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

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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 orthotopic 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 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.

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.\(^6\)\(^7\) 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.\(^1\)\(^0\) 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,\(^1\)\(^1\)\(^1\) 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\(\geq50\%) or <1\%. Thirty-one patients received 200mg tislelizumab plus platinum-based chemotherapy (carboplatin area under the curve in combination with 500mg/m\(^2\) pemtrexed) 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 CD11\(\alpha\) (#26703, CST) overnight. Fluorophore-conjugated secondary antibody (#A-11008, #A-11004, #A48255, #32733, Invitrogen) was then applied for 1hour. 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-I 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 cells×100%.

**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 ( Fol-I), and intratumoral lymphoid follicles with a germinal center ( Fol-II) based on the stage of maturity. 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, located in
the geriminal 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 geriminal 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 xenogeneic 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 life-long 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 (lymphocytes), CD31+ cells (endothelial cells), EpCAM+ cells (epithelial cells), NG2+ cells (pericytes), and SPARC+ cells (fibroblasts) (Figure 3A). These were cultured in a cigarette smoke extract (CSE) medium for 48 hours. The cell supernatant was then analyzed by ELISA, which revealed that malignant epithelial cells (tumor cells) secreted a higher amount of CCL21 compared with the other cell types (Figure 3B). Moreover, the results of single-cell RNA-sequencing (scRNA-seq) showed that malignant epithelial cells positive for EPCAM, NAPSA, and KRT7 derived from currently smoking patients with LUAD expressed more CCL21 than those derived from non-smokers (Figure 3C). This finding suggests that CCL21 induced by tobacco smoke exposure is mostly secreted by tumor cells.

It has previously been reported that the CCL21-CCR7 axis promotes CD4+ T cell homing and primes the adhesion of CD11a to ICAM-1 on the surface of antigen-presenting cells (APCs).18 Interestingly, we found a positive correlation between the expression of CCL21 and CD11a in the TCGA-LUAD database (Figure 3D). Moreover, a reanalysis of scRNA-seq data conducted in a previous study,19 from three currently smoking patients and three life-long never smoking patients with LUAD revealed an increasing interaction between T cells and various APCs (including B cells) in currently smoking patients (online supplemental figure S4A-E), which implicates that tumor topical T cells derived from currently smoking patients may have adhesion to APCs. It is important to elucidate the role of CCL21 in the chemotaxis and assembly of TLS subpopulations. Thus, we hypothesized that CCL21 may promote the CD11a expression of CD4+ T cells, which increases the adhesion of CD4+ T cells to B cells in the germinal center of TLSs. To test this hypothesis, we extracted CD4+ T cells from peripheral blood mononuclear cells and co-cultured them with A549 cells (online supplemental figure S4F) simultaneously treated with CCL21. The results indicate that treatment with CCL21 significantly increased the expression of CD11a on CD4+ T cells (Figure 3E). In the same way, we used a CSE medium and co-cultured CD4+ T cells with A549 cells that had been treated with neutralizing antibodies against CCL21 (αCCL21). We observed that under the condition of tobacco smoke exposure, the expression of CD11a was attenuated by treatment with anti-CCL21 antibodies (Figure 3F). Subsequently, the ability of T cells to adhere to B cells was evaluated using ICAM-1-coated polystyrene plates (online supplemental figure S4G). The results indicate that treatment with CCL21 significantly increased the adhesion of CD4+ T cells to ICAM-1 (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 on T cells and ICAM-1 on B cells, CD4+ T cells, and B cells were directly co-cultured, which was followed by immunofluorescence staining. The number of CD11a positive clusters, as well as the co-location of CD11a and ICAM-1 clusters, were found to be significantly increased following treatment with CCL21 (Figure 3I). The co-immunoprecipitation assay also demonstrated that CCL21 increased the interaction between CD11a and ICAM-1 co-cultured cells (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 SS5A). 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=100 µm). (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=100 µm). (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=100 µm). (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=50 µm). 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.20 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 non-response (figure 6B). The association between the therapeutic efficacy and clinical characteristics of 29 patients treated with tislelizumab analyzed by multivariate logistic analyses is shown in figure 6C. The random forests method was used to determine the predictive weight of the efficacy of immunotherapy combined with chemotherapy (figure 6D). The area under the curve (AUC) of serum CCL21 was 0.899, indicating a strong correlation between serum CCL21 and the clinical benefit of TPC therapy (figure 6E). The ROC curve analysis of PD-L1 TPS had an AUC of 0.554, indicating a moderate association with the clinical benefit of TPC therapy (figure 6F).
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 chemotherapy 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. 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. TLSs have been recognized as key components of antitumor immunity and their formation improves the immunotherapeutic effect of ICIs. 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. 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. 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. 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. 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. 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. 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. Hu et al's study pointed out that after tobacco exposure, T cell exhaustion may be caused by specifically activating the ADAM12+ Treg subset. 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. On the other hand, smoking can also improve the efficacy of immunotherapy by mediating PD-L1 expression in tumor cells and increasing immunogenic mutations. 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 CD29+B 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.

Acknowledgments

Data availability statement

Data are available upon reasonable request.

Supplemental material

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Competing interests

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