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ORIGINAL RESEARCH

Thymosin α1 suppresses migration and invasion of PD-L1 high-expressing non-small-cell lung cancer cells via inhibition of STAT3–MMP2 signaling

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Background: Thymosin α1 (Tα1) is one of the most commonly used immunomodulators for metastatic non-small-cell lung cancer (NSCLC) patients in many countries. Despite the identification of the direct suppression on cancer cell proliferation, little is known about its effect on metastasis and metastasis-related signaling such as matrix metalloproteinases (MMPs) and programmed cell death ligand 1 (PD-L1).

Materials and methods: NSCLC cells with distinguishing PD-L1 expression levels were treated with Tα1. siRNAs were used to knockdown PD-L1. Cell migration and invasion abilities were evaluated by wound-healing and transwell assays. The xenograft model by BALB/c nude mice was constructed to test the inhibitory effect of Tα1 on metastasis in vivo. The expression levels of metastasis-related signaling pathways and key molecules were assessed by Western blot (WB) and quantitative reverse transcriptase PCR (qRT-PCR).

Results: Tα1 significantly suppressed cell migration and invasion in PD-L1 high-expressing H1299, NL9980, and L9981 cells but not in PD-L1 low-expressing A549 or SPC-A-1 cells. This difference was demonstrated by mouse model in vivo as well. Knocking down of PD-L1 significantly impaired the inhibition of cell migration and invasion caused by Tα1 treating in PD-L1 high-expressing cells. Besides, Tα1 inhibited the activation and translocation of STAT3 and the expression of MMP2 in PD-L1 high-expressing NSCLC cells. Moreover, the treatment of STAT3 activator colivelin could partly reverse the Tα1-induced MMP2 suppression and the migration phenotype.

Conclusion: Tα1 significantly suppresses migration and invasion in PD-L1 high-expressing NSCLC cells compared with PD-L1 low-expressing NSCLC cells in vitro and in vivo, through the downregulation of STAT3–MMP2 signaling. These different responses to Tα1, together with the depiction of Tα1-induced signaling changes, suggest a potential benefit of Tα1 for PD-L1-positive NSCLC patients, enlightening the combination of Tα1 with target therapy or immune checkpoint inhibitors.

Keywords: matrix metalloproteinase 2, non-small-cell lung cancer, programmed cell death ligand 1, STAT3, thymosin α1

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Introduction

Lung cancer is the leading cause of both new cancer diagnoses and cancer-related deaths worldwide.[1] Non-small-cell lung cancer (NSCLC) comprises 85%–90% of all lung cancers.[2] Despite the increasing therapeutic means such as targeted drugs and stereotactic body radiation therapy, the 5-year overall survival rate of NSCLC remains less than 15%.[3] This is largely because of the strong invasive and metastatic potentials of NSCLC. Recent data show that approximately 90% of NSCLC patients die due

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to distant metastases rather than primary tumor;[4,5] hence, there is an urgent need to restrain tumor metastasis in NSCLC.

Thymosin α1 (Tα1), found in highest concentrations in the thymus, is a 28-amino-acid peptide first described and characterized by Goldstein et al.[6,7] In 1966, Goldstein et al purified a calf thymus extract termed thymosin fraction V (TFV), which can be divided into α-thymosin, β-thymosin, and γ-thymosin according to their isoelectric points.[7,8] Tα1 is the most abundant α-thymosin in TFV and has an immunomodulatory activity 10–1,000 times as potent as TFV.[9] The immunomodulatory effects of Tα1 include enhancing dendritic cell and antibody responses, modulating cytokine and chemokine production, and blocking steroid-induced apoptosis of thymocytes, but it is centered primarily on the augmentation of T-lymphocyte function.[10,11] T lymphocytes play a pivotal role in tumor immune surveillance and pathogen clearance; hence, Tα1 is now in clinical trials worldwide for the treatment of several types of malignancies including NSCLC.[10,12] In China, Tα1 has been approved and the oncologists often use it as a complementary treatment during chemo- or radiotherapy, the improved quality of life after which has been observed.[13]

Interestingly, besides immunomodulatory effect, Tα1 has shown to act directly on the tumor cells. Moody et al[14] first described the antigrowth effect of Tα1 on NCI-H1299 NSCLC cell line in vitro and in vivo. Subsequent studies further revealed the antiproliferative and apoptosis-inducing effects on lung cancer, breast cancer, and leukemia cells.[9,15,16] However, little is known about the effect on metastasis. Furthermore, when it comes to the impact of Tα1 on the pathway of tumor cells, all the aforementioned studies only outlined the relationship with the PI3K/AKT pathway.[15]

In the present study, we investigated the migration and invasion of NSCLC cells treated with Tα1, as well as the related signaling pathway changes in NSCLC cells. First, we observed the distinguishing reductions in migration and invasion in the NSCLC cells with different PD-L1 expression levels: PD-L1 high-expressing NSCLC cells (H1299, NL9980, and L9981) showed a significant suppression of metastasis after Tα1 treatment, while PD-L1 low-expressing cells (A549 and SPC-A-1) did not. The phenotypic changes after knocking down of PD-L1 verified the relationship between different responses and PD-L1 levels in NSCLC. Additionally, STAT3–matrix metalloproteinase 2 (MMP2) pathway was found to be associated with the metastatic changes in PD-L1 high-expressing NSCLC cells, which was further confirmed by the treatment of STAT3 activator. Our study depicted a more comprehensive molecular diagram of NSCLC cells treated with Tα1, enlightening the combination of Tα1 and targeted therapy in the future trials.

Materials and methods

Agents and antibodies

Tα1 was generously provided by Dr Qinghua Zhou (West China Hospital, Sichuan University, Chengdu, China). Tα1 powder was dissolved in the standard solvent mannitol and used for cell treatment immediately. Colivelin (# sc-361153) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), and LY294002 (# S1105) was purchased from Selleck Chemicals (Houston, TX, USA). Colivelin and LY294002 were dissolved in dimethyl sulfoxide (DMSO), stored in 1.5 mL sterile plastic tubes at –20°C, thawed, and used on the day of the experiment.

The antibodies used in this study include PD-L1 (# 13684; Cell Signaling Technology, Boston, MA, USA), phosphorylated-STAT3 (Tyr705) (p-STAT3; # 9145; Cell Signaling Technology), STAT3 (# 9139; Cell Signaling Technology), MMP2 (# 4022; Cell Signaling Technology), MMP9 (# 2270; Cell Signaling Technology), β-actin (AF0003; Beyotime Institute of Biotechnology, Shanghai, China), and PCNA (# 13110; Cell Signaling Technology).

Cell cultures

Human bronchial epithelial Beas-2B cells and human NSCLC cells (95C, 95D, A549, NCI-H1299, SPC-A-1, NCI-H460, and YTMLC-90) were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China), while the sub-cell lines, high-metastatic L9981 and low-metastatic NL9980, were isolated and established from a human lung large cell carcinoma cell line by Dr Qinghua Zhou.[17] Luciferase-expressing L9981 (L9981-luc) and A549 (A549-luc) cells were established by Tianjin Lung Cancer Institute (Tianjin, China). All the cells were cultured at 37°C in the Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a humidified atmosphere of 5% CO_2.

