Tobacco exposure primes the secretion of CCL21 positively associated with tertiary lymphoid structure and response to immunotherapy

Xuwen Yin,1,2,3 Hui Wang,1,2,4 Rutao Li,1,2,4 Xuming Song,1,2,4 Te Zhang,1,2,4,5 Yingkuan Liang,1,2 Yu-Zhong Chen,1,2,4 Xinnian Yu,1,2,4 Qixing Mao,1,2 Wenjie Xia,1,2 Bing Chen,1,2 Lin Xu,1,2,4,6 Gaochao Dong,1,2 Feng Jiang1,2

ABSTRACT

Background It has been reported that smoking history as a predictor of immunotherapy efficacy in patients with advanced lung cancer, however, the underlying mechanisms of this phenomenon remain largely unknown.

Methods The patients with lung adenocarcinoma’s (LUAD) cohort and the orthotopically transplanted mouse model were used to explore the correlation between smoking status and tertiary lymphoid structure (TLS) and chemokine CCL21, respectively. Cell adhesion and co-immunoprecipitation assays were performed to explore the interaction between CD4+ T cells and CD20+B cells under tobacco exposure. Chromatin immunoprecipitation-PCR was used to dissect the mechanism of upregulated CCL21 secretion in tobacco treatment. Serum CCL21 level was recorded in patients with LUAD treated with immunotherapy.

Results Here we observed that individuals with a smoking history exhibit an increased quantity and maturation level of TLS compared with non-smokers, along with higher levels of CCL21 secretion. Tobacco exposure promoted CCL21 expression in an epithelial cell-intrinsic manner, of which BaP, the main component of tobacco, facilitated the nuclear retention of the aryl hydrocarbon receptor that occupied the promoter of CCL21. Additionally, the activated CCL21/CCR7 axis increased the CD11a expression of CD4+T cells, boosting the interaction with CD20+B cells dependent on ICAM1, which potentially induced the TLS formation. Patients with elevated serum levels of CCL21 benefited more from immunotherapy.

Conclusions Patients with a smoking history exhibited higher levels of TLS via the CCL21-dependent mechanism, serum CCL21 was identified as a reliable biomarker for predicting the efficacy of immunotherapy.

INTRODUCTION

The first-line and second-line treatment options used for advanced non-small cell lung cancer (NSCLC) are both forms of immunotherapy based on immune checkpoint inhibitors (ICIs).1 Nevertheless, only a minority of people experience a durable response and long-term survival under these ICI-based therapies. To address this issue, the programmed death-ligand (PD-L1) tumor proportion score (TPS) has been used as a biomarker to predict the effectiveness of various ICIs in NSCLC.2 Despite this effort, the predictive value of PD-L1 TPS is still inadequate for patients who are PD-L1 negative or have PD-L1 TPS less than 50%.3

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Smoking history has been reported to be associated with favorable responses for patients with non-small cell lung cancer (NSCLC) treated with immunotherapy. The positive correlation between tertiary lymphoid structures (TLSs) and treatment with immune checkpoint inhibitors among patients with NSCLC has been established. Nonetheless, the association between tobacco exposure and TLSs is not fully comprehended.

WHAT THIS STUDY ADDS

⇒ We identified that tobacco exposure positively correlated with the abundance and maturation of TLSs, dependent on boosting the CCL21 secretion, through an epithelial cell-intrinsic manner. BaP, the main component of tobacco, promoted the nuclear transportation of aryl hydrocarbon receptor to increase the CCL21 transcription. In addition, we also observed that for patients with advanced NSCLC with the programmed death-ligand 1 tumor proportion score less than 50%, those with elevated serum CCL21 level had more response rate for immunotherapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our study provided a potential mechanism for patients with smoking history to benefit from immunotherapy. Furthermore, we suggest that serum CCL21 levels can serve as a valuable non-invasive biomarker to assess the presence of TLSs in patients with advanced NSCLC undergoing immunotherapy, as biopsy may not be possible.
Therefore, there is a pressing need to identify reliable predictors of treatment efficacy in the field of immunotherapy to aid medical professionals in their clinical decision-making.

Several scientific studies have demonstrated a positive correlation between tertiary lymphoid structures (TLSs) present in the immune microenvironment and the prognosis of patients suffering from tumors, as well as the effectiveness of immunotherapy interventions. $^{1, 5}$ TLSs are characterized by the inclusion of lymphoid aggregates in the contact region between the B cell zone and the adjacent T cell zone, providing a critical local microenvironment for the production of antitumor cells and humoral immune responses. $^{4}$ Recent work has also shown that memory B cells, such as germinal center B cells and plasma cells, can cooperate with TLSs and other key immune components to modify the activation state of T cells. $^{6}$ The development of TLSs has been provided to depend on an analogous chemokine and cytokine network. $^{7}$ In addition, TLS density has been correlated with the density of CD4+ T cells in tumors. $^{8}$ One study demonstrated that treatment with anti-PD-1 could induce a population of circulating T cells with an enhanced capacity to activate B cells, increase the number of TLSs and CCL21 in tumors, and promote the production of antitumor antibodies, thereby impairing tumor growth. $^{9}$ In summary, a specific set of cells and chemokine CCL21 are involved in the formation of TLSs in tumors.

Smoking has been shown to induce chronic inflammation in the lung, leading to the accumulation of oncogenic driver mutations and ultimately contributing to the development of lung cancer. $^{10}$ Recently, the clinical trials CheckMate 057 and KEYNOTE-001 showed that patients with NSCLC with a smoking history had a better outcome from ICIs therapy when compared with non-smoking patients, $^{11, 12}$ suggesting that patients with LUAD with a long history of smoking may have altered molecular pathways that affect the efficacy of ICIs. The possible impact of smoking-induced inflammation on the effectiveness of ICIs by stimulating the generation and development of TLSs remains uncertain.

Here, our findings highlight that the abundance and maturation of TLSs are higher in patients with a smoking history compared with non-smokers, which is dependent on the CCL21 secretion. We observed that BaP, the main component in tobacco, promoted the transcription of CCL21 in an aryl hydrocarbon receptor (AhR)-dependent manner. In addition, the active CCL21/CCR7 axis increased the expression of CD11a in CD4+ T cells, priming the interaction with CD20+B cells dependent on ICAM1, which potentially induced the TLS formation. In particular, serum CCL21 levels can be used as a positive independent predictor for patients with advanced NSCLC treated with tislelizumab combined with chemotherapy.

