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RESEARCH

Nicotine promotes M2 macrophage polarization through α5-nAChR/SOX2/CSF-1 axis in lung adenocarcinoma

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Abstract

α5-nicotinic acetylcholine receptor (α5-nAChR) plays a vital part in lung adenocarcinoma (LUAD). However, it is not comprehensively understood that how the α5-nAChR affects LUAD. Through diverse bioinformatics analyses and immunohistochemistry, the expressions of α5-nAChR and SOX2 as well as their relations were dissected. α5-nAChR regulated the differentiation of monocytes into M2 macrophages by targeting the STAT3/SOX2/CSF-1 signaling in the coculture system by western blotting and ChIP. α5-nAChR-mediated macrophage-mediated LUAD cell migration via SOX2/CSF-1 signaling in the cocultured medium. Correlations of α5-nAChR, SOX2 and M2 phenotype tumor-associated macrophages (TAMs) were validated in mouse LUAD models and clinical samples. α5-nAChR expression was connected to SOX2 expression, smoking and bad prognosis of LUAD among clinical samples. Nicotine-induced SOX2 expression was mediated by α5-nAChR via STAT3. Additionally, SOX2-mediated macrophage colony-stimulating factor (CSF-1) expression contributed to LUAD progression in vitro. Furthermore, α5-nAChR expression was strongly linked to pSTAT3, SOX2 and M2 macrophage marker CD206 expression and negatively correlated with M1 macrophage marker CD86 expression in vivo. It is indicated that M2 macrophages are mediated by the new α5-nAChR /SOX2/CSF-1 axis in nicotine-related LUAD, which is a potential therapeutic strategy for cancer.

Keywords α5-nicotinic acetylcholine receptor (α5-nAChR) · SOX2 · Macrophage colony-stimulating factor (CSF-1) · M1/M2 polarization · LUAD

Introduction

In terms of lung cancer cases, non-small cell lung cancer (NSCLC) occupies 80% in which lung adenocarcinoma (LUAD) is the most common and its incidence is increasing annually [1, 2]. Despite advances in diagnosing and treating LUAD, the five-year overall survival rate of LUAD patients is less than 20% [3]. Consequently, it is excessively imperative to seek for the new targets for LUAD pathogenesis and therapy.

In fact, smoking becomes one of the primary reasons for lung cancer. Nicotine, the primary addiction causing ingredient in tobacco smoke [4], acts primarily at nicotinic acetylcholine receptors (nAChRs) [5]. Functioning nAChRs are α-subunit homopentamers or heteropentamers with a minimum of a α-subunit or a β-subunit. On the part of mammals, eight α subunits (CHRNA2-CHRNA7, CHRNA9, and CHRNA10) have already been reported, as well as four β subunits (CHRNB1-CHRNB4), and one each of the δ (CHRND), ε (CHRNE), and γ (CHRNG) subunits [6]. In genome-wide association studies (GWAS), it was discovered that CHRNA5 (encoding α5-nAChR) was highly and consistently associated with the risk of lung cancer induced by smoking [7]. Through gene set

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variation analysis (GSVA) and functional enrichment analysis, researchers have also discovered other signaling pathways related to immune evasion in LUAD, such as the IFN-γ/α response, IL-2/STAT5 signaling pathway, IL-6/JAK/STAT3 signaling pathway, allograft rejection, and inflammatory response [8]. In our recent work, it has been proven that α5-nAChR affects the progression of nicotine-related lung cancer via STAT3, Ly6E, NLRP3 and FHIT [9–11] and participates in immune activities of T and NK cells by regulating STAT3/Jab1/PD-L1 axis in LUAD [12]. It also regulates the tumor-killing activity of TAMs by promoting the expression of the CD47 protein [13].

It recently became clear that nicotine promotes the stem-like cell self-renewing [14]. Nicotine is capable of upregulating SOX2; additionally, that is crucial to self-renew NSCLC cells and maintain the stemness [15]. SOX2 is critical for the development of embryos and maintains the stemness of adult stem cells and embryos [16]. SOX2 expression retains cancer stem cell (CSC)-like features, which is connected to worse outcome of LUAD. SOX2 expression is correlated with a wide variety of tumors and has been proven with positive impacts on tumor cell characteristics such as the ability to proliferate, migrate, invade and metastasize [17]. SOX2 has a significant role in the recruiting of tumor-associated macrophages (TAMs) by activating several cytokine expressions, which include ICAM, CCL3 and MIP-1α [18]. Tumor progression is mediated by recruitment of TAMs into the tumor microenvironment (TME).

As for host defence and adaptive immunity, macrophages play an irreplaceable role [19]. There have been two main polarization statuses M1 and M2 in macrophages. The most plentiful immunocytes are macrophages infiltrating the cancer, which are generally known as TAMs. Numerous investigations have demonstrated that macrophages in tumors display a predominantly M2-like phenotype [20]. In comparison, M1 macrophages are characterized by pro-inflammation and cytotoxicity. Additionally, M2 macrophages are characterized by immunosuppression and boost tissue regeneration as well as vascularisation. It has been demonstrated that M2 macrophages enhance cancer cell proliferation and viability and promote angiogenesis and metastasis [21]. M2 TAMs are key players in the promotion of tumor growth and invasion at primary and metastatic cancer sites, and high levels of M2 TAMs have been associated with aggressive tumor phenotypes and disease development among multiple human cancers [22]. In lung cancer, M2 TAMs interact with tumor cells to promote proliferation, chemo-resistance, distant metastasis and survival [23]. In addition, M2 TAMs produce cytokines and chemokines that cause T cell exhaustion and promote the development of immunosuppressive TME [24]. Tobacco smoking influences the immunological milieu by affecting macrophage polarization and phagocytosis via the JAK2/STAT3 pathway [25]. However, the connection among α5-nAChR, SOX2 and TAMs in LUAD is not yet clear.

In the present research, α5-nAChR and SOX2 expressions in vivo were explored in LUAD. Additionally, it was showed that α5-nAChR expression is related to SOX2 expression, smoking, along with decreased survival. α5-nAChR mediates the phenotypic transformation of M1 and M2 TAMs via SOX2/CSF-1 signaling and facilitates LUAD cell transfer. To our understanding, it is the earliest research on the α5-nAChR/SOX2/CSF-1 axis regarding M2 macrophage polarization in LUAD.

Materials and methods

CHRNA5 and SOX2 expression and clinical characteristics using relevant databases

CHRNA5 and SOX2 expression properties in TCGA LUAD ($n=483$) and normal ($n=347$) were dissected by means of GEPIA (http://gepia.cancer-pku.cn/help.html). Correlations between CHRNA5 and SOX2 were determined using the Tumor Immune Estimation Resource 2.0 (http://cistrome.shinyapps.io/timer/), a platform applied to the systematic research on cancer gene expression correlations. Using KM plotter (http://kmplot.com/analysis), the CHRNA5 expression or SOX2 expression and LUAD overall survival of patients with correlation were analyzed: patients with clinical data on primary tumor sample classification and use of the UALCAN (http://ualcan.path.uab.edu/analysis.html), which is an interactive database accessible to tumor OMICS analysis, to generate box plots of CHRNA5 and SOX2 expression levels in LUAD based on patients' smoking habits and individual cancer stages [26].

We also analyzed LUAD datasets in TCGA ($n=574$) by Gene Ontology (GO) and Gene Set Enrichment Analysis (GSEA). In addition, GSEA was conducted to detect signaling pathways. GO enrichment analyses were performed to detect immune cell infiltration and CHRNA5 and SOX2 expression in LUAD [27].

Tissue specimens and cell culture

Tissue microarrays (#OD-CT-RsLug04-003, approval no. SHXC2021YF01) were supplied by Shanghai Outdo Biotech Co., Ltd (Shanghai, China) and contained 55 LUAD and 53 paraneoplastic specimens (at the age of 45–82 years, and the average age was 64.2). In 55 specimens, there existed 15 male non-smokers with adenocarcinoma, 15 male smokers with adenocarcinoma, 15 female non-smokers with adenocarcinoma, and 10 female smokers with adenocarcinoma. Samples with a clear clinical diagnosis and a pathological pattern consistent with adenocarcinoma of the lung were

included. Clinical LUAD samples were obtained from patients with lung adenocarcinoma at Jinan Central Hospital, affiliated to Shandong First Medical University (approval no. R202303060078).