Wound-healing assay

The NSCLC cells were seeded into six-well plates and cultured to 90% confluence. A sterile 1 mL pipette tip (Corning Incorporated, Corning, NY, USA) was used to scratch straight lines in the cell layer to create wounds. Then, the cells were washed with PBS twice and cultured in the RPMI-1640 medium with Tα1 of different concentrations. Wounds were

observed at different time points within the scrape lines, and representative points were marked and photographed at five individual fields. The widths of wound gaps were measured using the ImageJ software.

Migration and invasion assays

A transwell apparatus with 8 μm filter inserts (# 3422; Corning Incorporated) was used to measure cell migration ability. The apparatus with 50 μL of matrigel coated at the bottom of the upper chamber was used to measure invasion ability. Six hundred microliters of medium with 10% FBS was added into the lower chamber, and 5×10^4 cells after treatment were added into the upper chamber without FBS. After an incubation of 18 h (migration assay) or 24 h (invasion assay) at 37°C, the inner side of the chamber was wiped with a cotton swab to remove the cells and the outer side was fixed by isopropanol for 20 min and then stained by 0.05% crystal violet for 20 min. The stained cells of each chamber were photographed in five randomly selected fields and subjected to cell counting. The average cell number in the five fields was recorded, and each experiment was repeated at least three times.

Cell counting kit-8 (CCK-8) assay

A CCK-8 (CK04-3000T; Dojindo Laboratories Co., Ltd, Kumamoto, Japan) was used to assess cell viability. The cells (7,000 cells/well) were seeded in 96-well plates. After 24 h of incubation, the cells were further treated with Tα1 for 12, 24, 36, or 48 h. Cell viability was detected by adding 10 μL of CCK-8 dissolved in 90 μL of RPMI-1640 medium, and the absorbance at 450 nm was recorded using a multi-label counter after 2 h incubation.

Colony formation assay

Briefly, 500 cells were seeded in 12-well plates. After 24 h of incubation, the cells were treated with 45, 90, or 180 μM Tα1 for 12–16 days. The cells were fixed with 4% formaldehyde for 20 min at room temperature (RT), and the colonies were stained with 0.5% crystal violet and manually counted. Colonies with at least 100 cells were considered to represent viable cells.

In vivo assay

All animal experiments were approved by the ethics committee of Tianjin Medical University. Immunodeficient BALB/c mice (aged 5–7 weeks, female) were purchased from the Institute of Zoology (Chinese Academy of Sciences, Beijing, China) and housed with sterilized cages under 12 h light/dark cycle, 18°C–22°C, and 50%–60% relative humidity. Food and drinking water were provided ad libitum. After 1 week adaptation, each mouse was inoculated with 0.1 mL of tumor cell suspension (9×10^6 A549-luc or 1×10^7 L9981-luc) at the right posterior flank and in vivo imaged using an IVIS200 (Xenogen; Caliper Life Sciences, Hopkinton, MA, USA). These data were identified at 0 weeks. When tumors became visible (50–80 mm^3), the mice were subcutaneously injected with Tα1 dissolved in mannitol (20 μg/g body weight, once a day), while the control mice were injected with mannitol only. Luciferase activity was measured every 7 days and analyzed by the Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

Protein extraction and Western blotting

Cells after treatment were washed with ice-cold PBS for three times. RIPA lysis buffer (P0013C; Beyotime Institute of Biotechnology) with 1% phenylmethanesulfonyl fluoride solution (PMSF) was used for cell lysis and total protein collection. For nuclear and cytoplasmic protein extraction, Nuclear and Cytoplasmic Protein Extraction Kit (P0027; Beyotime Institute of Biotechnology) was used and the protocol is as follows. First, cells in 12-well plates were scraped off and collected by centrifugation. Each cell pellet was dissolved in 100 μL cytoplasmic protein extraction agent A with 1% of PMSF by 5 min vortex and 15 min incubation on ice. Next, 5 μL of cytoplasmic protein extraction agent B was added, followed by another 5 min vortex and 1 min incubation on ice. Then, the sample was centrifuged at 16,000× *g* for 5 min at 4°C, and the supernatant containing cytoplasmic protein was frozen immediately for further analysis. The pellet was re-suspended in 25 μL of nuclear protein extraction agent with 1% of PMSF, followed by 30 s vortex and 30 min incubation on ice. Finally, the supernatant containing the nuclear protein was obtained after another centrifugation at 16,000× *g* for 10 min at 4°C. Protein lysates were boiled at 100°C for 10 min, separated with 10% SDS-PAGE, and then transferred into the nitrocellulose (NC) membrane (EMD Millipore, Billerica, MA, USA). Membrane was blocked by skim milk dissolved in 5% Tris-buffered saline with Tween-20 buffer (TBST) at RT for 1 h. After the incubation with primary antibodies overnight at 4°C, the blots were then incubated with corresponding goat antirabbit (A0208, 1:2,500; Beyotime Institute of Biotechnology) or goat antimouse (A0216, 1:2,500; Beyotime Institute of Biotechnology) IgG horseradish peroxidase-conjugated secondary antibody for 1 h at RT. After that, the membrane was washed with TBST five times for 7 min at each time. The signals were visualized with electrochemiluminescence substrates (EMD Millipore).

Table 1 Primer sequences for real-time PCR analysis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
PD-L1	GGTGCCGACTACAAGCGAAT	TAGCCCTCAGCCTGACATGTC
MMP2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC
MMP9	TTGACAGCGACAAGAAGTGG	GCCATTCACGTCGTCCTTAT
GAPDH	CCATCTTCCAGGAGCGAGATC	GCCTTCTCCATGGTGGTGAA

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; PD-L1, programmed cell death ligand 1.

Quantitative reverse transcriptase PCR (qRT-PCR)

The total RNA of NSCLC cells was extracted using the TRIzol reagent (Thermo Fisher Scientific) and reversely transcribed to single-strand complementary DNA with the Reverse Transcriptase M-MLV (RNase H-) (Takara, Shiga, Japan). Primers of PD-L1, MMP2, MMP9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by the Beijing Genomics Institute (Beijing, China). Reactions were amplified in a 7900 Fast RT-PCR System (Thermo Fisher Scientific) and carried out under the following conditions: initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 10 s, and polymerization at 72°C for 30 s. The cycle threshold (CT) values of the target genes were identified, and mRNA levels were calculated as a ratio of normalized GAPDH level according to the $2^{-\Delta\Delta CT}$ method. Gene-specific primers are listed in Table 1.

siRNA interference and transfections

PD-L1-siRNA was purchased from GenePharma (Shanghai, China). The targeting sequences are shown in Table 2. H1299, NL9980, L9981, A549, and SPC-A-1 cells were cultured overnight to a confluence of 50%. Transfections without serum were conducted with 80 nM PD-L1 siRNAs or negative control siRNAs using the Lipofectamine 2000 reagent (Thermo Fisher Scientific) for 6 h. After culturing with serum for additional 42 h, the cells were trypsinized and divided evenly for Western blot (WB), qRT-PCR, and Tα1 treatment experiments.