### METHODS AND MATERIALS

#### Patient samples

Tissues were selected randomly from patients undergoing thoracoscopic radical resection of lung cancer and who were diagnosed with LUAD in Jiangsu Cancer Hospital from August 2020 to August 2021. Among them, only lifelong never smokers or current smokers who did not smoke more than 50 packages per year were enrolled. Summarily, 80 life-long non-smoking patients and 56 currently smoking patients were enrolled. Their clinical information is shown in online supplemental table 2. Peripheral blood samples were randomly selected retrospectively from 67 patients who were treatment-naive for either histologically confirmed locally advanced (stage IIIB) or metastatic (stage IV) LUAD, as classified by the seventh edition of the American Joint Committee on Cancer Staging Manual, in Jiangsu Cancer Hospital from August 2021 to December 2021. These peripheral blood samples were extracted at initial diagnosis and stored in the biobank of Jiangsu Cancer Hospital. Among the patients, 23 were withdrawn for carrying sensitizing EGFR mutations or ALK rearrangements, as determined by tissue-based analyses. Thirteen patients were withdrawn for having a PD-L1 TPS≥50% or <1%. Thirty-one patients received 200mg tislelizumab plus platinum-based chemotherapy (carboplatin area under the curve in combination with 500mg/m² pemetrexed) once every 3 weeks intravenously. The patients were then assessed for the effectiveness of this treatment after either three or four cycles. The evaluation of the treatment effect was conducted independently by two senior oncologists via radiological or serological tests and medical records, retrospectively. Two patients had interrupted treatment and were excluded. All of the above were retrospective studies, such that the findings did not affect the treatment decisions of the specialists. The clinical information of the included patients is shown in online supplemental table 3.

#### Immunofluorescence and confocal microscopy

Cells were sequentially fixed, permeabilized, and blocked. The primary antibodies applied included anti-CCL21 (#AF366, R&D), ICAM1 (#Ab222736, Abcam), and CD11α (#26703, CST) overnight. Fluorophore-conjugated secondary antibody (#A-11008, #A-11004, #A48255, #32733, Invitrogen) was then applied for 1 hour. After washing the cells with phosphate-buffered saline (PBS), they were stained with 4,6-diamidino-2-phenylindole (DAPI) (#C1005, Beyotime). The resulting cells were finally captured under a Zeiss LSM 710 confocal microscope.

#### Luciferase assay

The wild-type positive control group (pGL3-CCL21 WT luciferase reporter gene plasmid) and the motif mutant group (pGL3-CCL21 MT# 1, pGL3-CCL21 MT#2 luciferase reporter gene plasmids) were constructed by Nanjing BioGot. All plasmids then co-transfected with
a pRL-TK plasmid into cells using Lipofectamine 3000 (#L3000075, Thermo) in triplicate. After 48 hours of co-transfection, luciferase activity was measured using the Dual-Glo Luciferase Assay System (#DD1205-01, Vazyme) according to the manufacturer’s guidelines. The obtained data are represented as the mean±SD of three experiments.

T cell static adhesion assay
Washing solution was formulated in PBS containing 2 mM MgCl₂ (#M434105, Aladdin) and 1 mM CaCl₂ (#C431203, Aladdin). Coating solution (ICAM-1 coating) containing ICAM-1 recombinant protein (#720-IC-200, R&D Systems) was diluted in PBS solution to a final concentration of 10 µg/mL. Adhesion solution was prepared in PBS adhesion solution containing 0.5% bovine serum albumin (BSA), 2 mM MgCl₂, and 1 mM CaCl₂, and then added CD4⁺ T cells stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) at a concentration of 5 µM. Finally, the fluorescence intensity of adhered CD4⁺ T cells was measured using a plate reader. The percentage of adhesion was calculated as the mean fluorescence intensity of remaining cells/starting cellsx100%.

Chromatin immunoprecipitation-quantitative real-time PCR
Chromatin immunoprecipitation (ChiP) was performed according to the manufacturer’s instructions using the Magna ChiP A kit (#17–610; MilliporeSigma, Burlington, Massachusetts, USA). Anti-AhR, (#83 200S) were purchased from CST. The quantitative PCR analysis was performed as described above. Results represent the mean±SD of three experiments.

Statistical analysis
All statistical analyses were performed using RStudio (V.4.1.2) and GraphPad Prism (V.8.4.0) software. For continuous variables following a normal distribution, the Student’s t-test was used. For non-normally distributed data, a non-parametric test was used. Differences between groups were compared using analysis of variance when non-parametric tests were used. Correlation analysis was performed using the Pearson correlation coefficient method. All results are presented as the mean±SD. All statistical tests were two-way and p<0.05 was considered statistically significant.

Other methods and abbreviations, please refer to online supplemental file 2 for further details.

RESULTS
Tobacco smoke exposure positively correlated with the formation of TLSs
To explore whether there were differences in the enrichment of TLSs between smokers and non-smokers in patients with LUAD, we analyzed the levels of 12 TLS-related chemokines between life-long non-smoking patients with LUAD (82 patients) and patients who smoked more than 50 packs per year (116 patients) derived from 506 patients with LUAD in The Cancer Genome Atlas (TCGA) database. The analysis indicated that most of the TLS-related chemokines were increased in smokers when compared with non-smokers, including CXCL9, CXCL10, CCL4, CCL5, CXCL11, CCL21, and CCL8 (figure 1A). TLSs were classified into lymphocyte aggregation (AGG), intratumoral lymphoid follicles without a germinal center (Fox-I), and intratumoral lymphoid follicles with a germinal center (Fox-II) based on the stage of maturity.13 14 The H&E sections were obtained from the TCGA database to evaluate the number and maturation of the identified TLSs (online supplemental table S1), which showed that smoking patients had more mature TLSs than non-smoking patients (figure 1B). We also retrospectively enrolled 136 patients with LUAD in Jiangsu Cancer Hospital, who were divided into two groups based on their smoking status. The abundance and maturation of the TLSs were also assessed from the H&E sections (online supplemental table S2), which showed that smoking patients had abundant TLSs both in the tumor and adjacent tissues (figure 1C). Furthermore, the number of TLSs was increased in smokers when compared with non-smokers (figure 1D). We also assessed the maturation stages of TLSs from the sections of 136 patients with LUAD of Jiangsu Cancer Hospital (hereafter referred to as JCH-LUAD cohort), which revealed that smoking patients had more mature TLSs than non-smoking patients (figure 1E). Finally, we explored the correlation between smoking status and TLS abundance in an animal model, which was established as follows. We orthotopically transplanted luciferase-tagged LLC cells into the lungs of C57BL/6J mice. These mice were then randomly assigned to the experimental group (cigarette smoke exposure) and the control group (clean air) (figure 1F). Even with no statistical difference in malignant progression of tumors, more TLSs were identified in the lungs of the smoking group, while few were identified in the control group (figure 1G,H). These results suggest that tobacco smoke exposure promotes the generation of TLSs.