A549 and H1299 cell lines were supplied by the Cell Resource Center of Chinese Academy of Sciences (Shanghai, China). They were incubated in RPMI-1640 culture medium (#CM10041, MACGENE, China) added 10% fetal bovine serum (FBS, #164210-500, Procell, China) and 1% penicillin/streptomycin (#p1400, Solarbio, China). Cell culture was performed in moist medium containing 5% CO_2 at the temperature of 37 degrees. As described before, the LUAD cells were cultured with 1 μM nicotine (#54-11-5, Sigma, USA) for sixteen hours [28]. THP-1 acute monocytic leukemia cells (#ZQ0086, Shanghai Zhong Qiao Xin Zhou Biotechnology Co.,Ltd. China) were hatched within RPMI-1640 medium with the addition of 10% FBS, 1% P/S and 0.05 mM β-mercaptoethanol as per production instructions. The cell lines were within continuous culture for less than 30 days and were periodically checked for the presence of mycoplasma.

Immunohistochemistry

For analysis by immunohistochemistry, tissue slices (4 μm thick) were deparaffinized, hydrated, antigen occluded, and processed with an endogenous peroxidase repressor for 10 min. Subsequently, they were incubated using mouse monoclonal α5-nAChR primary antibody (1:200, #66363-1-Ig, Proteintech), SOX2 antibody (1:200, #23064, CST), phospho-STAT3 antibody (1:200, #AP0070, Abclonal), CD86 (M1 macrophage marker [29], 1:200, #YT7823, Immunoway) and CD206 (M2 macrophage marker [29], 1:50, #sc-70586, SANTA) at 4 °C during the night. The slices were hatched using enhancer-marked goat anti-rabbit (#pv-9001, China) or anti-mouse (#pv-9002, China) IgG polymers for 3 min; it was then stained with 3,3′-diaminobenzidine (DAB) and restained with hematoxylin. The slices without primary antibody were used as negative controls [12]. Two independent investigators blinded to the clinical data performed expression analysis by microscopy.

This scoring system was utilized: unstained denoted 0, yellow denoted 1, and brown denoted 2. The proportions of cancer cells were graded as below: $<1\%$ denoted 0, 1–25% denoted 1, 26–75% denoted 2, and 76–100% denoted 3. Positive staining was indicated when the outcome of two indicators multiplied by ≥ 4.

Western blot analysis

Monolayers of A549 and H1299 were homogenized in RIPA buffer (Cwbio, China) supplemented with phosphatase blocker cocktail (Cwbio) and phenylmethylsulfonyl fluoride (Beiotime, China). Total proteins were segregated by SDS-PAGE on an 8% gel and blotted on a nitrocellulose membrane (0.45 μm, Millipore, USA) for western blot analysis. Then, those proteins were hatched at 4 °C for 8–12 h using antibodies against α5-nAChR (1:2000, #66363-1-Ig, Proteintech), pSTAT3 (1:1000, #AP0070, Abclonal), STAT3 (1:2000, #4904, CST), SOX2 (1:2000, #3032, CST) and GAPDH (1:10000, #10494-1-AP, Proteintech). The levels of the GAPDH expression were regarded as one internal reference. Visualize and analyze proteins using FluorChem E (ProteinSimple, USA).

Plasmid and lentiviral infection

The SOX2 overexpression plasmid and control were purchased from Tsingke Biological Technology. A549 and H1299 were plated in 6-well plates once those cells were at 60–70% confluence. Moreover, lipofectamine 2000 (Invitrogen, USA) was used with the aim of adding the plasmid to an ultimate concentration of 2 μg/ml as per product descriptions.

Human CHRNA5 cDNAs (MOI = 10) were transfected into the pGV-puro lentiviral puromycin resistance vector. The overexpression of the α5-nAChR protein was obtained in A549 (designated α5-nAChR OE). The transfected cells were cultured using puromycin (2 μg/ml, #P8230, Solarbio) for a minimum of 1 week.

Chromatin immunoprecipitation (ChIP)

The chromatin immunoprecipitation experiment was performed with the reagents provided with the SimpleChIP@ Plus Kit (#9004S, CST), in light of the manufacturer's certificate. Briefly, the cells were added with 1% formaldehyde and cultured to approximately 1×10^7 cells. The chromatin of the samples was then digested with microsphere nuclease (#10011, CST). The DNA was segmented into 150–900 bp. Detection was done by means of Super DNA Marker (#CW2583, CWBIO). Chromatin immunoprecipitation was executed by means of either control IgG or SOX2 (1:50, #3032, CST) primary antibody. Forward primer 5′-TAAAAT CTGGCCTTGGGACTC-3′ and reverse primer 5′-TCTCTC TGACCAAGGTGTTATG-3′ were constructed for the sake of targeting a -236/-226 area of the human CSF-1 promoter associated with the transcriptional starting point, which included the SOX2 binding point. Non-immunoprecipitated fragments of chromatin were used as input controls.

Luciferase assay

In order to evaluate the activation of the CSF-1 promoter, luciferase reporter systems were constructed using mutant CSF-1 and wild-type promoters. Additionally, the systems

owned one single dual-luciferase reporter plasmid pGL3 [30]. A 24-well plate was seeded with a total of 2×10^4 A549 cells per well and co-transfected with a combination of 780 ng pGL3-CSF-1 (RiboBio, China) and 200 ng pcDNA-SOX2 (Tsingke Biotech, China) expression plasmids [31], and transfection with lipofectamine 2000 (#11668019, Life Technologies, CA, USA). Two days after transfected, firefly and renilla luciferase activation (#E1980, Promega) was determined by means of one dual-luciferase reporter trial. The luciferin-luciferase chemiluminescence method was used to measure luminescence on a Promega GloMax Navigator. All transfectant experiments were conducted three times with independent replicates.

Macrophages cocultured with tumor cells

8×10^5 THP-1 cells were processed with 100 ng/mL PMA (#HY-18739, MedChemExpress) in each well for 48 h [32]. Macrophages (2×10^5) and LUAD cells (5×10^5) were cocultured in 6-well plates for 24 h, and cell coculture supernatants were gathered for subsequent assays: effective to target (E/T) rate of 2.5:1.

Cell migration assay

Cell transfer was determined by means of 24-well transwell plates with 8-µm membrane filters inserted (#3422, Corning). In brief, 1×10^5 LUAD cells were isolated again within non-serum supplemented medium and plated in upper chambers (Corning, USA). THP-1-derived macrophages and tumor cells (NC/SOX2 +) coculture supernatants were seeded into the lower chamber (RPMI-1640 medium with 10% FBS) [33], or 4×10^4 macrophages were placed in the lower chamber (24-well plates). Upper chamber cells were eliminated using one cotton swab, immobilized using methanol, and stained with 1% crystal violet. Migrating cells were counted using ImageJ software (Version 1.53e).

Immunofluorescence (IF) assay

IF was conducted with THP-1 derived macrophages and paraffin sections from clinical patients. It was the immobilized cells that were treated with 4% paraformaldehyde for 15 min, permeable with 0.3% Triton X-100 for 3 min at 4 °C, and inhibited with 10% goat serum for 30 min. Tissue slides (4 µm thick) were deparaffinized, hydrated, and vulnerable to an antigen extraction procedure. Those slides were subsequently fixed using goat serum for 45 min, after which they were treated with primary antibodies against CD86 (1:200, Immunoway, #YT7823), CD206 (1:50, SANTA, #sc-70586), SOX2 (1:200, CST, #23,064) and α5-nAChR (1:200, Proteintech, #66,363–1-Ig) and incubated overnight at 4 °C. In addition, secondary antibodies largely included goat antirabbit Alexa Fluor 488 and antimouse Alexa Fluor 594. Nuclear staining was performed by means of DAPI.

Enzyme-linked immunosorbent assay (ELISA)

The CSF-1 level in cell culture supernatants from A549 and H1299 was determined using the human CSF-1 ELISA Kits (Multi Sciences, China) as described in the manufacturer's guidelines. Subsequently, the cell culture fluid was gathered. Furthermore, they were centrifuged at 300 × g for 10 min at the temperature of 4 degrees. In cell culture supernatants, the CSF-1 cytokine level was measured with ELISA. One microplate reader was utilized with the intention of measuring the absorbance at 450 nm (Thermo Scientific, USA). The concentration of CSF-1 was determined from the standard curve generated by diluting the CSF-1 standard in the assay device.