Statistical analysis

Data in bar graphs are expressed as the mean ± SD from at least three independent experiments. Statistical analysis was performed using the Student's unpaired *t*-test or one-way ANOVA. All statistical analyses were performed with the GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). A *P*-value of ≤ 0.05 was considered statistically significant.

Results

Tα1 suppresses migration and invasion in PD-L1 high-expressing NSCLC cells but not in PD-L1 low-expressing NSCLC cells

First, the study revealed the distinguishing levels of PD-L1 transcription and expression in different NSCLC cell lines. Compared with the normal bronchial epithelial cells (Beas-2B), H1299, NL9980, and L9981 presented high PD-L1 levels, while A549 and SPC-A-1 presented a lower expression (Figure 1). These five NSCLC cell lines were selected for the following experiments. In wound-healing assay, the migration abilities of H1299, NL9980, and L9981 cells were attenuated by Tα1 in both time- and dose-dependent manners (Figure 2A, $P<0.001$). Transwell assay further showed the significant suppression of migration and invasion after 48 h treatment with 90 and 180 μM Tα1 in H1299, NL9980, and L9981 (Figures 2B and 3A–C, $P<0.001$). However, as for PD-L1 low-expressing cells, A549 and SPC-A-1, the migration and invasion reductions were not significant in the quantitative assessment of transwell assay (Figures 2B and 3D and E, $P>0.05$). Additionally, though CCK-8 and colony formation assays revealed a significant suppression of proliferation by Tα1 in both PD-L1 high-expressing NSCLC cells (H1299, NL9980, and L9981) and PD-L1 low-expressing NSCLC cell (A549) as reported,[18] there was still a tendency that the suppression was stronger in PD-L1 high-expressing cells (Figure S1). In summary, NSCLC cells with high

Table 2 Target sequences of PD-L1 siRNA

Gene	Sense target sequence (5′–3′)	Anti-sense target sequence (5′–3′)
si-PD-L1-1	GGAGAAUGAUGGAUGUGAATT	UUCACAUCCAUCAUUCUCCTT
si-PD-L1-2	GGCACAUCCUCCAAAUGAATT	UUCAUUUGGAGGAUGUGCCTT
NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Abbreviations: NC, negative control; PD-L1, programmed cell death ligand 1.

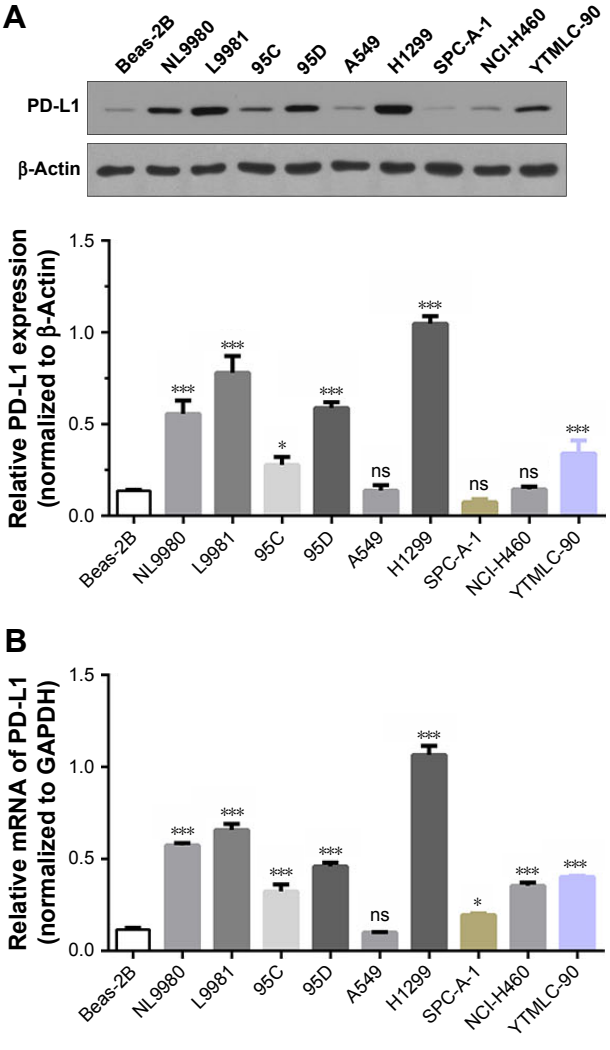

Figure 1 The different protein expression (**A**) and mRNA levels (**B**) of PD-L1 in human bronchial epithelial cells and a series of NSCLC cell lines.
Note: After quantitative evaluation of Western blotting and RT-PCR assays, PD-L1 high-expressing NSCLC cell lines, namely H1299, L9981, and NL9980, and PD-L1 low-expressing NSCLC cell lines, namely A549 and SPC-A-1, were selected for the subsequent experiments. *$P<0.05$ and ***$P<0.001$ vs control group.
Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NSCLC, non-small-cell lung cancer; PD-L1, programmed cell death ligand 1; RT-PCR, reverse transcriptase PCR; ns, not statistically significant.

PD-L1 expression have a distinct response of migration and invasion to Tα1.

Tα1 attenuates metastases of PD-L1 high-expressing NSCLC but not low-expressing NSCLC in nude mice

PD-L1 high-expressing cell L9981-luc was inoculated at the right posterior flank of eight nude mice. Four mice received Tα1 treatment, while the other four mice were administrated with the solvent. The same treatment was performed in the other two groups of nude mice inoculated with PD-L1 low-expressing cell A549-luc. Among those inoculated with L9981-luc, significantly fewer metastatic lesions appeared in the Tα1 treatment group compared with the control group. Inconsistently, mice inoculated with A549-luc displayed similar amount of metastatic lesions between control and Tα1 treatment groups and there was no significant difference in the fluorescence intensity between the metastatic lesions of the two groups as well (Figure 3F).

Knocking down of PD-L1 impairs the inhibitory effects of cell migration and invasion caused by Tα1 treating in PD-L1 high-expressing NSCLC cells

To verify the relationship between different responses and PD-L1 levels, PD-L1 was knocked down by siRNAs in H1299, NL9980, and L9981 cells (Figure 4A–C). PD-L1-silenced cells with negative control cells were treated with or without 180 μM Tα1 for 48 h. Similar to A549 and SPC-A-1 cells, the migration and invasion suppression of PD-L1-silenced cells could not be observed (Figure 4D–F, $P>0.05$), while the negative control cells remained a significant suppression (Figure 4D–F, $P<0.001$).

Tα1 inhibits the activation of STAT3 and suppresses the expression of MMP2 in PD-L1 high-expressing NSCLC cells

To explore the mechanism whereby Tα1 suppressed the migration and invasion in PD-L1 high-expressing cells, H1299, NL9980, and L9981 cells were treated with 180 μM Tα1 for 48 h. Western blotting and qRT-PCR showed that the mRNA and protein levels of MMP2 were decreased significantly (Figure 5A and B), along with the inhibited activation (Figure 5A, C, and D) and the reduced translocation (Figure 5E) of STAT3. Subsequently, STAT3 activator colivelin was applied combining with or without Tα1. Colivelin-induced activation of STAT3 was accompanied by the elevated MMP2 expression (Figure 5F). Moreover, the MMP2 suppression caused by Tα1 exposure could partly be reversed by colivelin (Figure 5F), and similar results were observed in the phenotype change in migration (Figure 5G). Overall, in PD-L1 high-expressing NSCLC cells, the STAT3–MMP2 pathway is the target, whereby Tα1 suppresses migration and invasion.