Tobacco smoke exposure activated the CCL21-CCR7 axis
To investigate the mechanism by which tobacco smoke exposure promotes the generation of TLS, we analyzed the expression of 12 TLS-related chemokines in the JCH-LUAD cohort. The results indicate that among the chemokines, the expression of CCL21 was ranked first in the smoking patients (figure 2A). In addition, CXCL9 and CXCL11 also showed significant increase in current smoking patients. TLSs were identified in 232 patients and absent in 126 patients in TCGA LUAD tissues, revealing that the expression of CCL21 in TLS-present tissues (TLS⁺) was significantly higher than that in TLS-absent tissues (figure 2B). Interestingly, CXCL9 also showed significant increase in TLS⁺, but not CXCL11 (online supplemental figure S1A,B). Further, CCL21 expression was positively associated with the infiltration of B cells, which identified via MCP-counter,15 located in...
the germinal centers of TLSs in patients with a smoking history in TCGA-LUAD (figure 2C). These results suggest that tobacco smoke exposure promotes the expression of CCL21. The interaction of CCL21 with its receptor CCR7 is a major determinant of lymphoid migration and the lymph node homing of immune cells.16 To verify whether tobacco smoke exposure is regulated by the CCL21-CCR7 axis, mouse in situ injection models were subjected to exposure to either tobacco or fresh air. ELISA was conducted to reveal that CCL21 protein levels in the sera of mice in the smoking group were significantly increased compared with the non-smoking group (figure 2D). Consistently, flow cytometry showed that CCR7 positive (CCR7+) cells were significantly increased in the peripheral blood lymphocytes of the tobacco smoke-exposed mice (figure 2E, online supplemental figure S1C). Moreover, using H&E dyeing and anti-CD20 to mark TLS while marking the germinal center within mature TLS via anti-CD23, anti-AID, anti-BACH2, and anti-BCL6 immunohistochemistry (IHC) (figure 2F, online supplemental figure S2). Notably, CCR7+ TLSs showed more frequent in current smoking patients (figure 2F,G, online supplemental table S2). The percentage of CXCR3+ TLSs showed no statistical difference between the two groups, although the proportion of CXCR3+ TLS was high in both groups (online supplemental figure S3A,B). Anti-CXCR3 IHC of aforementioned mice pulmonary xenograft tumors observed consistent results (online supplemental figure S3C,D). These findings indicate that the CCL21-CCR7 axis, rather than the CXCL9-CXCR3 axis, might regulate the formation and assembly of TLSs. Furthermore, we used the previously constructed tobacco smoke-exposed LUAD model with the addition of CCL21 neutralizing antibody (figure 2H). Bioluminescence imaging revealed the malignant progression of the xenogeneic tumor impervious regardless of tobacco exposure or CCL21 neutralizing antibody (figure 2I).

Lung tissues were harvested from the euthanized mice to assess the number of TLS (identified with H&E and anti-CD20 IHC) and CCL21 expression levels. The results...
Figure 2  Tobacco smoke exposure promoted CCL21 secretion by regulating the CCL21-CCR7 axis. (A) Analysis of 12 TLS-related chemokine expression by qRT-PCR in tissues from 136 patients with LUAD at Jiangsu Cancer Hospital. (B) CCL21 expression in TLS+ and TLS− tumor tissues from TCGA-LUAD database. (C) Correlation of CCL21 expression levels with germinal center B cells in tobacco-exposed patients using TCGA-LUAD database. (D) LLC cells were injected into the lungs of C57BL/6J mice for 1 week, then exposed to tobacco smoke for 3 weeks, and finally sacrificed. The expression of CCL21 in serum was detected by ELISA, and (E) the expression of CCR7 in peripheral blood lymphocytes was detected by flow cytometry. (F) Tissues of 136 patients with LUAD from Jiangsu Cancer Hospital. H&E staining low-power field of view (scale bar=2000 µm) and high-power field of view (scale bar=100 µm) and IHC of lung tissue sections using anti-CD20, anti-CD23, and anti-CCR7 antibodies. (G) Histograms showing the percentage of CCR7+TLSs (identified by H&E stain and CCR7 IHC) in all TLSs from current smokers with LUAD compared with lifelong non-smokers with LUAD. (H) Orthotopic implantation models of LLC-Luciferase cells. One week after orthotopic implantation, intravenous tail injections with or without CCL21 neutralizing antibody were administered, and then the mice were exposed to tobacco smoke for 3 weeks before being sacrificed. (I) LLC cells were injected into the lungs of C57BL/6J mice for 1 week, then exposed to tobacco smoke for 3 weeks, and finally sacrificed, the representative images of mouse bioluminescence imaging at Week 1 and Week 4 (left panel) and the corresponding quantification analysis (right panel). (J) Representative image of IHC assay of lung tissue sections using anti-CD20 and anti-CCL21 antibodies, and (K) quantified results. Data are presented as the mean ± SD. ns, not significant. **p<0.01, ***p<0.001, two-tailed Student’s t-test. IHC, immunohistochemistry; LUAD, lung adenocarcinoma; qRT-PCR, quantitative real-time PCR; TCGA, The Cancer Genome Atlas; TLSs, tertiary lymphoid structures; TLS(+), TLS-present tissues; TLS(−), TLS-absent tissues.
revealed that the expression of CCL21 and the number of TLS increased under tobacco smoke exposure, while decreasing with the addition of CCL21 neutralizing antibody (figure 2H,1). These data suggest that TLSs are induced by tobacco smoke exposure in a CCL21-dependent manner.

CCL21 activated the CD11a-ICAM1 axis to promote the interaction between CD4+ T cells and B cells

To explore the various cell subsets that secrete CCL21 under tobacco smoke exposure, we isolated the five major subsets from fresh LUAD tissues, including CD45+ cells under tobacco smoke exposure, we isolated the five major subsets17 from three currently smoking patients, and CD11a in the TCGA-positive correlation between the expression of CCL21 and CD11a was attenuated by treatment with CCL21. We observed that the expression of CCL21 and the number of TLS increased under tobacco smoke exposure, while decreasing with the addition of CCL21 neutralizing antibody (figure 3G). Additionally, we observed that the adhesion of ICAM-1 to CD4+ T cells could be blocked by anti-CCL21 antibodies in the CSE medium (figure 3H). To demonstrate whether the activation of CCL21 promoted the direct interaction between CD11a and ICAM-1 observed in the tissues of smoking patients (figure 3K). Finally, IHC staining showed that CD20, CD4, CD11a, and ICAM-1 were significantly colocalized in the tissues of smoking patients (figure 3L). These results jointly suggest that the tobacco smoke exposure-induced activation of the CCL21-CCR7 axis in tumor cells promotes the interaction between CD4+ T cells and B cells.