Xenograft tumor model

All animal studies were approved by the Institutional Animal Care and Use Committee of Central Hospital Affiliated with Shandong First Medical University (approval no. JNCH2021-77). BALB/c thymus-free nude mice at the age of 4–6 weeks were supplied by Beijing HFK Bioscience (Beijing, China). 2×10^6 A549 cells were suspended in 100 µl of Dulbecco's modification of Eagle's medium (#CM15019, MACGENE, China). Mice were injected subcutaneously into the right abdomen to achieve xenograft ($n = 6$). The tumor size was measured by standard formula for calculating its volume: length × width^2/2. After 19 days, those mice were killed humanely; additionally, the tumor was harvested, fixed in 4% paraformaldehyde, and subjected to paraffin embedding, histologic examination and immunohistochemical staining.

Statistical analysis

The statistic data were construed by means of GraphPad Prism 9.0 and SPSS v26.0, which was presented as mean ± standard deviation. Each experiment was conducted independently a minimum of three times. Sample numbers (n) are indicated in the figure legends. Student's t-test was applied to compare the two groups. Differences between those groups were analyzed by one-way analysis of variance (ANOVA) and Dunnett's post hoc test. Pearson's Chi-square test was employed to dissect the association between α5-nAChR and SOX2 in LUAD. Statistical significance was determined using p-values < 0.05 ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$), which were considered statistically significant in all figures, while ns was not significant.

Results

CHRNA5 and SOX2 expression correlates with poor prognosis and smoking status in lung adenocarcinoma from databases

Through the GEPIA, analysis uncovered the higher expression levels of CHRNA5 and SOX2 in TCGA regarding LUAD tissues ($n = 483$) than those in controls ($n = 347$) (Fig. 1a). The TIMER 2.0 online assessment revealed that CHRNA5 expression positively correlated with SOX2 expression (Fig. 1b). LUAD patients with a high level of CHRNA5 or SOX2 expression had a shorter life than those with a low level of CHRNA5 or SOX2 expression (Fig. 1c, d). CHRNA5 and SOX2 expressions were more elevated in LUAD smokers than those in nonsmokers based on the UALCAN (Fig. 1e, f). Expressions of CHRNA5 and SOX2 were linked to the cancer stage (Fig. 1g, h). Lastly, it was suggested that CHRNA5 and SOX2 might be linked to LUAD progression.

CHRNA5 and SOX2 inhibitory effect on macrophage immunity using GSEA and GO enrichment analysis

Through the GSEA, it was indicated that the TNF-α signaling pathway was negatively correlated with CHRNA5 and SOX2 expressions, besides, the pathway was statistically significant (Fig. 1i, j). The results of GO analysis showed that both CHRNA5 and SOX2 expression were related to macrophage infiltration in LUAD (Fig. 1k, l). TNF-α is a key cytokine for polarization of the M1 macrophage phenotype and tumor killing [34]. As a result, it was suggested that CHRNA5 and SOX2 might be associated with TAMs.

α5-nAChR expression correlated with SOX2, CD86 and CD206 expression in LUAD samples

Since α5-nAChR expression was linked to SOX2 and macrophage immunity using database analysis, we further assessed α5-nAChR, CD86 and CD206 expression. α5-nAChR and SOX2 expressions were measured by IHC in LUAD tissue microarray (OD-CT-RsLug04-003). α5-nAChR (65%, 36/55) and SOX2 (61.8%, 34/55) expressions were markedly increased in LUAD tissues compared to paracarcinoma tissues (Fig. 1m, n). Pearson's chi-square analysis revealed a positive association between α5-nAChR and SOX2 in LUAD (Fig. 1o). Expressions of α5-nAChR and SOX2 were associated with smoking, but not with patient age or sex (Table 1).

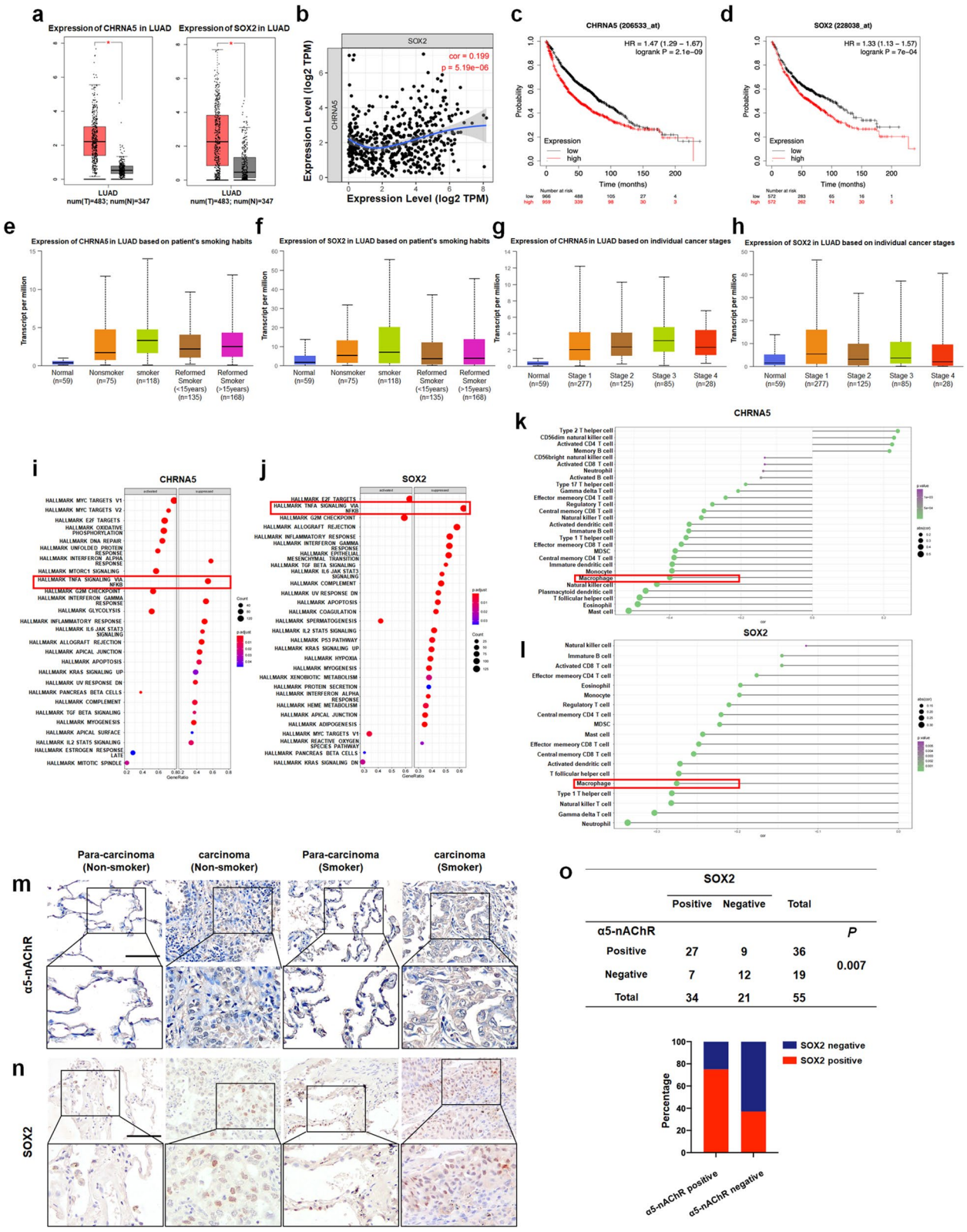
In addition, CD86, CD206, α5-nAChR, and SOX2 expressions were checked in clinical LUAD samples using IF staining. α5-nAChR and SOX2 expression raised during stage III-IV by comparison with stage I-II tumor tissues (Fig. 2a, b). Expressions of α5-nAChR and SOX2 were upregulated among the smokers by comparison with the nonsmokers in LUAD tissues. Meantime, the M1/M2 (CD86/CD206) ratio decreased during stage III-IV tumor tissues by comparison with stage I-II LUAD tissues (Fig. 2c, d). These showed that α5-nAChR was significantly connected to SOX2 and inversely associated with M1/M2 in LUAD, which suggested SOX2 and macrophage immunity were correlated to α5-nAChR expression.