PD-L1 low-expressing NSCLC cells present a significantly lower expression of MMP2, which could not be suppressed by Tα1

To investigate why Tα1 could not suppress the migration and invasion of PD-L1 low-expressing NSCLC cells significantly,

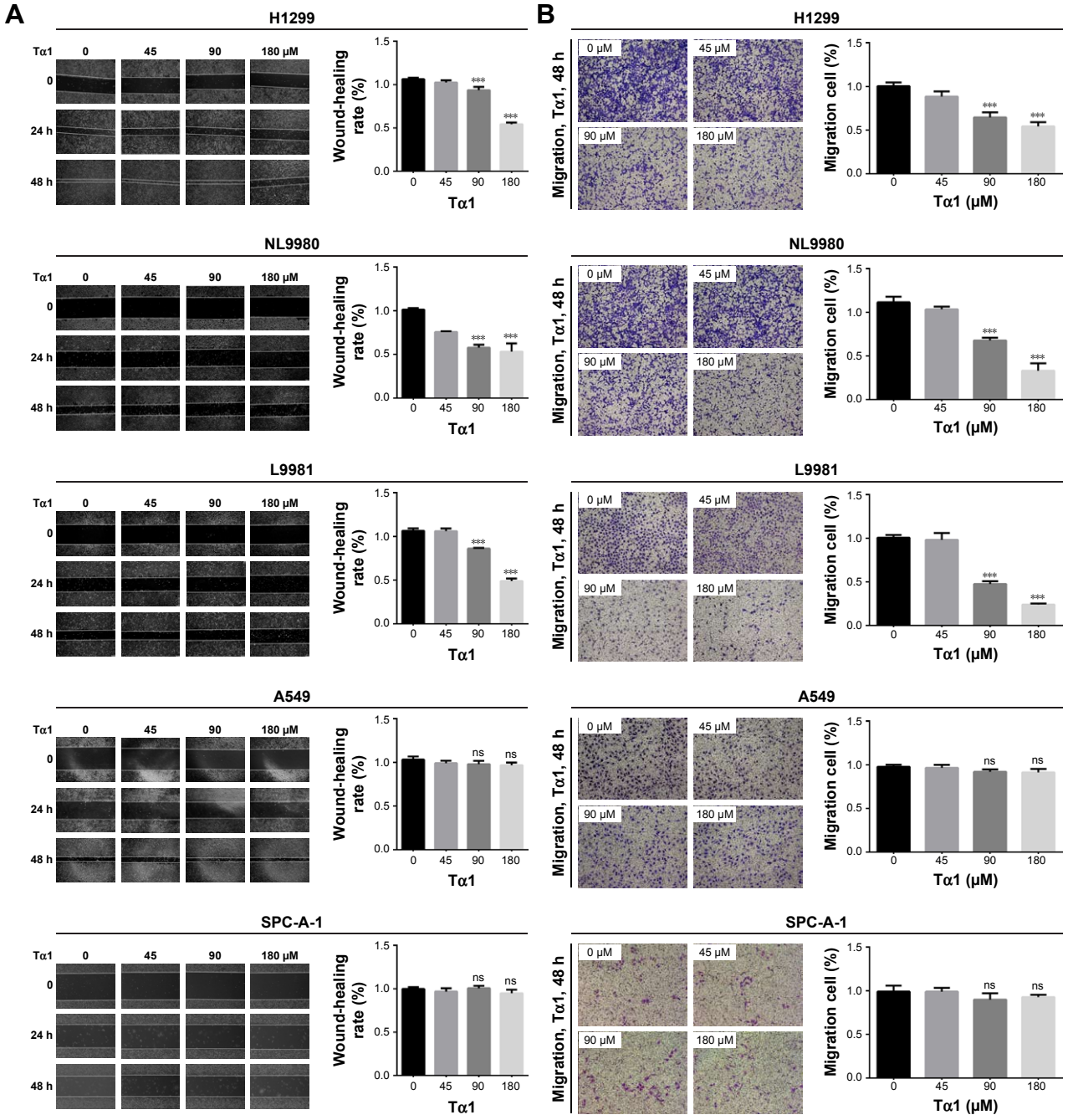

Figure 2 Tα1 suppressed the migration of PD-L1 high-expressing NSCLC cells but not PD-L1 low-expressing NSCLC cells.

Notes: (**A**) For wound-healing assays of H1299, NL9980, and L9981, the wound widths after 48 h were significantly wider in 90 and 180 μM Tα1-treated groups than in control group, but this phenomenon disappeared in PD-L1 low-expressing NSCLC cell lines A549 and SPC-A-1. (**B**) For transwell assay, the transmembrane capacities of H1299, NL9980, and L9981 were significantly suppressed by 90 and 180 μM Tα1 treatment for 48 h, but A549 and SPC-A-1 presented insignificant response to Tα1. ***$P<0.001$ vs control group. Magnification: 200×.

Abbreviations: NSCLC, non-small-cell lung cancer; PD-L1, programmed cell death ligand 1; Tα1, thymosin α1; ns, not statistically significant.

A549 and SPC-A-1 were exposed to 180 μM Tα1 for 48 h. However, the mRNA and protein levels of MMP2 (Figure 5A and B), as well as the activation of STAT3 (Figure 5A), were not inhibited by Tα1. Besides, the expression of STAT3, MMP2, and PD-L1 was assessed in H1299, L9981, A549, and SPC-A-1 cells with or without knocking down of PD-L1. A significantly lower expression of p-STAT3 and MMP2 was positively correlated with the lower PD-L1 expression in A549 and SPC-A-1 (Figure 5H). Moreover, knocking down of PD-L1 led to the decreased protein levels of p-STAT3 and MMP2 (Figure 5H). Similar correlation was demonstrated in mRNA levels between MMP2 and PD-L1 (Figure 5I).