BaP promoted CCL21 expression in LUAD cells both in vitro and in vivo

The expression levels of CCL21 in the normal lung epithelial cell line HBE and several LUAD cell lines were analyzed in CSE medium culture. The results showed that the expression of CCL21 was significantly upregulated in both A549 and H1975 cells (figure 4A,B) and was highest at the tobacco medium concentration of 50% (figure 4C,D, online supplemental figure S5A). To investigate which precise component of tobacco increased the secretion of CCL21, we focused on four of its main components, including BaP, NNK, DbA, and BzP. The results indicate that BaP alone was responsible for significantly increasing the expression of CCL21 (figure 4E,F, online supplemental figure S5B–F), which was further confirmed by the results of immunofluorescence staining (online supplemental figure S5G). We then further validated that BaP increased the expression of CCL21 in vivo. LLC cells were injected into the lungs of C57BL/6j mice, who were then treated with BaP for 4 weeks (figure 4G). As expected, bioluminescence imaging revealed that the malignant progression of the xenogeneic tumor was not disturbed by BaP (figure 4H). This orthotopic lung mouse model revealed that the expression of serum CCL21 increased under BaP treatment, as did the number of TLSs and the proportion of peripheral blood CCR7 positive cells (figure 4I–K). Finally, A549 cells were treated with BaP and anti-CCL21 antibodies, which was then followed by co-culturing with CD4+ T cells. The results revealed that the expression of CD11a and the adhesion of CD4+ T cells to ICAM-1 was increased under BaP treatment, and conversely, were dampened when simultaneously treated with anti-CCL21 antibodies (figure 4L,M). These results indicate that BaP, the main component of
Figure 3  CCL21 activated the CD11a-ICAM1 axis. (A) Five major cell subsets (CD45+ cells, CD31+ cells, EpCAM+ cells, NG2+ cells, and SPARC+ cells) were sorted and cultured from fresh LUAD tissues using flow cytometry. (B) Determination of CCL21 secretion by CD45+, CD31+, EpCAM+, NG2+, and SPARC+ cells cultured in CSE medium. The supernatant from cultured cells was collected and assayed using ELISA. (C) The t-SNE plots showed epithelial cell subpopulations in subcluster analysis, according to current smoker (above) and non-smoker (below) single-cell RNA sequencing. (D) Correlation between CCL21 and CD11a in the TCGA-lung database. (E) CD4+ T cells were extracted by magnetic beads and co-cultured with A549 cells treated either with or without CCL21 for 48 hours. The expression of CD11a in peripheral blood lymphocytes was assayed by flow cytometry. (F) CD4+ T cells were extracted by magnetic beads and co-cultured with A549 cells treated with or without CSE or CSE+αCCL21 for 48 hours. The expression of CD11a in peripheral blood lymphocytes was assayed by flow cytometry. (G) CD4+ T cells were extracted by magnetic beads and co-cultured with A549 cells treated either with or without CCL21 for 48 hours. B cell and T cell interactions were detected by the T cells static adhesion assay (scale bar=100μm). (H) CD4+ T cells were extracted by magnetic beads and co-cultured with A549 cells treated with or without CSE or CSE+αCCL21 for 48 hours. B cell and T cell interactions were detected by the T cells static adhesion assay (scale bar=100μm). (I) B cells co-cultured with CD4+ T cells treated with CCL21 recombinant protein schematic. (J) Immunofluorescence staining of B cells co-cultured with CD4+ T cells using anti-CD20, anti-CD4, anti-CD11a, and anti-ICAM-1 antibodies (scale bar=100μm). (K) The co-immunoprecipitation assay demonstrated the interaction between CD11a and ICAM1. (L) Representative immunofluorescence images of stained lung tissue derived from smoking and non-smoking patients using an anti-CD20, anti-CD4, anti-CD11a, and anti-ICAM-1 antibodies (scale bar=50μm). Data are presented as the mean±SD. ns, not significant. *p<0.05, **p<0.001, two-tailed Student’s t-test.

CSE, cigarette smoke extract; LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas; t-SNE, t-distributed stochastic neighbor embedding.
tobacco, promotes the expression of CCL21 in tumor cells.

BaP-mediated CCL21 secretion was dependent on AhR

BaP exposure has been reported to activate the AhR signaling pathway and its associated downstream factors. In this study, the knockdown of AhR significantly reduced both the messenger RNA and protein expression levels of CCL21, and this effect was not rescued by BaP addition (figure 5A–C), suggesting that BaP increases CCL21 expression in an AhR-dependent manner. Next, we used CH23191, an AhR antagonist, to further verify the AhR-dependent role of BaP-mediated CCL21 secretion (online supplemental figure S5H). We observed a positive correlation between the expression of AhR and CCL21 in the GTEx database (figure 5D). The expression of AhR was also significantly higher in the presence
of TLSs from the TCGA-LUAD database (figure 5E).

As expected, bioluminescence imaging revealed the malignant progression of the xenogeneic tumor was not disturbed by AhR. The knockdown of AhR also decreased the number of BaP-induced TLSs as well as the proportion of CCR7+ and CCL21+ cells in peripheral lymphocytes in vivo (figure 5F–I, online supplemental figure S6A–C). These data indicate that the BaP-mediated expression of CCL21 and number of TLS is dependent on AhR.

Previous studies have shown that activated AhR can be transported to the nucleus as a transcription factor. Interestingly, in the current study, BaP significantly increased the localization of AhR from the cytoplasm to the nucleus (figure 5J–K). To investigate whether the expression of CCL21 is regulated by AhR, we analyzed the CCL21 gene sequence using the ENCODE database and found two sequences located in the promoter region with strong chromatin opening potential:
5'-GCGCG-3' (region #1) and 5'-GCGCG-3' (region #2) (online supplemental figure S6D). ChIP-PCR assays were performed to further verify the binding ability of AhR to these two predicted conserved binding sequences. The results indicate that AhR can bind to region #2 in the CCL21 promoter (figure 5L). We also designed a CCL21 wild-type luciferase plasmid (WT) and two mutant luciferase plasmids (MT#1 and MT#2) to confirm the binding of AhR to the CCL21 promoter (figure 5M,N). From this, we observed significant upregulation of luciferase activities in both the WT and MT#1 plasmids with the overexpression of AhR or the treatment of BaP, but not in MT#2, indicating that AhR binds to the ‘CGCAC’ sequence (figure 5O). This demonstrates that BaP treatment promoted the transcription of CCL21 by activating the binding of AhR to chromatin region #2 in the promoter of CCL21 (figure 5P).