α5-nAChR regulates SOX2 expression through pSTAT3 in LUAD cells

For the purpose of better comprehending the potential connection between α5-nAChR and SOX2, we examined the levels of pSTAT3 and SOX2 protein expression by western blotting in α5-nAChR knockdown and overexpression as well as in nicotine-treated cells. A549 and H1299 were chosen for the following experiments. SOX2 interacts with other factors, such as STAT3, to regulate tumor cell progression [35]. Our prior study showed that α5-nAChR participated in nicotine-induced LUAD progression via the JAK2/STAT3 pathways [28]. Western blot analysis revealed that after steady knockdown of α5-nAChR by lentivirus, expressions of SOX2, pSTAT3, and α5-nAChR were reduced significantly by comparison with the control group (Fig. 3a). Meanwhile, α5-nAChR overexpression and nicotine treatment increased the expression of α5-nAChR, pSTAT3, and SOX2 (Fig. 3b, c). When STAT3 phosphorylation was inhibited by niclosamide, SOX2 expression decreased (Fig. 3d). These results indicate that nicotine modulates SOX2 expression via the α5-nAChR/pSTAT3 axis.

α5-nAChR regulates CSF-1 expression via SOX2 in LUAD cells

As a protein important for the recruitment and function of TAMs, macrophage CSF-1 regulates the survival, differentiation, and proliferation of haematopoietic cell lines, like promonocytes, osteoclasts, monocytes, monoblasts, and macrophages [36]. Previous studies have shown elevated CSF-1 levels in patients' sera with disparate categories of lung cancer [37]. Consensus binding sites for SOX2 are enriched in fragments of the CSF-1 core promoter. Through the analysis on transcription elements (http://jaspar.genereg.net), it was expounded that CSF-1 promoter contained SOX2 binding points (Fig. 3e). The combination of anti-SOX2 antibody and CSF-1 gene promoter was investigated via CHIP trial in sh-CHRNA5 and A549 NC groups. A549 cells treated with sh-CHRNA5 significantly reduced the quantity of SOX2 integrating with the CSF-1 promoter, by



comparison with the NC group (Fig. 3f). We also confirmed the SOX2 binding site in the CSF-1 promoter using a luciferase reporter. Furthermore, it was discovered that SOX2 overexpression improved the relative luciferase activation of CSF-1 versus the binding site mutant group (Fig. 3g). Taken together, it is denoted that SOX2 is capable of transactivating

◂**Fig. 1** Expression of CHRNA5 and SOX2 in LUAD patients in the TCGA database. **a** Expression of CHRNA5 in LUAD. **b** Expression of SOX2 in LUAD. **c** The association of CHRNA5 expression with clinical outcomes in LUAD. **d** The association of SOX2 expression with clinical outcomes in LUAD. **e** The expression of CHRNA5 based on patient's smoking habits in LUAD. **f** The expression of SOX2 based on the patient's smoking habits in LUAD. **g** The expression of CHRNA5 based on individual cancer stages in LUAD. **h** The expression of SOX2 based on individual cancer stages in LUAD. **i**, **j** Expression of CHRNA5 and SOX2 correlates negatively with the TNF-α pathway in GSEA enrichment based on TCGA LUAD samples. **k** Associations between CHRNA5 expression and 28 immune cells in LUAD from the TCGA database in GO analysis. **l**Associations between SOX2 expression and 28 immune cells in LUAD from the TCGA database in GO analysis. **m** α5-nAChR expression in LUAD and paracarcinoma samples from smokers and nonsmokers. Scale bar = 100 μm. **n** SOX2 expression in LUAD and paracarcinoma samples from smokers and nonsmokers. Scale bar = 100 μm. **o** Correlation between α5-nAChR and SOX2 expression in 55 adenocarcinoma specimens. $P < 0.05$

the CSF-1 gene through integrating with the areas between − 236 bp and − 226 bp of the promoter. Meanwhile, ELISA results showed that CSF-1 secretion levels significantly decreased after sh-CHRNA5, and SOX2 overexpression significantly reversed the α5-nAChR knockdown-mediated inhibition of CSF-1 in A549 and H1299 (Fig. 3h). It is indicated that α5-nAChR is involved in mediating CSF-1 expression through SOX2 in LUAD cells.

SOX2/CSF-1 axis involved in the differentiation of monocytes into M2 macrophages in the cocultured system

Myeloid progenitors differentiating into heterogeneous population are driven by CSF-1 signal transduction via CSF-1R, including bone-resorbing osteoclasts, macrophages, dendritic cells and monocytes. Within the systemic circulation, macrophage transfer, growth, activity and viability are regulated by CSF-1 [38]. The medium of SOX2-overexpressed A549 and H1299 cells significantly decreased the M1/M2 polarization of macrophages. The reduced ratio was abolished through adding anti-CSF-1 antibody to A549 and H1299 (Fig. 4a, b). What's more, it was suggested that the SOX2/CSF-1 axis affected the monocyte differentiating into M2 macrophages during LUAD.

α5-nAChR-mediated macrophage-mediated LUAD cell migration via SOX2/CSF-1 signaling in the cocultured system

A549 and H1299 were cultured in the upper chamber of transwell plates. THP-1-derived macrophages, which were conditioned with 100 ng/ml PMA for 48 h, were placed to the lower chamber of the aforementioned plates. α5-nAChR knockdown decreased the metastasis capability of A549 and H1299, whereas SOX2 overexpression partially restored the metastasis capability of LUAD cells within the coculture system (Fig. 5a, b). To further verify the function of CSF-1, the influence of CSF-1 was blocked by adding a CSF-1 antibody to the conditioned medium, which was cocultured with macrophages and tumor cells in the lower chamber, and the migration effect of the LUAD cell line was further examined. Macrophages were stimulated with conditioned medium from NC- or SOX2-overexpressing LUAD cells. The CSF-1 antibody group showed significantly inhibited migration of LUAD cells by comparison with the blank control and IgG groups (Fig. 5c, d). Additionally, it was demonstrated that α5-nAChR-mediated macrophage-mediated cell transfer via SOX2/CSF-1 signaling in LUAD.

Expression levels of α5-nAChR, pSTAT3, SOX2, CD86, and CD206 are related in LUAD xenograft tissues of BALB/c mice

Since it was found that α5-nAChR expression was connected to SOX2 and M1/M2 macrophage ratio in vitro, CD86, CD206, α5-nAChR, SOX2, and pSTAT3 expressions were then evaluated in LUAD xenograft tissues. Tumors originated in CHRNA5-inhibiting A549 clones, which were smaller than those originating in control cells (Fig. 6a). We further evaluated α5-nAChR, pSTAT3, SOX2, CD86 and CD206 expression in LUAD xenograft tissues. Expressions of α5-nAChR, pSTAT3, SOX2, and CD206 were more elevated; however, CD86 expression was lower in NC group than that in KD group (Fig. 6b). Furthermore, expressions of α5-nAChR, pSTAT3, SOX2 and CD206 were more elevated in the nicotine-treated xenograft tissues than those in the NC group tissues, whereas CD86 expression was lower. The discovery also emerged in both KD and nicotine-treated KD groups. Moreover, it was indicated that α5-nAChR-mediated M2 macrophage polarization via SOX2 signaling in LUAD.

Discussion

Smoking is one of the primary risk elements for lung cancer [39]. As the main addictive part of cigarettes, nicotine has recently been shown to promote lung tumor initiation and development by binding to nAChRs and activating downstream signaling pathways [40]. By suppressing both innate and adaptive immune reactions, nicotine exerts an immunosuppressive effect [41]. The effect of nicotine on macrophage polarization is primarily reflected in its ability to promote macrophage polarization toward the M2 type, which is commonly associated with anti-inflammatory responses, tissue repair, and immunosuppression [42, 43]. Nicotine achieves this effect by activating specific signaling pathways. aQP3 regulates M2-type macrophage polarization through the

Table 1 Correlations of α5-nAChR and SOX2 expression with clinical parameters in lung adenocarcinoma specimens

Clinical pathology	Case ($n=55$)	α5-nAChR		P	SOX2		P
		Positive	Negative		Positive	Negative	
Sex							
Male	30	20	10	0.530	19	11	0.509
Female	25	16	9		15	10	
Age (years)							
≤60	19	12	7	0.511	9	10	0.095
>60	36	24	12		25	11	
Smoking history							
No	30	15	15	0.008	12	18	<0.001
Yes	25	21	4		22	3	

PPAR-γ/NF-κB axis and influences LUAD progression [44]. Nicotine may also modulate macrophage polarization through the α7-nAChR, which activates the α7-nAChR/SIRT1 axis, which in turn modulates THP-1 M0 macrophage polarization through cholinergic anti-inflammatory pathways [45]. In the present study, our experimental results indicate that nicotine promotes LUAD progression and macrophage M2 polarization through the α5-nAChR/SOX2/CSF-1 axis. Nicotine has a complex role in macrophage regulation in lung adenocarcinoma, and our results partially explain that nicotine promotes tumor progression and immune escape of TAMs in lung adenocarcinoma by modulating the mutual crosstalk between tumor cells and macrophages.