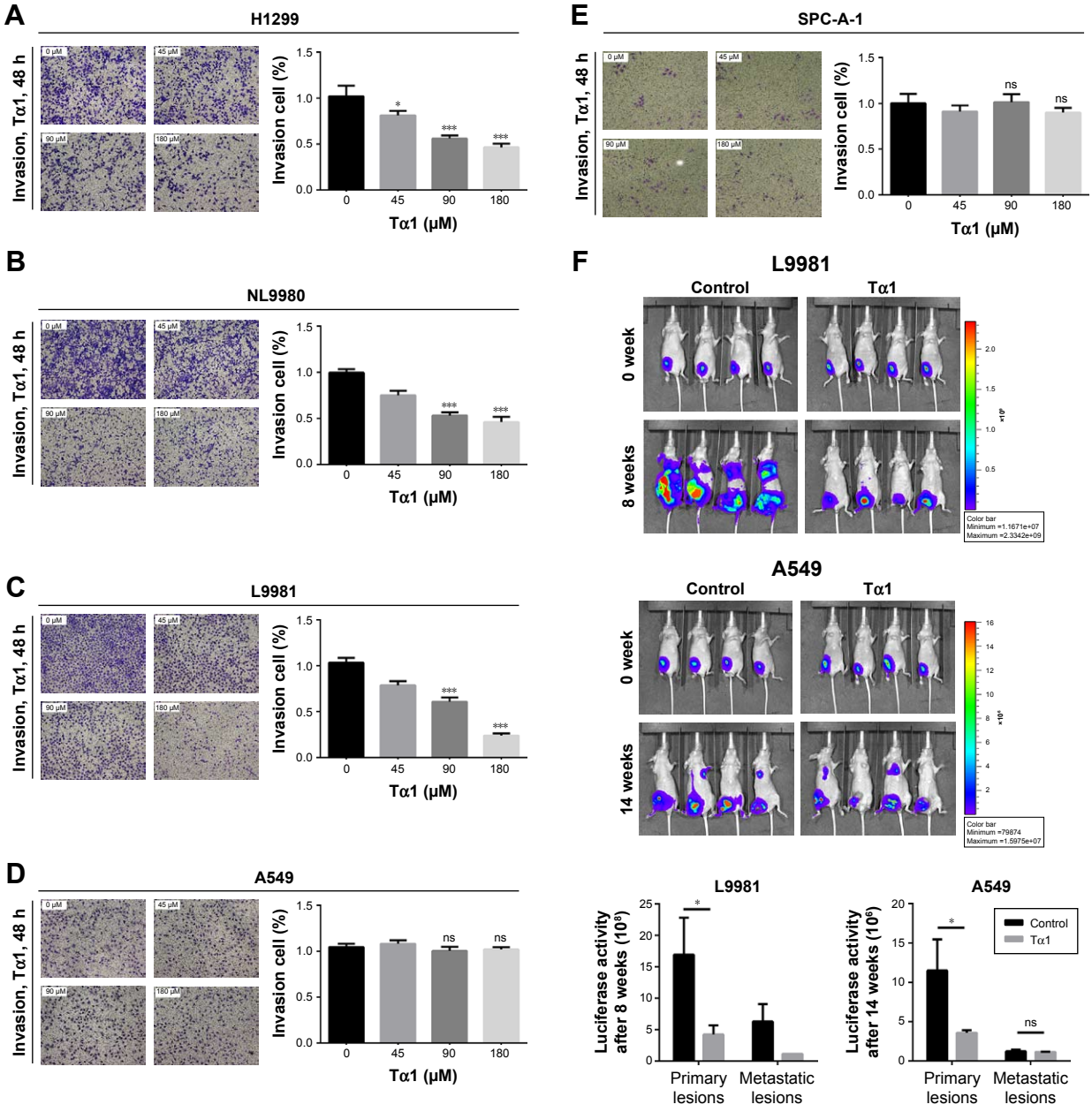

Figure 3 Tα1 suppressed the invasion as well as in vivo metastasis of PD-L1 high-expressing NSCLC cells but not PD-L1 low-expressing NSCLC cells.

Notes: For transwell assay, the transmembrane invasion abilities of H1299 (**A**), NL9980 (**B**), and L9981 (**C**) were significantly inhibited after 90 and 180 μM Tα1 treatment for 48 h, while the invasion abilities of A549 (**D**) and SPC-A-1 (**E**) were not significantly influenced by Tα1. (**F**) The nude mice model by L9981-luc and A549-luc cells were constructed, followed by the administration of Tα1 (20 μg/g body weight, once a day) or the solvent mannitol. Among mice inoculated with L9981-luc, significantly fewer metastatic lesions appeared in the Tα1 treatment group compared with those in the control group after indicated weeks, while mice inoculated with A549-luc displayed same number of metastatic lesions with similar fluorescence intensity between control and Tα1 treatment groups. *$P<0.05$ and ***$P<0.001$ vs control group. Magnification: 200×. A549-luc, luciferase-expressing A549; L9981-luc, luciferase-expressing L9981.

Abbreviations: NSCLC, non-small-cell lung cancer; PD-L1, programmed cell death ligand 1; Tα1, thymosin α1; ns, not statistically significant.

LY294002 has been reported to downregulate the PD-L1 expression in various NSCLC cells,[19,20] and the PD-L1 protein in L9981 and A549 could be downregulated by LY294002 effectively, which was also accompanied by the decreased protein level of MMP2 (Figure 5J). Overall, both intrinsic and induced low expression of PD-L1 are associated with the low expression of p-STAT3 and MMP2, indicating that Tα1 only effects on STAT3–MMP2 pathway with high levels of molecule expression.

Discussion

Tα1 is one of the most commonly used immunomodulators for metastatic NSCLC patients in many countries.[13,21] In this study, we investigated the direct effect of Tα1 on

cell metastasis of NSCLC in vitro and in vivo and explored the signaling changes associated. We found that Tα1 could significantly suppress the migration and invasion in PD-L1 high-expressing cells. On the contrary, it did not function as metastatic suppressor in either PD-L1 low-expressing cells or PD-L1-silenced NSCLC cells. Furthermore, this suppressive effect in PD-L1 high-expressing NSCLC cells was demonstrated to be associated with the downregulation of STAT3–MMP2 signaling. Schematic model proposed in Figure 6 summarized the regulating process.

PD-L1 is a coinhibitory molecule expressed mainly on macrophages, dendritic cells, activated T and B cells, and tumor cells.[22] The PD-L1 expressing on cancer cells, when binding to its receptor programmed cell death 1 (PD-1) expressing on activated T cells, can impair the anticancer function of T cells.[22,23] High expression of PD-L1 in tumor tissues has been reported as a poor prognostic biomarker in NSCLC patients,[24–26] and quite a few evidence showed a positive association between PD-L1 expression and metastatic ability of NSCLC cells.[27–30] Taking epithelial–mesenchymal transition (EMT) for example, data in Figure S2 revealed a protein profile facilitating EMT in PD-L1 high-expressing NSCLC cell lines, while Asgarova et al[28] showed a reversible upregulation of PD-L1 during cytokine-driven EMT in