The serum CCL21 level was associated with the clinical benefit of tislelizumab combined with chemotherapy

We demonstrated that CCL21 promotes the recruitment of CD4+ T cells to B cells to facilitate the formation of TLSs. We then conducted a retrospective study of 31 patients with LUAD (without druggable mutation and PD-L1 TPS>1% while<50%) treated with immunotherapy combined with chemotherapy (tislelizumab+pemetrexed+carboplatin, TPC) to verify the correlation between peripheral blood serum CCL21 (bCCL21) levels and the clinical benefit of TPC therapy. Twenty-nine of these patients received four courses of treatment, with complete follow-up, and had their levels of bCCL21 detected before starting treatment (figure 6A). After treatment, the median and mean expression values of bCCL21 were significantly higher in patients with partial response than those in patients with stable disease or progression.
progressive disease (figure 6B, online supplemental table S3). The OR value calculation also showed that the bCCL21 level was most significantly associated with clinical benefit (figure 6C). Moreover, random forest results suggested that the CCL21 level had the highest predictive weight for the immunotherapy response among all variables, including serum CCL21, PD-L1 TPS, age, gender, and metastasis/hydrothorax (figure 6D). Receiver operating characteristic (ROC) analysis also showed that bCCL21 was more sensitive to TPC treatment, indicating that bCCL21 has a more significant predictive ability than PD-L1 TPS (figure 6E,F). In conclusion, serum CCL21 could effectively predict the efficacy of the PD-1 inhibitor combined with chemotheraphy in patients with a TPS between 1% and 50%.

DISCUSSION

It should be noted that the smoking history of patients with lung cancer is significantly correlated with the immunotherapy response.21,22 In addition, cigarette smoke may trigger extensive innate and adaptive immunity in the lungs, thereby shaping a local chronic inflammatory environment to provide conditions conducive to the generation of TLSs.23-25 TLSs have been recognized as key components of antitumor immunity and their formation improves the immunotherapeutic effect of ICI.36 Therefore, in this study, we explored the association between smoking and TLSs. We found that the abundance and maturity of TLSs in lung cancer tissues of smokers were higher than that in non-smokers (figure 1). Interestingly, most of the 12 chemokines associated with TLSs were found to be upregulated in lung tissues of smoking patients, among which, CCL21 mediates the formation of TLSs (figure 2). In general, CCL21 is considered crucial for the recruitment of lymphocytes and the formation of TLSs.

Here, we found a novel mechanism by which CCL21 induces TLS formation. CCL21 is already known to be expressed by the high endothelial venules of secondary lymphoid organs, while its signaling through CCR7 is also known to recruit and activate T cells during an inflammatory response.27,28 In this study, we found that tobacco-induced CCL21 can activate integrin CD11a on the surface of CD4+ T cells and promote its interaction with ICAM1 on the surface of B cells (figure 3). The germinal center consists of a dark zone (DZ) and a light zone (LZ). In the DZ region, B cells proliferate and undergo somatic hypermutation; in the LZ zone, B cell receptors carrying relatively high-affinity receptors capture and process more antigens, and by interacting with Tfh cells, the positive selecting B cells of the LZ return to the DZ.29 In this study, naïve B cells exhibited adhesion to CD4+ T cells, thereby promoting the high-affinity selection of B cell germinal centers under the action of CD11a-ICAM1. Moreover, we identified a new upstream pathway by which tobacco activates CCL21 to promote the formation of TLSs. BaP, a member of the polycyclic aromatic hydrocarbons family, is a major carcinogen and environmental pollutant that is present in cigarette smoke.30 The activated AhR of BaP can transfer from the cytoplasm to the nucleus to play a role as a transcription factor, thereby regulating the transcription of target genes and inducing an immune response.31 As expected, we elucidated the mechanism by which BaP activates the AhR receptor to bind to the CCL21 promoter region and promote the transcription of CCL21 (figure 5). In addition, although we demonstrated that tobacco-induced CCL21 was secreted by malignant epithelial cells, it has not been ruled out that other cells may also secrete CCL21.

We showed evidence for the increased clinical benefit of immunotherapy in long-term smokers due to the formation of TLSs, though it is also worth noting that smoking remains the most important factor in the induction of lung cancer.10 Moreover, the complex spectrum of compounds in cigarettes can promote the malignant progression of tumors through multiple aspects. Correspondingly, we found that the migration and invasion abilities of tumor cells cultured in a CSE medium were increased, while pseudopodia representing tumor cell invasion and metastasis were formed (online supplemental figure S7A,B). After treatment with the CCL21 recombinant protein, the migration and invasion ability of tumor cells was increased, and the expression levels of the pseudopodia-associated proteins FASCIN, VASP, and CDC42 were also significantly increased (online supplemental figure S7C-E). This is consistent with previous reports that CCL21 can also mediate tumor cell escape from immune surveillance or avoid apoptosis by activating AKT phosphorylation to play a tumor-promoting role.32-34 In other words, CCL21 seems to play a double-edged role in the occurrence and development of tumors, so it is meaningful to correctly understand the nuance of its function in antitumor immunity. The association between chronic inflammation induced by smoking and exhausted T cells has been widely reported.35 Hu et al’s study pointed out that after tobacco exposure, T cell exhaustion may be caused by specifically activating the ADAM12+ Treg subset.36 The above single-cell transcriptome study results are consistent with the FOXP3 IHC results in our JCH-LUAD cohort, aforementioned. In summary, we speculate that smoking can promote T cell exhaustion by mediating chronic inflammation and recruiting Treg, respectively. However, the association between smoking and the benefits of tumor immunotherapy is complex. On the one hand, T cell exhaustion and negative immune microenvironment reprogramming mediated by smoking will counteract the efficacy of immunotherapy.37 On the other hand, smoking can also improve the efficacy of immunotherapy by mediating PD-L1 expression in tumor cells38 and increasing immunogenic mutations.39 Our study also found that CCL21-dependent TLS driven by tobacco exposure is associated with the benefits of immunotherapy in LUAD. Given the complexity of benefits from tumor immunotherapy, we advocate comprehensively integrating published
immunotherapy efficacy biomarkers and clinical treatments in larger immunotherapy cohorts to balance the beneficial factors and detrimental factors of ICI therapy, thereby more comprehensively and accurately predicting the efficacy of immunotherapy in patients with LUAD. In conclusion, although smoking history can serve as an independent predictor, the notion that tobacco exposure confers advantages to immunotherapy warrants cautious interpretation. The therapeutic course of lung cancer must also consider various determinants to devise a more evidence-based and advantageous management strategy for patients.