Nicotine and its constituents have been shown to promote the self-regeneration of lung adenocarcinoma stem-like cells [46]. Nicotine is capable of upregulating SOX2, which is crucial to self-renew NSCLC cells and maintain the stemness [15]. The transcription factor SOX2 is important to embryonic progression and even becomes a key player in maintaining multiple adult stem cell populations and embryonic stem cells [47]. In contrast, abnormal SOX2 expression is linked to a wide range of cancers, and SOX2 positively influences tumor cell properties, including proliferation, invasion and transfer [48]. There is also growing evidence that SOX2 conferred resistance to established cancer treatments and is expressed in cancer stem cells [49]. In our previous study, it has been proven that α5-nAChR affects the progression of nicotine-related lung cancer via STAT3 [28]. In this study, α5-nAChR and SOX2 expressions are associated by modulating STAT3 phosphorylation in nicotine-related lung cancer. Otherwise, SOX2 promotes CSF-1 to facilitate M2 macrophage polarization in TAMs, which promotes LUAD progression. It suggests that acetylcholine and analogues interplay with the cell surface α5-nAChR to activate pSTAT3/SOX2/CSF-1 signaling pathway, which promotes M2 macrophage polarization and is involved in lung cancer.

CSF-1R-mediated signaling is critical in the survival and differentiation of mononuclear phagocytes, particularly macrophages [50]. Previous studies have supported targeting the CSF-1/CSF-1R axis as a prospective therapeutic approache of pancreatic ductal adenocarcinomas or malignant meningeoma [51]. It was discovered in this research that blocking CSF-1/CSF-1R signaling in lung adenocarcinoma reduced M2-type macrophage polarization and slowed tumor progression. The data suggested that CSF-1/CSF-1R signaling might be an efficient therapeutic target for reprogramming the immunosuppressive microenvironment of human LUAD tumors and enhancing the efficacy of immunotherapy.

TAMs are critical members of the TME and have been implicated in modulating cancer cell proliferation, metastasis, angiogenesis, extracellular matrix remodeling, immunosuppression, chemotherapeutic resistance and checkpoint inhibition immunotherapy [52]. In contrast, when properly stimulated, macrophages mediate the phagocytosis of cancer cells and cytotoxic tumor death [53] and have effective bidirectional interactions with various elements of the innate and adaptive immune response. Consequently, they have become therapeutic targets in cancer treatment [54]. Targeting macrophages includes inhibiting cytokines and chemokines involved in the recruiting and polarization of tumor-promoting myeloid cells, as well as the activation of their antitumor and immunostimulatory functions [55]. Previous clinical studies have shown that targeting negative regulators of myeloid cell function (checkpoints) may actually have antitumor potential [56]. Macrophage-based strategies hold the promise of complementing and synergizing with the available toolkit in oncology. Our results suggest that CSF-1 secreted by cancer cells can effectively stimulate the macrophage polarization to the M2 type. Accordingly, the migratory ability of tumor cells and progression were promoted. These results provide an important theoretical basis for treating smoking-related lung adenocarcinoma by targeting macrophages.

SOX2 is a key player in regulating embryo growth and maintaining stem cell differentiation capability, which acts as one of stem cell markers [57]. SOX2 has a significant

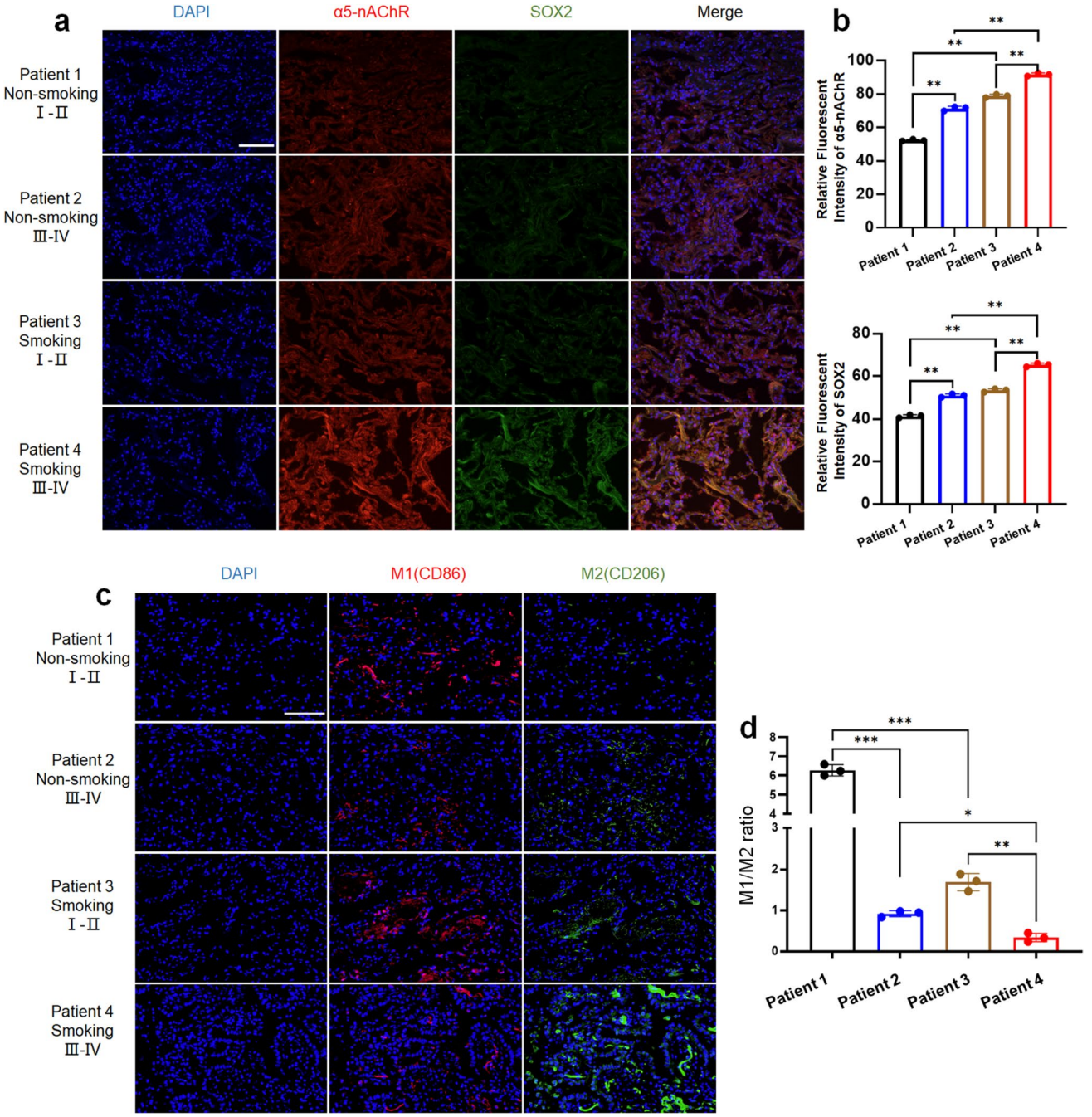
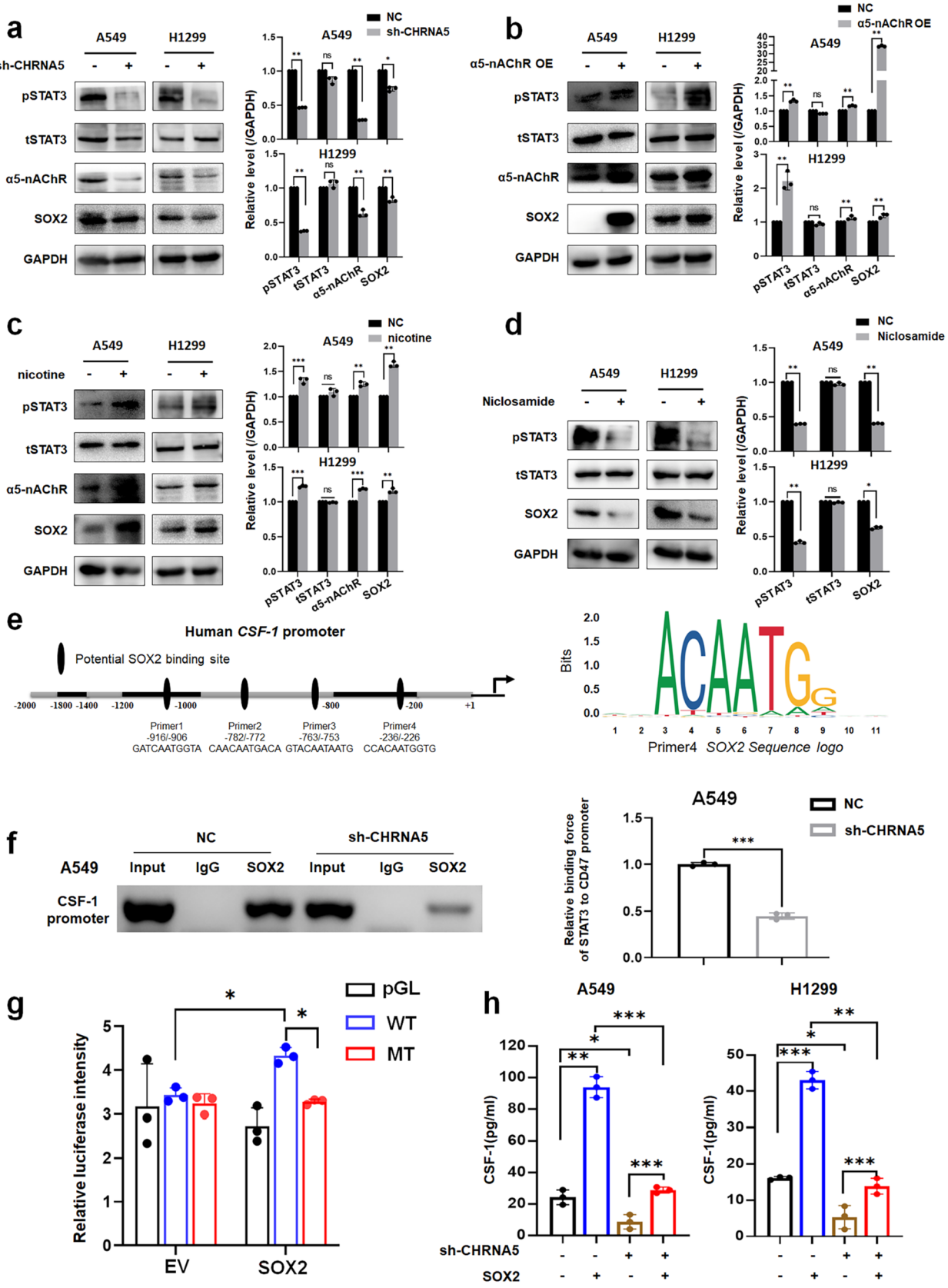