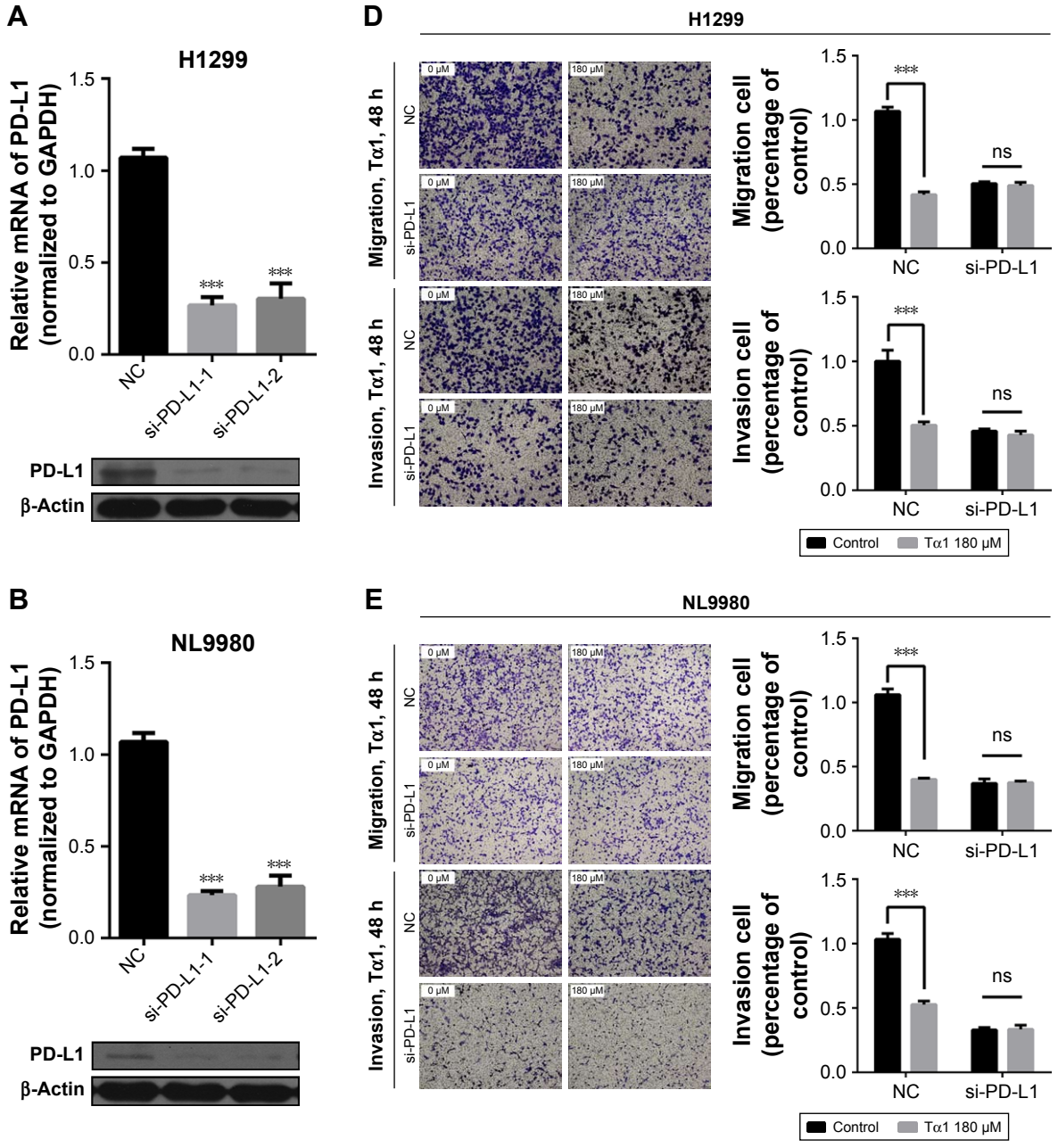

Figure 4 (*Continued*)

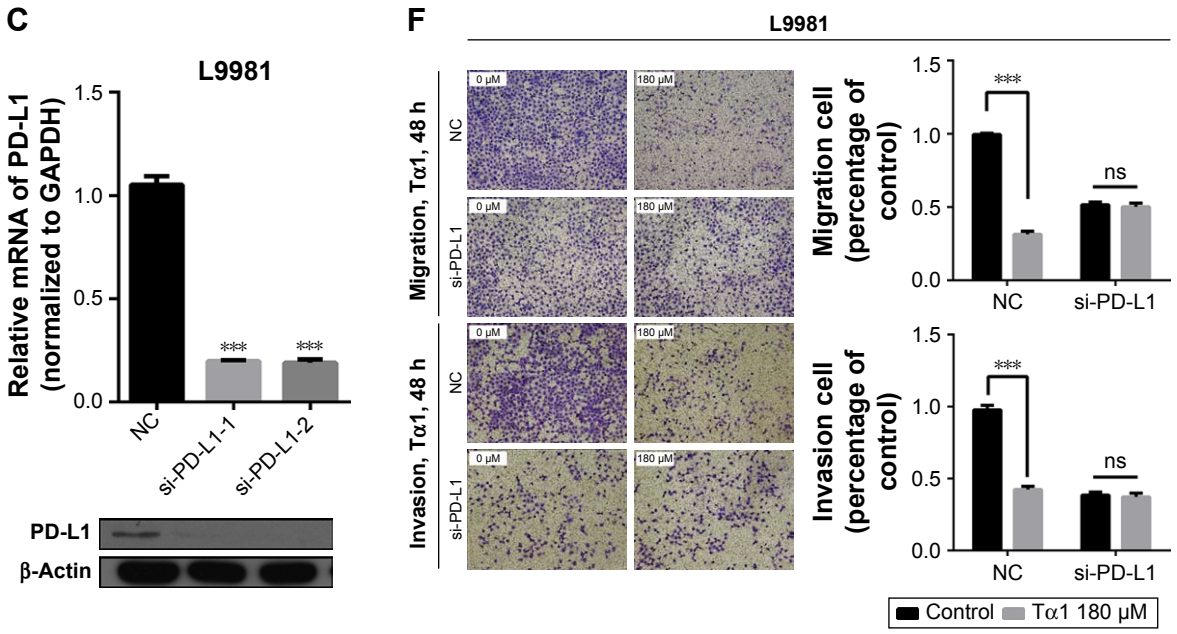

Figure 4 Knocking down of PD-L1 significantly impaired the inhibitory effects of cell migration and invasion caused by Tα1 treating in PD-L1 high-expressing NSCLC cells.
Notes: PD-L1 was effectively knocked down by siRNAs in PD-L1 high-expressing NSCLC cells H1299 (**A**), NL9980 (**B**), and L9981 (**C**). The PD-L1-silenced cells and negative control cells were treated with or without 180 μM Tα1 for 48 h (**D**–**F**). In transwell assay, the transmembrane migration and invasion abilities of H1299 (**D**), NL9980 (**E**), and L9981 (**F**) cells could not be significantly inhibited by Tα1 after knocking down of PD-L1, while the negative control cells remained a significant response to Tα1. ***$P<0.001$ vs control group. Magnification: 200×.
Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control; NSCLC, non-small-cell lung cancer; PD-L1, programmed cell death ligand 1; Tα1, thymosin α1; ns, not statistically significant.

NSCLC cells. In the present study, the lower expression of PD-L1 not only meant a significantly weak inhibitory effect of Tα1 on metastatic phenotypes and MMP2 expression but also signified a consistent lower protein expression on the STAT3–MMP2 pathway. These results suggest the association between PD-L1 and the metastasis-associated molecule MMP2 in NSCLC cells. Indeed, recent study showed that PD-L1 blockade decreased both the mRNA and protein levels of MMP2 in tumor tissues,[31] which is consistent with our results. In contrast, the positive correlation between PD-L1 and MMP2 may also explain, in turn, why Tα1 could not suppress the MMP2 expression and the metastatic