At present, several markers can be used to predict the efficacy of lung cancer immunotherapy, but they have limitations. The prediction of the efficacy of ICIs has mainly been based on the quantification of tumor PD-L1 expression. Current guidelines recommended the use of first-line monotherapy with PD-L1 inhibitors for advanced NSCLC only when the PD-L1 TPS is ≥50%. However, the necessity of biopsy, the treatment of tissues, the spatial heterogeneity of tumors, and the use of different analytical systems all limit the accuracy and consistency of this concomitant diagnostic method. Moreover, the tumor mutation burden (TMB) is an index used to evaluate the frequency of gene mutations. Although the TMB has some predictive prospects, there have been several concerns regarding its clinical use, not the least of which is the lack of standardized detection. The status of TLSs is related to the prognosis of tumor patients and the efficacy of immunotherapy, while it is independent of PD-L1 expression in evaluating immunotherapy. However, due to physical and other objective factors, not all advanced patients can undergo a biopsy, and it remains difficult to evaluate clinical markers directly in tumor tissue, such as TLSs and PD-L1. We evaluated the efficacy of immunotherapy in combination with chemotherapy (TPC) by measuring the expression level of CCL21 in peripheral blood serum to reflect the abundance of peritumor TLSs in patients. The results showed that CCL21 may serve as an independent predictor of ICI efficacy in advanced NSCLC (figure 6). For patients with advanced NSCLC and a PD-L1 TPS <50%, it is of guiding significance to find a stable predictor of immunotherapy’s clinical benefit to assist with clinical treatment selection. However, our study has the following limitations. The number of cohorts we studied was small, so our findings need to be validated in a larger prospective cohort study. In addition, for tumors with a poor immune microenvironment or in a state of exhaustion, the induction of TLSs may stimulate the infiltration of lymphocytes again, improve the recognition of tumor antigens, and thus restore the antitumor immune effect. There also lies good prospects in developing techniques or drugs to induce TLSs to improve the efficacy of immunotherapy.

In summary, we found that the abundance of TLSs and the secretion of CCL21 in smoking patients are higher than those in non-smoking patients. We observed that BaP, the main component in tobacco, promoted the transcription of CCL21 in an AhR-dependent manner. In addition, CCL21 is regulated by the CCL21/CCR7 axis, which increases the expression of CD11a in CD4+T cells and promotes the interaction with ICAM-dependent CD20+ cells, thus potentially inducing the formation of TLSs. Our study sheds light on the mechanism underlying the enhanced immunotherapy response in some patients with LUAD who have been smoking for a long time. Importantly, serum CCL21 can replace TLS as an independent predictor of the efficacy of ICIs in advanced NSCLC, which is conducive to the accurate screening of patients before immunotherapy.

Author affiliations
1Department of Thoracic Surgery, Nanjing Medical University Affiliated Cancer Hospital & Jiangsu Cancer Hospital & Jiangsu Institute of Cancer Research, Nanjing, People’s Republic of China
2Jiangsu Key Laboratory of Molecular and Translational Cancer Research, Cancer Institute of Jiangsu Province, Nanjing, People’s Republic of China
3Department of Pharmacy, Nanjing Stomatological Hospital, Affiliated Hospital of Medical School, Nanjing University, Nanjing, People’s Republic of China
4The Fourth Clinical College of Nanjing Medical University, Nanjing, People’s Republic of China
5Department of Biochemistry and Molecular Genetics, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA
6Collaborative Innovation Center for Cancer Personalized Medicine, Nanjing Medical University, Nanjing, People’s Republic of China

Contributors XY: Data curation; Investigation; Validation; Writing original draft. HW: Validation; Investigation; Visualization. RL: Investigation; Visualization; Methodology. XS: Formal analysis; Investigation; Methodology. TZ: Investigation; Methodology. YL: Investigation; Methodology; Y-2C. Resources. XHu: Resources. QM: Funding acquisition. WX: Funding acquisition. BC: Resources. LX: Resources; Writing review and editing. GD: Conceptualization; Funding acquisition; Supervision; Writing review and editing. FJ: Conceptualization; Project administration; Supervision; Writing review and editing. FJ accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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Patient consent for publication Not applicable.

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Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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ORCID iD
Feng Jiang http://orcid.org/0000-0001-6569-5956
REFERENCES


Title page

Tobacco exposure primes the secretion of CCL21 positively associated with tertiary lymphatic structure and response to immunotherapy

Additional file Figure S1-S7
Figure S1. (A) Expression levels of CXCL9 and (B) CXCL11 in TLS- and TLS+tissues were analyzed using the TCGA-LUAD database. (C) Gating strategy. Cell debris and doublets were excluded first. Subsequently, CD3 positive protocells were gated, then CD4 positive protocells were gated. Finally, CCR7 or CD11a positive cells are selected.
Figure S2. Representative images of lung tissue sections from LUAD patients were immunohistochemically stained using anti-BACH2, anti-BCL6, and anti-AID antibodies.
Figure S3. (A) Representative images of lung tissue sections from LUAD patients were immunohistochemically stained using anti-CXCR3 antibodies. (B) The graph shows the percentage of CCR7+TLSs in all TLSs of LUAD patients who were classified as current smokers or non-smokers. LLC Cells were injected into the lungs of C57BL/6J mice for 1 week, then exposed to tobacco smoke for 3 weeks, and finally sacrificed. (C) The representative immunohistochemical and (D) quantitative images of lung tissue sections stained with anti-CXCr3 antibodies.
**Figure S4.** (A) scRNA-seq data of lung adenocarcinoma primary lesion from 6 patients, including 3 current smokers and 3 life-long never smoking patients were clustered via UMAP method. (B) The major cytological subclasses were annotated according to Williams et al. previous study, which annotated all high-throughput sequenced cells into five main classes. The plot revealed the mean expression level of markers for each main class in each cluster. (C) UMAP plot revealed the classes partition for all clusters. (D) PCA plot revealed the effective discrimination for dividing T cell and B cell, using CD3D, CD3E, CD2, CD4, CD8A as T cell marker and CD79A, MZB1, MS4A1, CD79B as B cell marker according to Williams et al. previous study. (E) The plot showed the inter-cell type crosstalk in current smokers and life-long never smoking patients, respectively. (F) CD4+ T cells were extracted by magnetic beads and co-cultured with A549 cells treated with different ways for 48 h. (G) ICAM-1-coated polystyrene plates were used to adhere CD4+ T cells (CFSE staining).
Figure S5. (A) The A549 cells were treated with NNK, Dba and BzP at 0, 2.5 μM, 5 μM, 10 μM concentrations for 48 h, and the expression of CCL21 was assessed by qRT-PCR. (B) The A549 cells were treated with BaP at 0, 10%, 25%, 50% CSE medium for 48 h, and the expression of CCL21 was assessed by Western blot. (C-F) The A549 and H1975 cells were treated with BaP (C), NNK (D), Dba (E) and BzP (F) at 0, 2.5 μM, 5 μM, 10 μM concentrations for 48 h, and the expression of CCL21 was assessed by Western blot. (G) The A549 cells were treated with BaP at 5μM concentration for 48 h, and the expression of CCL21 was assessed by immunofluorescence assays (scale bar = 50 μm). (H) A549 cells were treated with CH23191 or BaP for 48h, detected by immunofluorescence assays.
Figure S6. (A) LLC Cells transferred with sh-AhR or sh-Ctrl for 48h, AhR knock-out efficiency was assessed by Western blot. (B) LLC Cells transferred with sh-AhR or sh-Ctrl, then injected into lung of randomized 57BL/6J mice for 1 week, then treated with BaP for 3 weeks and sacrificed. (C) the protein was extracted from lung tissue, and AhR was detected by Western blot. (D) The sequence of CCL21 gene was analyzed by ENCODE database, and the AhR binding site of the CCL21 promoter were founded two xenobiotic responsive elements (XREs).
Figure S7. (A) A549 cells were treated with ctrl medium or CSE medium for 48 h, detected by immunofluorescence assays. (B) A549 cells were treated with ctrl medium or CSE medium for 48 h, assessed by transwell migration and invasion assays. (C-E) A549 cells were treated with vehicle or CCL21 for 48 h, detected by immunofluorescence assays(C), transwell migration and invasion assays(D) and Western blot(E).
**Supplementary Table S4** Primer sets, Sequences of siRNAs and Oligonucleotide sets