Fig. 2 α5-nAChR, SOX2, CD86, and CD206 expression was correlated with clinical stage, smoking status, and macrophage polarization in lung adenocarcinoma. **a**, **b** Expression of α5-nAChR and SOX2 in smokers and non-smokers and different clinicopathological lung adenocarcinoma samples. Quantifcations are shown in the right panel. Scale bar = 100 μm; $n = 3$; $^{**}P < 0.01$. **c**, **d** Expression of CD86 and CD206 in smokers and non-smokers and different clinicopathological lung adenocarcinoma samples, the M1/M2 macrophage ratio is shown on the right. Quantifcations are shown in the right panel. Scale bar = 100 μm; $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

role in the recruiting of TAMs [18]. CSF-1 is known to be widely overexpressed in tumors and to promote tumor growth and invasiveness by stimulating the pro-tumorigenic activity of TAMs [58]. α5-nAChR and SOX2 expressions are associated by modulating STAT3 phosphorylation and nicotine interplays with α5-nAChR to activate pSTAT3/SOX2/CSF-1 signaling pathway and promotes M2 macrophage polarization involved in lung cancer. These results offer new understanding of the prospective α5-nAChR molecular systems underlying SOX2/CSF-1 axis-mediated M2 macrophage polarization in lung cancer.


a
A549
H1299
sh-CHRNA5
pSTAT3
tSTAT3
α5-nAChR
SOX2
GAPDH
NC
sh-CHRNA5
Relative level (/GAPDH)
b
α5-nAChR OE
NC
α5-nAChR OE
c
nicotine
NC
nicotine
d
Niclosamide
NC
Niclosamide
e
Human CSF-1 promoter
Potential SOX2 binding site
-2000 -1800 -1400 -1200 -1000 -800 -200 +1
Primer1 -916/-906 GATCAATGGTA
Primer2 -782/-772 CAACAATGACA
Primer3 -763/-753 GTACAATAATG
Primer4 -236/-226 CCACAATGGTG
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CSF-1 promoter
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CSF-1(pg/ml)
sh-CHRNA5
SOX2

◂**Fig. 3** α5-nAChR regulates CSF-1 expression through pSTAT3/SOX2 in A549 and H1299 cells. **a** expression of a5-nAChR, pSTAT3, and SOX2 after sh-CHRNA5 treatment in A549 and H1299 cells. $*P<0.05$; $**P<0.01$. **b** Expression, of a5-nAChR, pSTAT3, and SOX2 after α5-nAChR OE in A549 and H1299 cells. $**P<0.01$. **c** Nicotine promoted the expression of a5-nAChR, pSTAT3, and SOX2 in A549 and H1299 cells. $**P<0.01$; $***P<0.001$. **d** STAT3 silencing downregulates the expression of STAT3 and SOX2 in A549 and H1299 cells. $*P<0.05$; $**P<0.01$. **e** SOX2-binding site in the human CSF-1 promoter. **f** SOX2 bound to the CSF-1 promoter in A549 cells. $***P<0.001$. Data are presented as the mean ± SD of three independent experiments, ns not signifcantly different. **g** The transcriptional activation of the CSF-1 promoter regions by SOX2 was detected by using a dual-luciferase assay in case of binding sites mutation. $n=3$; $*P<0.05$. **h** CSF-1 levels in cell culture supernatants treated with sh-CHRNA5 or SOX2 overexpression. $n=3$; $*P<0.05$; $**P<0.01$; $***P<0.001$

There are some limitations of the present study that could be addressed in our following study. The clinical sample size needs to be expanded to evaluate the application of α5-nAChR/SOX2/CSF-1 in clinical practice. Studies by multiple research centers can increase the diversity of the sample, thereby enhancing the generalizability of the research findings. Collecting more data by following the same group of participants over time can help verify the stability and long-term validity of the initial research findings. The clinical value should be further confirmed in a larger cohort including preclinical intervention studies.

In a summary, our results suggest that the overexpression of the α5-nAChR/pSTAT3/SOX2 axis enhances M2 macrophage polarization by upregulating CSF-1 secretion, and the secreted CSF-1 increases M2 macrophages within tumors and thereby promotes tumor progression and LUAD cell migration (Fig. 7). It suggested that the α5-nAChR/SOX2/CSF-1 axis plays a role in the migration of cancer cells during M2 macrophage polarization. To comprehensively understand the role of the α5-nAChR/SOX2/CSF-1 signaling pathway, future research could consider the following aspects: (1) epigenetic regulation: explore the link between α5-nAChR genes and the epigenetic regulatory of SOX2 in LUAD; (2) role in the tumor microenvironment: examine how α5-nAChR/SOX2/CSF-1 signaling pathway