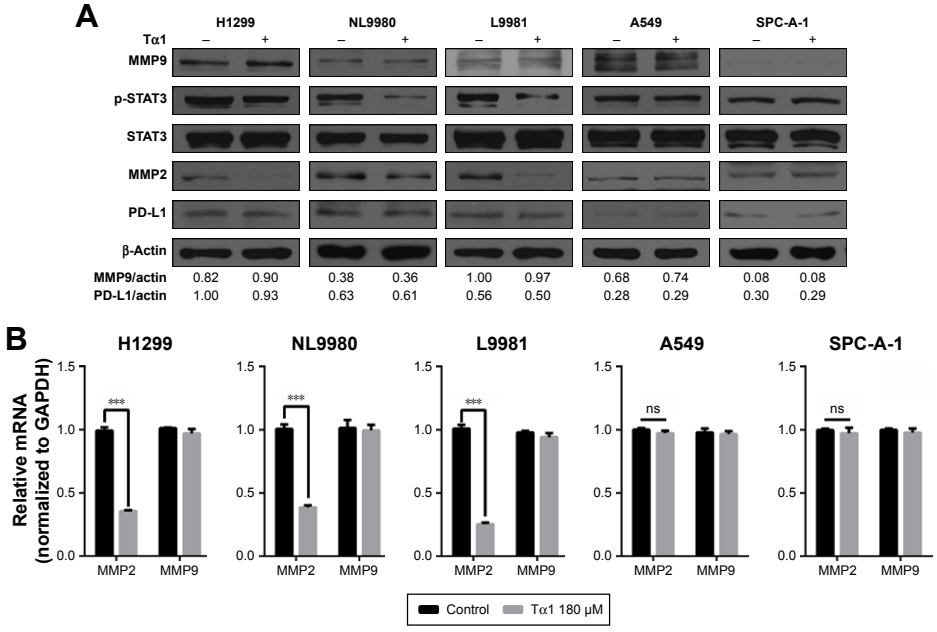

Figure 5 (*Continued*)

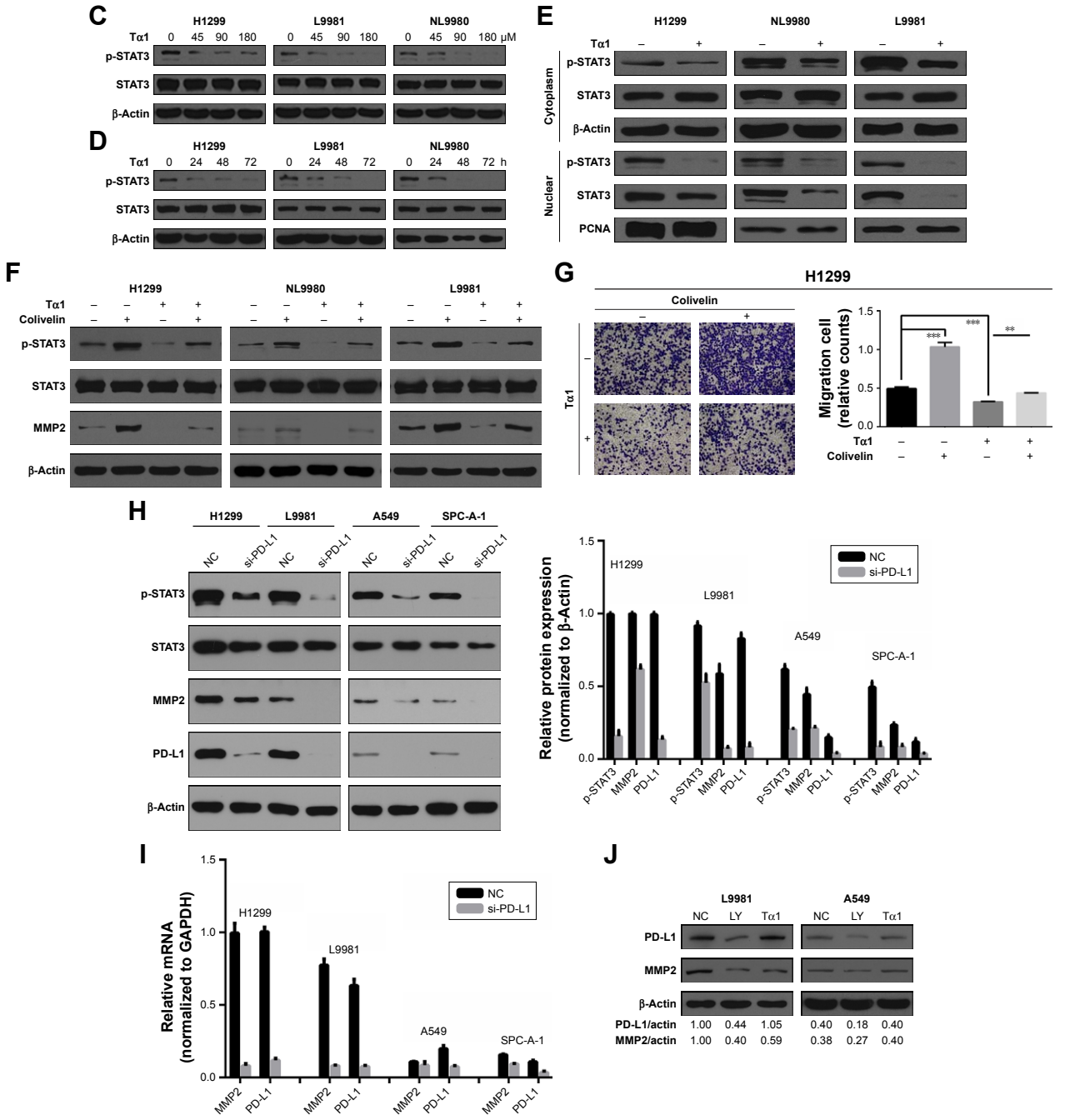

Figure 5 The suppression of migration and invasion by Tα1 in PD-L1 high-expressing NSCLC cells was associated with the inhibition of STAT3–MMP2 signaling.

Notes: (**A**) After treatment with 180 μM Tα1 for 48 h, the expression of p-STAT3 and MMP2 was decreased in H1299, NL9980, and L9981 cells but not in A549 or SPC-A-1 cells. (**B**) The mRNA levels of MMP2 in H1299, NL9980, and L9981 cells but not in A549 or SPC-A-1 cells were downregulated after treatment with 180 μM Tα1 for 48 h. (**C**) H1299, NL9980, and L9981 cells were treated with 45, 90, and 180 μM Tα1, respectively, for 48 h, and the expression of p-STAT3 was decreased in dose-dependent manners. (**D**) H1299, NL9980, and L9981 cells were treated with 180 μM Tα1 for 24, 48, and 72 h, and the expression of p-STAT3 was decreased in time-dependent manners. (**E**) After treatment with 180 μM Tα1 for 48 h, the expression of p-STAT3 and total-STAT3 in cytoplasm and nucleus of H1299, NL9980, and L9981 was assessed by Western blot. The expression of p-STAT3 and total-STAT3 in nucleus was downregulated markedly, and the translocation of STAT3 in H1299, NL9980, and L9981 was inhibited by Tα1. (**F**) H1299, NL9980, and L9981 cells were pretreated with 0.5 μM colivelin (STAT3 activator) or DMSO for 1 h, followed by treatment with or without 180 μM Tα1 for another 72 h. The Tα1-induced suppression of MMP2 and p-STAT3 could be partly reversed by colivelin. (**G**) The Tα1-induced suppression of migration in H1299 cells could be partly reversed by colivelin as well. (**H**) The expression of STAT3, MMP2, and PD-L1 was assessed by Western blot in H1299, L9981, A549, and SPC-A-1 cells with or without knocking down of PD-L1. A significantly lower expression of p-STAT3 and MMP2 was positively correlated with the lower PD-L1 expression in A549 and SPC-A-1, and knocking down of PD-L1 resulted in decreased protein levels of p-STAT3 and MMP2 in H1299, L9981, A549, and SPC-A-1 cells. (**I**) Positive correlation between the mRNA of MMP2 and PD-L1 was observed in H1299, L9981, A549, and SPC-A-1 cells, and knocking down of PD-L1 led to decreased mRNA level of MMP2. (**J**) L9981 and A549 cells were treated with 10 μM LY (used as PD-L1 inhibitor) or 180 μM Tα1 for 48 h, and the expression of PD-L1 was downregulated by LY but not by Tα1, while the expression of MMP2 was decreased along with PD-L1 in the LY group. $^{**}P<0.01$ and $^{***}P<0.001$ vs control group. Magnification: 200×.