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**Sequences of siRNAs**

- sh-AhR: AAGTCGGTCTCTATGCCGCT

**Oligonucleotide sets**

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## Supplementary Table S5 Anti-body

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Supplementary methods

Public data analysis

RNA-seq raw counts for TCGA lung adenocarcinoma dataset were downloaded using the “TCGAbioliinks” R package\textsuperscript{13}. According to the clinical information, life-long non-smoking LUAD patients (82 patients) and patients who smoked more than 50 packs per year (116 patients); including current and former smokers; were selected for differential expression analysis. Differential expression analysis was subsequently carried out using the “DESeq2” R package\textsuperscript{14}. The pathological slide images from TCGA-LUAD project were downloaded from the NIH Genomic Data Commons (https://gdc.cancer.gov/), which included 358 LUAD patients, in turn comprised of 53 life-long non-smoking LUAD patients and 60 patients who smoked more than 50 packs per year (Table S1). We evaluated the abundance, presence, location, and subtype of TLSs by hematoxylin and eosin (H&E) staining. Two pathologists independently reviewed the stained TLSs without having been provided any clinical information of the patients. TLSs observed within the tumor tissue, around the tumor tissue, or in the peritumoral area were included in the statistics. TLSs were divided into three categories according to the morphology of H&E staining: (1) lymphocyte aggregation (AGG), which included small, quasi-circular clusters of lymphocytes; (2) TLS follicles I (FoL-I), which encompassed intratumoral lymphoid follicles without a germinal center; and (3) TLS follicles II (FoL-II), which included intratumoral lymphoid follicles with a germinal center. This classification method was used in our previous study\textsuperscript{15}. The “MCPcounter” R package was used to evaluate the abundance.
of various cell types in the tumor microenvironment from bulk RNA-seq samples. Single-cell RNA-seq (scRNA-seq) data were published by Nayoung et al. in a previous study. The “Seurat” R package was used for processing the scRNA-seq data, including steps for quality control, normalization, the removal of the batch effect, clustering, and annotation. The cell subtype markers were chosen according to previous studies by Williams et al. and Nayoung et al. The “CellCall” R package was used for analyzing cell-cell crosstalk. The data of ATAC-seq derived from A549 cells and DNase-seq derived from PC-9 cells were downloaded from the Encyclopedia of DNA Elements (ENCODE). These data were then visualized by IGV 2.9.4.

**H&E staining and Immunohistochemistry**

LUAD tissue was fixed in 10% formalin, embedded in paraffin, and serially sectioned. The slides were cut into 4-μm-thick sections for histopathological studies. After dewaxing, hydration, and heat-induced epitope retrieval, the paraffin slides were incubated overnight with the following primary antibodies: anti-CD20 (CST, #70168, 1:500), anti-CD23 (Abcam, #Ab92495, 1:400), anti-CCR7 (Abcam, #Ab221209, 1:500), anti-CXCR3 (Abcam, #Ab288446, 1:500), anti-BCL6 (Abcam, #Ab272859, 1:500), anti-AID (Invitrogen, #39-2500, 1:100), anti-BACH2 (Invitrogen, #PA5-100792, 1:100). This was followed by incubation with the secondary antibodies (#BS13278 & #BS12478, BioGot) at 37°C for 1.5 h. The slides were then treated with 3,3-diaminobenzidine solution (#P0202, Beyotime) before being scanned and digitized.
Enzyme-linked immunosorbent assay (ELISA) to measure CCL21, CXCL9, CXCL11 in the serum and culture medium supernatant

The peripheral blood of LUAD patients and C57BL/6 mice were respectively collected. The samples were then separated in PBMC separator tubes (#FMS-900012, FCMACS) and the serum was collected after centrifugation at 1200 g for 10 minutes. The culture medium supernatant was similarly collected after centrifugation at 1200 g for 10 min to remove apoptotic cells and sediment. These samples were then diluted into sample diluents and assayed using the Human CCL21/6Ckine ELISA Kit (#PC138, Beyotime), Mouse CCL21a ELISA Kit (#ab208985, Abcam), Human CXCL9 ELISA Kit (#ab219047, Abcam), Human CXCL11 ELISA Kit (#ab289695, Abcam), and the OD value was recorded at 450 nm with a spectrophotometer.

Cell samples

All cell lines used in this study were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and identified by short tandem repeat sequence analysis. A549, H1975, and PC9 cells were cultured in 1640 medium containing 10% fetal bovine serum. HBE, H1299, and LLC cells were cultured in DMEM medium containing 10% fetal bovine serum. Cigarette smoke extract (CSE) medium was prepared using the modified Carp & Janoff method. The benchtop vacuum pump, sterile rubber tube, and 50 mL centrifuge tube were connected. The rubber interface was sealed. Two unfiltered 1R6F reference cigarettes were continuously blended into 50 mL of serum-free 1640 high-glucose medium.
using 0.5 MPa of air pressure. The medium was then filtered using a 0.2 μm filter membrane. CD4$^+$ T cells and B cells were extracted by CD4 MicroBeads (#130-045-101, Miltenyi) and CD20 MicroBeads (#130-091-104, Miltenyi) according to the manufacturer’s protocol. The peripheral blood samples used for extraction were drawn from healthy volunteers and written authorization was obtained from all volunteers. Primary CD4$^+$ T cells were cultured in TexMACS Medium (#130-097-196, Miltenyi). Primary B cells and other primary cells extracted via fluorescence-activated cell sorting were cultured in serum-free medium, 1 ml of mammosphere media (DMEM/F12, #11320-082, Gibco), 1:50 B27 (# 17504-044, Invitrogen), 500 ng/ml hydrocortisone (#CC-403, Lonza), 40 μg/ml, insulin (#12585-014, Gibco), 20 ng/ml EGF (#C029, Novoprotein), 100 U/ml penicillin, and 100 μg/ml streptomycin (#C0222, Beyotime). All cells were cultured under 5% carbon dioxide and a constant temperature of 37°C in an incubator.