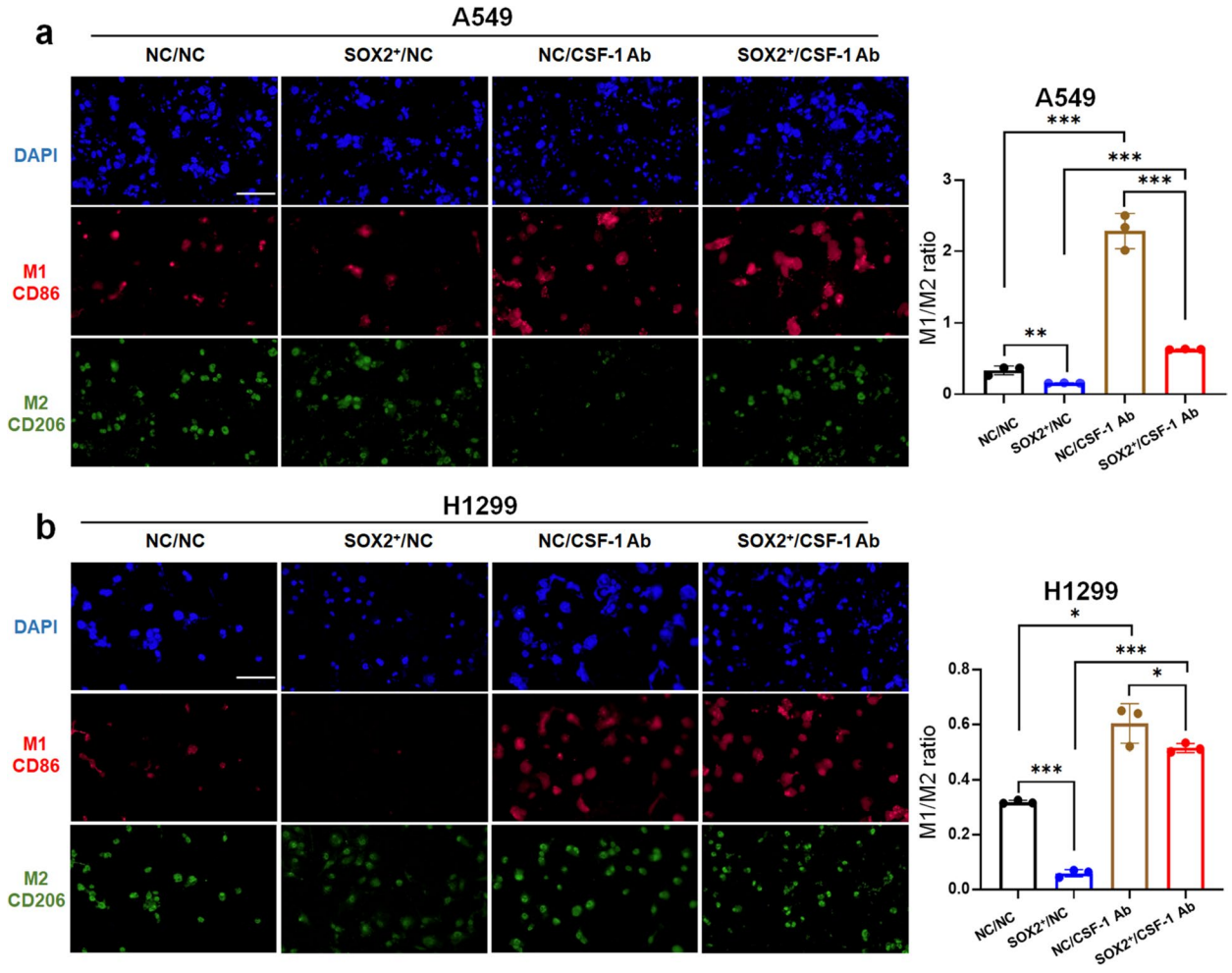

Fig. 4 SOX2/CSF-1 axis contributed to the differentiation of monocytes into M2 macrophages. **a**, **b** Polarization of M1 and M2 macrophages in the coculture system. M1/M2 ratio results were quantified. Scale bar = 100 μm; $n=3$; $*P<0.05$ $**P<0.01$; $***P<0.001$

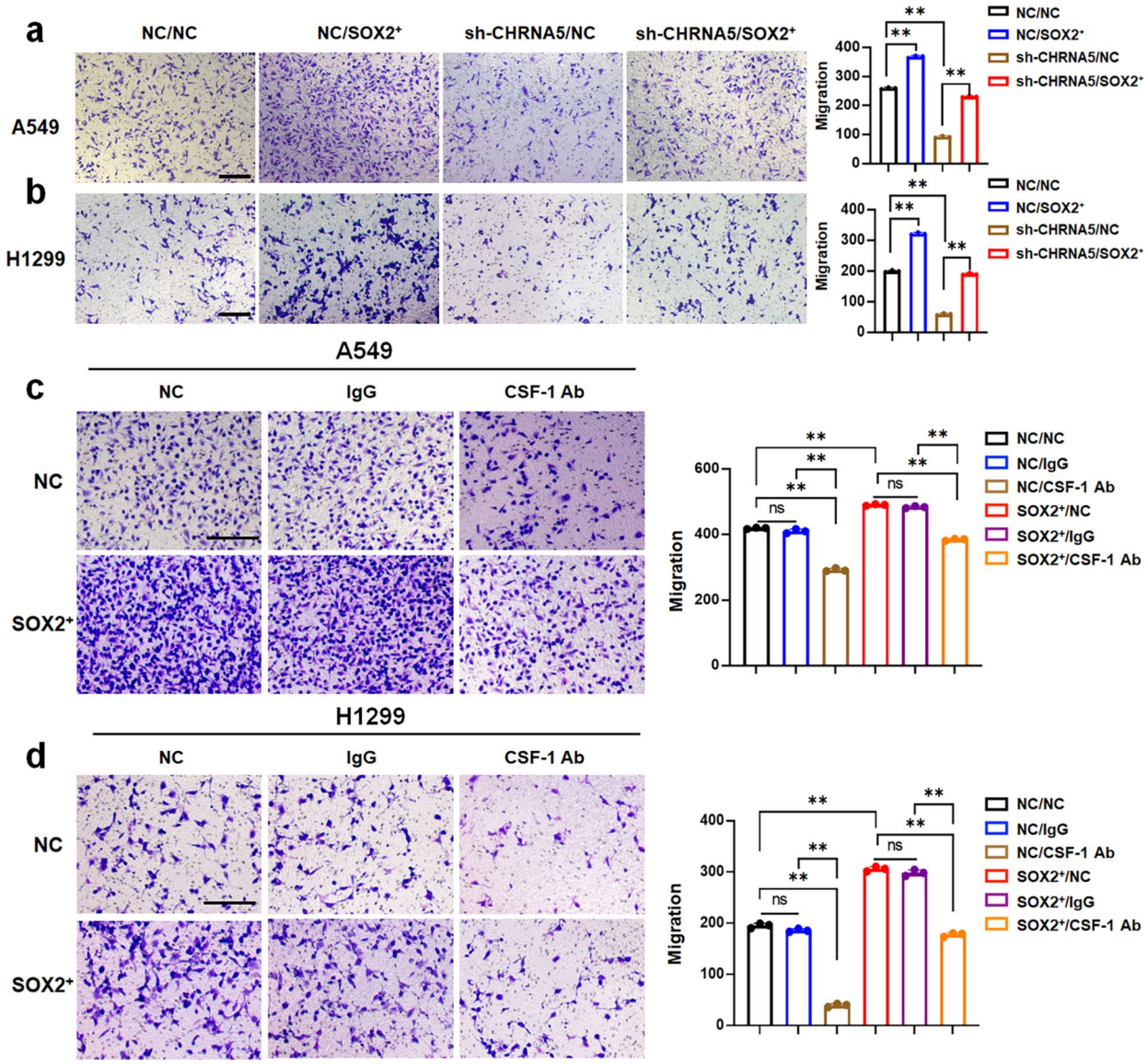

Fig. 5 The α5-nAChR/SOX2/CSF-1 signaling pathway involved in macrophage-mediated cancer cell migration. **a**, **b** Transwell assay showed that sh-CHRNA5 or SOX2 overexpression regulates macrophage-mediated migration of A549 and H1299 cells. Scale bar = 100 μm; $n = 3$; $^{**}P < 0.01$. **c**, **d** Transwell assays showed that SOX2 overexpression or CSF-1 Ab conditioned medium regulates the migration of A549 and H1299 cells. Scale bar = 100 μm; $n = 3$; $^{**}P < 0.01$

regulates the function of immune cells in the tumor microenvironment (such as macrophages); (3) validation with clinical samples: use clinical samples for validation studies to analyze the expression patterns and functional differences of the α5-nAChR/SOX2/CSF-1 signaling pathway in different LUAD patients, as well as the relationship between these differences and patient prognosis, treatment response, and immune evasion.

