCCGGGAGTCAT
CXCL2	ATCCCTTGGACATTTTATGTCTTTC	TCTCTGCTCTAACACAGAGGGA
GAPDH	AAAGCCTGCCGGTGACTAAC	AGGAAAAGCATCACCCGGAG