**Experimental animals**

This study employed animal models, specifically 4-6-week-old female C57BL/6 mice, which were procured from GemPharmatech (Nanjing, China). The animal studies were conducted following the protocols approved by the Animal Ethics Committee and Institutional Review Board of the Institute of Zoology, Nanjing Medical University. The mice were exposed to cigarette smoke generated by the DSI’s Buxco Smoke Generator (Buxco, NC, USA) inside a perspex box at a frequency of 12 cigarettes per day for 4 weeks, five days a week. Each cigarette smoke exposure lasted for three minutes, followed by a 15-minute period of fresh air. The whole body cigarette smoke exposure per cigarette lasted for 3 minutes, which was followed by a 15-minute period of fresh air. In other experiments, mice were treated with Benzo(a)pyrene (BaP) at 100 mg/kg (diluted in corn oil) twice a week for a total of 4
weeks. We also investigated the effect of CCL21 neutralization on the mice by administering intraperitoneal injections containing either 10 μg/g of body weight of anti-mouse CCL21 (#AF457, R&D systems) or control IgG (#AB-108-C, R&D systems) diluted in InVivoPure pH 7.0 Dilution Buffer. The lung tissue of euthanized mice was homogenized to extract interstitial fluid, which was subsequently cleaned with cold saline to remove any visible blood or mucus. The drained tissue was cut, grounded, and filtered through a 20μm nylon mesh, and 5 μL of the resulting interstitial fluid from each sample was used in subsequent experiments.

**Western blot**

Cells were washed with PBS, lysed on ice with a mixed solution of RIPA (#89901, Thermo) and protease inhibitor (#A32963, Thermo), centrifuged, added with 1:5 SDS-PAGE Sample Loading Buffer (#P0015F, Beyotime), and heat-denatured to prepare the protein samples. The protein concentration was detected by the BCA Protein Assay Kit (#P0010S, Beyotime). Protein samples were separated by electrophoresis using a 10% SDS-PAGE gel before being transferred to a PVDF membrane, blocked with skim milk, and subjected to incubation with primary antibodies overnight. The primary antibodies were anti-CCL21 (#Ab9851, Abcam), anti-CD11a (#26703, CST), anti-ICAM-1 (#Ab222736, Abcam), anti-β-actin (#3700, CST), anti-Fascin (#54545, CST), anti-VASP (#3132, CST), anti-Cdc42 (#2462, CST), and anti-HSP90 (#4877, CST). Fluorophore-conjugated secondary antibody (#611-145-002 & #610-144-002, Rockland) was then applied for 1.5 hours. Finally, the PVDF membrane was imaged using the Odyssey CLx (LICOR) instrument.

**Flow cytometry assay**
Fresh lung adenocarcinoma samples were processed in a gentle MACS automatic tissue processor (Miltenyi) to prepare single-cell suspensions. The cells were then stained in cell staining buffer (#420301, Biolegend) for 30 min before being blocked with anti-human EpCAM (#Ab223582, Abcam), anti-human CD45 (#Ab10558, Abcam), anti-human CD31(#Ab9498, Abcam), anti-human NG2 (#Ab255811, Abcam), and anti-human SPARC (#Ab225716, Abcam). After blocking, the cells were incubated with fluorophore-conjugated antibodies. Finally, the cells were run on flow cytometric analyzers. Five major microenvironmental component cell subsets were sorted by the FACS Aria cell sorting system using the five channels APC, FITC, Alexa Fluor 594, Alexa Fluor 674, and Brilliant Violet 421.

CD4 T cells obtained from healthy volunteers’ peripheral blood mononuclear cells (PBMCs) were isolated using a Human CD4 T Cell Isolation Kit (CD4 MicroBeads, #130-045-101, Miltenyi). The cells were then stained in cell staining buffer for 30 min before being blocked with PerCP/Cyanine5.5 anti-human CD11a antibody (#301229, BioLegend). The samples were subsequently run on a BD LSRII Flow Cytometer for analysis. The gating strategy was in Figure S1. The data were analyzed using FlowJo software (v10.7). The antibodies used in flow cytometry are listed in the antibodies resources; Table S5.

**Quantitative reverse transcription-PCR (qRT-PCR) assay**

Total RNA was extracted using Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. The qRT-PCR assay was then performed to detect target gene expression using PowerUp SYBR Green Master Mix (Applied Biosystems) in a
StepOne Plus real-time PCR system (Applied Biosystems). The sequences of the primer sets used for various target genes are listed in Table S4. The expression levels of the target genes were normalized to the β-actin gene.

Staining of filopodia in the cell membrane

A549 cells from different treatment groups were seeded at a density of $1 \times 10^5$ on coverslips pre-coated with fibronectin and left for 24 hours. They were then fixed with 4% paraformaldehyde, permeabilized (0.25% Triton-X), blocked with 1% bovine serum albumin, and finally stained with Actin-Tracker Green-488 (#C1033, Beyotime) and DAPI.

Co-Immunoprecipitation

Co-IP was performed according to the manufacturer’s instructions using the Magna ChIP A kit (#88804, Thermo). Cells were lysed in immunoprecipitation lysis buffer by spinning the cell lysate on a rotor wheel for 10 minutes at 4 °C. All supernatants were pooled after centrifugation of the samples at 15,000 g. The immunoprecipitation input was removed, and the samples were incubated with appropriate magnetic beads for 3 hours at 4 °C with rotation. Afterwards, the beads were washed three times with immunoprecipitation wash buffer and eluted with Laemmli buffer. The antibodies were used anti-CD11a (#26703, CST), anti-ICAM-1 (#Ab222736, Abcam).

Nuclear cytoplasmic isolations
Cells from the different treatment groups were treated by a Pairs Kit (#AM1921, Thermo) to isolate the contained proteins. The nuclear and cytoplasmic protein fractions were used to detect the expression levels of target genes by Western blot. GAPDH was used as the cell plasma localization reference and H3 was used as the nuclear localization reference to normalize the expression levels of target genes and to calculate the ratios of nuclear proteins to cytoplasmic proteins.
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>lung adenocarcinoma</td>
<td>LUAD</td>
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<tr>
<td>tertiary lymphoid structure</td>
<td>TLS</td>
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<tr>
<td>non-small cell lung cancer</td>
<td>NSCLC</td>
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<tr>
<td>immune checkpoint inhibitors</td>
<td>ICIss</td>
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<td>aryl hydrocarbon receptor</td>
<td>AhR</td>
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<td>programmed death-ligand tumor proportion score</td>
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<td>Enzyme-linked immunosorbent assay</td>
<td>ELISA</td>
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<td>TCGA database</td>
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