Our study provides valuable insights into the role of α5-nAChR/SOX2/CSF-1 in antitumor immune response. Targeting the α5-nAChR/SOX2/CSF-1 axis for lung cancer treatment and combination therapy may provide new avenues for the development of novel therapies to modulate immune responses and inhibit lung cancer growth. The expression of α5-nAChR is strongly correlated with smoking status, suggesting that smokers may respond better to therapeutic strategies targeting this receptor: development of small molecule inhibitors or antibodies targeting α5-nAChR or gene therapeutics to reduce SOX2 expression and, in addition, to enhance immune responses in the tumor microenvironment by modulating CSF-1 signaling. Further studies are needed to elucidate the underlying mechanisms by which α5-nAChR/SOX2/CSF-1 expression affects tumor biology and its therapeutic efficacy in targeted therapies for

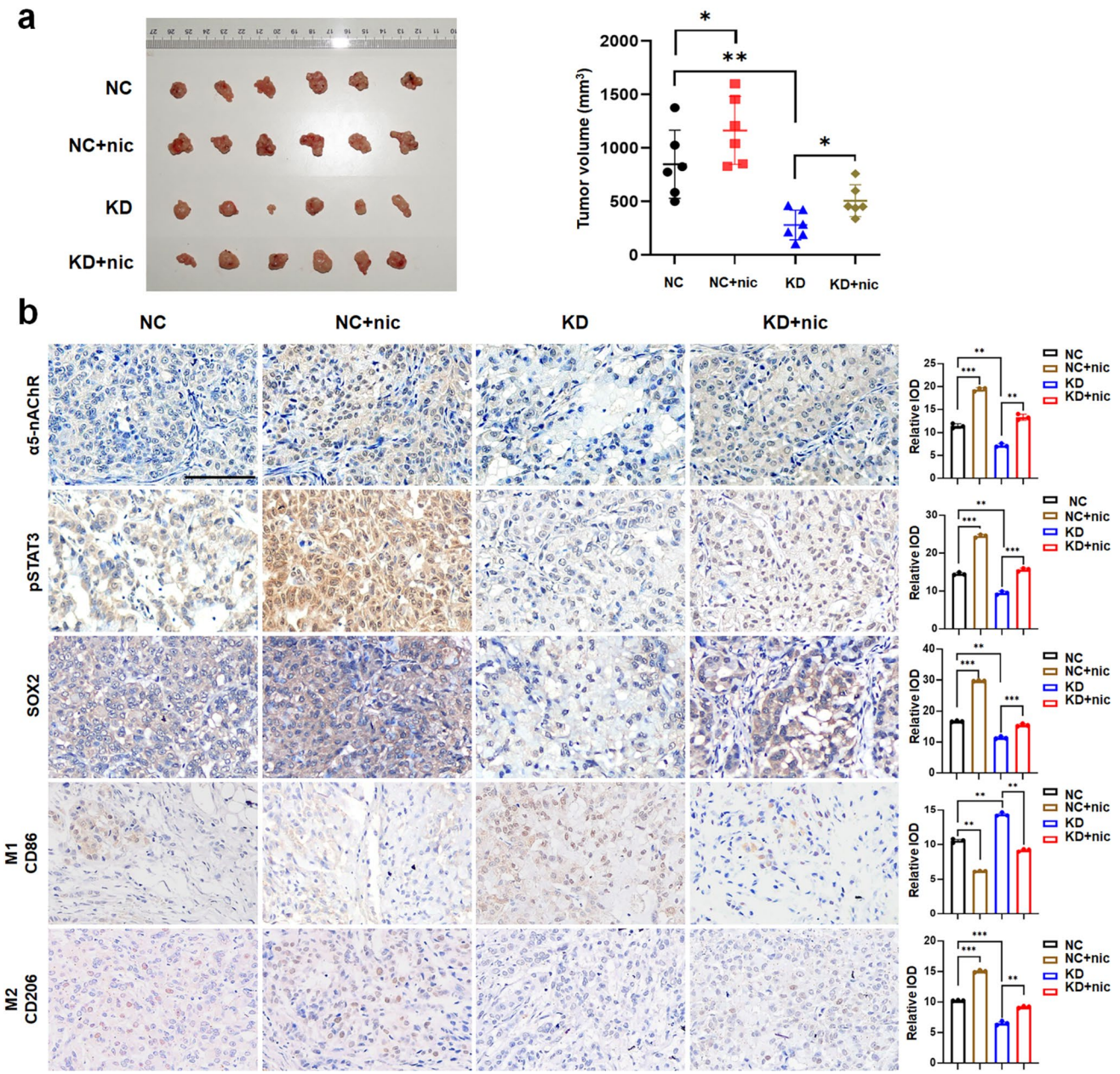

Fig. 6 α5-nAChR, pSTAT3, SOX2, CD86, and CD206 expression in LUAD tumor xenograft tissues. **a** The morphology of tumor xenografts from each mouse. $n=6$; $^{*}P<0.05$; $^{**}P<0.01$. **b** α5-nAChR, pSTAT3, SOX2, CD86 and CD206 expression in the NC, NC + nic, KD, and KD + nic groups. Scale bar = 50 μm; $n=3$; $^{**}P<0.01$; $^{***}P<0.001$

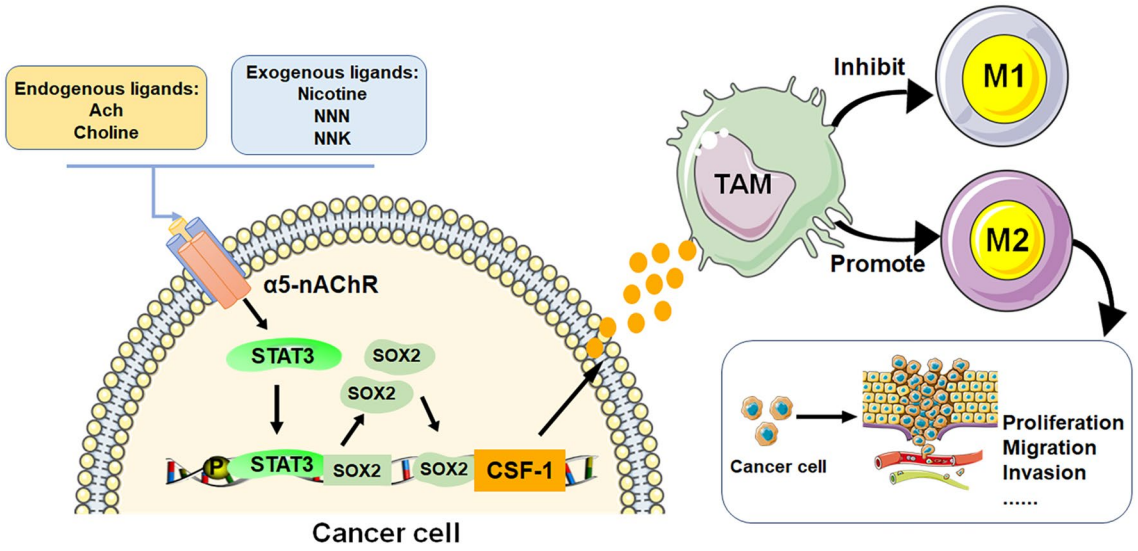

Fig. 7 The α5-nAChR and SOX2 signaling cascade is involved in lung cancer progression and immune escape. Nicotine and cell surface α5-nAChR bind and interact to activate the STAT3/SOX2 signaling pathway, which promotes macrophage M2 polarization by promoting CSF-1 secretion in tumor cells, further promoting the metastatic ability of tumor cells

lung cancer patients, and to explore its therapeutic potential in a broader range of cancers.

Author contributions Xiaoli Ma and Guiyu Kang contributed to conceptualization; Guiyu Kang, Hui Song, Qi Liu, and Pan Pan contributed to data curation; Lei Bo, Qiang Li, Jingtan Li, and Jingting Wang contributed to formal analysis; Xiaoli Ma and Haiji Sun contributed to funding acquisition; Pan Pan contributed to investigation; Guiyu Kang and Pan Pan contributed to methodology; Yanfei Jia contributed to resources; Qi Liu, Qiang Li, Jingting Wang contributed to software; Guiyu Kang supervised the study; Hui Song contributed to validation; Lei Bo contributed to visualization; Xiaoli Ma and Guiyu Kang contributed to writing—original draft; Xiaoli Ma contributed to writing—review & editing. All authors reviewed the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations Conflict of interest

The authors declare that there is no conflict of interests.

Ethics approval The study was approved by the Ethics committee of the Institutional Animal Care and Use Committee of Central Hospital Affiliated to Shandong First Medical University and conformed to the Declaration of Helsinki (approval no. JNCH2021-77). The study involving the usage of human tissues was performed in accordance with the Declaration of Helsinki and with the approval of the ethics committee of Shanghai Outdo Biotech and Central Hospital Affiliated to Shandong First Medical University (approval no. SHXC2021YF01 and R202303060078).

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