Abbreviations: DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LY, LY294002; MMP2, matrix metalloproteinase 2; NSCLC, non-small-cell lung cancer; NC, negative control; PD-L1, programmed cell death ligand 1; p-STAT3, phosphorylated-STAT3; Tα1, thymosin α1; ns, not statistically significant.

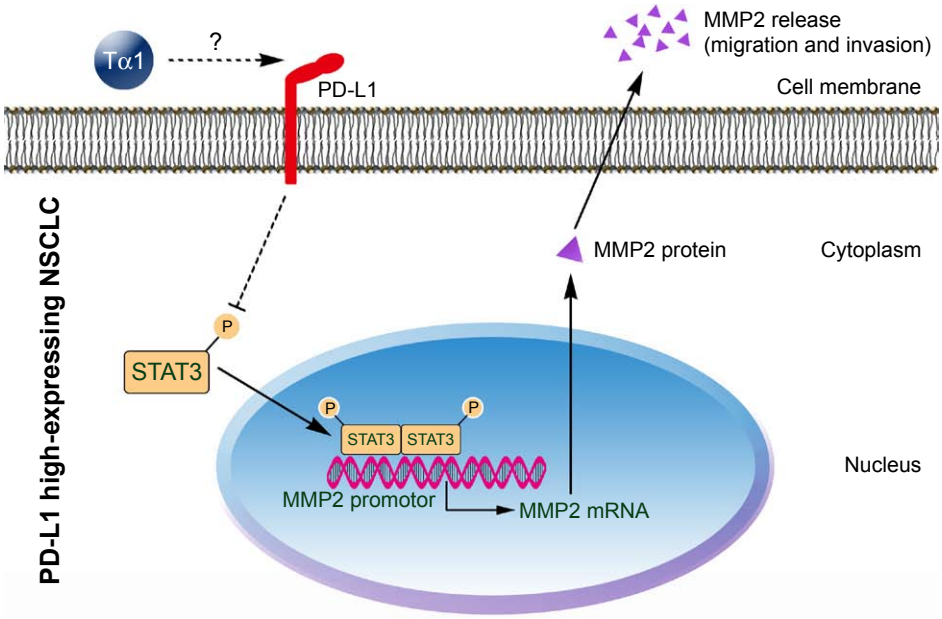

Figure 6 Schematic models of the proposed molecular mechanisms of Tα1-induced metastatic suppression in PD-L1 high-expressing NSCLC cells.
Notes: Tα1 suppresses the phosphorylation of STAT3, inhibiting the translocation of p-STAT3 and the transcription of MMP2, which causes the decrease in MMP2 protein and the suppression of metastatic ability in PD-L1 high-expressing NSCLC cells. Given that all these processes were only observed in PD-L1 high-expressing cells and the positive correlation between PD-L1 and STAT3–MMP2 signaling has been demonstrated, it is speculated that Tα1 may inhibit STAT3–MMP2 signaling through stimulating PD-L1.
Abbreviations: MMP2, matrix metalloproteinase 2; NSCLC, non-small-cell lung cancer; PD-L1, programmed cell death ligand 1; p-STAT3, phosphorylated-STAT3; Tα1, thymosin α1.

phenotypes in PD-L1 low-expressing cells. One most probable explanation is that the protein level of MMP2 in PD-L1 low-expressing cells is too low to be suppressed significantly by Tα1. Another possibility is that PD-L1 is exactly the intermediary molecule between Tα1 and STAT3–MMP2 pathway. Therefore, adequate amounts of PD-L1 in PD-L1 high-expressing cells can effectively respond to Tα1, though it was not decreased by Tα1.

Discontinuously expressed in normal tissues, MMPs are often highly expressed on malignant cells, degrading extracellular matrix protein and ultimately invading other tissues.[32,33] MMP2 is one of the key enzymes in the activation of other MMPs,[34] and the overexpression of which contributes to the metastasis and angiogenesis of lung cancer.[35,36] Several immunomodulators, including triticumoside, mushroom-derived polysaccharide, and timosaponin AIII, have been found to suppress MMP2 expression, thereby inhibiting the invasion and migration of NSCLC cells.[37–39] As for Tα1, little is known about its effect on MMPs. We investigated the antimetastasis effect of Tα1, observing a significant downregulation of MMP2, but not MMP9, on both mRNA and protein levels. To explore whether the simultaneously observed inactivation of STAT3 had influenced the transcription of MMP2, H1299, NL9980, and L9981 were co-treated with STAT3 activator colivelin and Tα1. The Tα1-induced suppression of both MMP2 protein and cell migration phenotype was partly reversed by STAT3 activator colivelin, suggesting an association between the inhibited STAT3 and the decreased MMP2. Several studies have showed that STAT3–MMPs' regulation plays important roles in the antimetastasis effect of many biological extracts such as silibinin and plumbagin.[40,41] Our results support these findings and further define the MMP2, rather than MMP9, as the target of STAT3 in the Tα1 treatment of NSCLC cells.

The involved molecules of this study enlighten the following three combination modes for advanced NSCLC: 1) Tα1 plus STAT3 inhibitors, a novel target therapy on the way from trial to clinic; 2) Tα1 plus pembrolizumab, an immune checkpoint (PD-1/PD-L1) inhibitor for the first- and subsequent-line treatment of NSCLC; and 3) Tα1 plus bisphosphonate, a classic inhibitor of MMPs for the clinical management of bone metastases. Among them, the combination of Tα1 with pembrolizumab is promising. A PD-L1 expression level of ≥50% is an indicator for first-line pembrolizumab therapy in advanced NSCLC patients,[42] while the antimetastasis role of Tα1 is specifically effective in PD-L1 high-expressing NSCLC cells. Furthermore, as a safe immunopotentiator, Tα1 may augment anticancer efficiency

of the immune checkpoint inhibitor in vivo. The combination of the two drugs is expected in animal experiments and future clinical trials.

Conclusion

Tα1 can significantly suppress cell migration and invasion in PD-L1 high-expressing cells rather than in low-expressing NSCLC cells in vitro and in vivo. This suppression was associated with the inhibition of STAT3–MMP2 signaling. These results not only depict a more comprehensive molecular diagram of NSCLC cells treated with Tα1 but also enlighten the combination of Tα1 and immune checkpoint inhibitors in the future trials.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

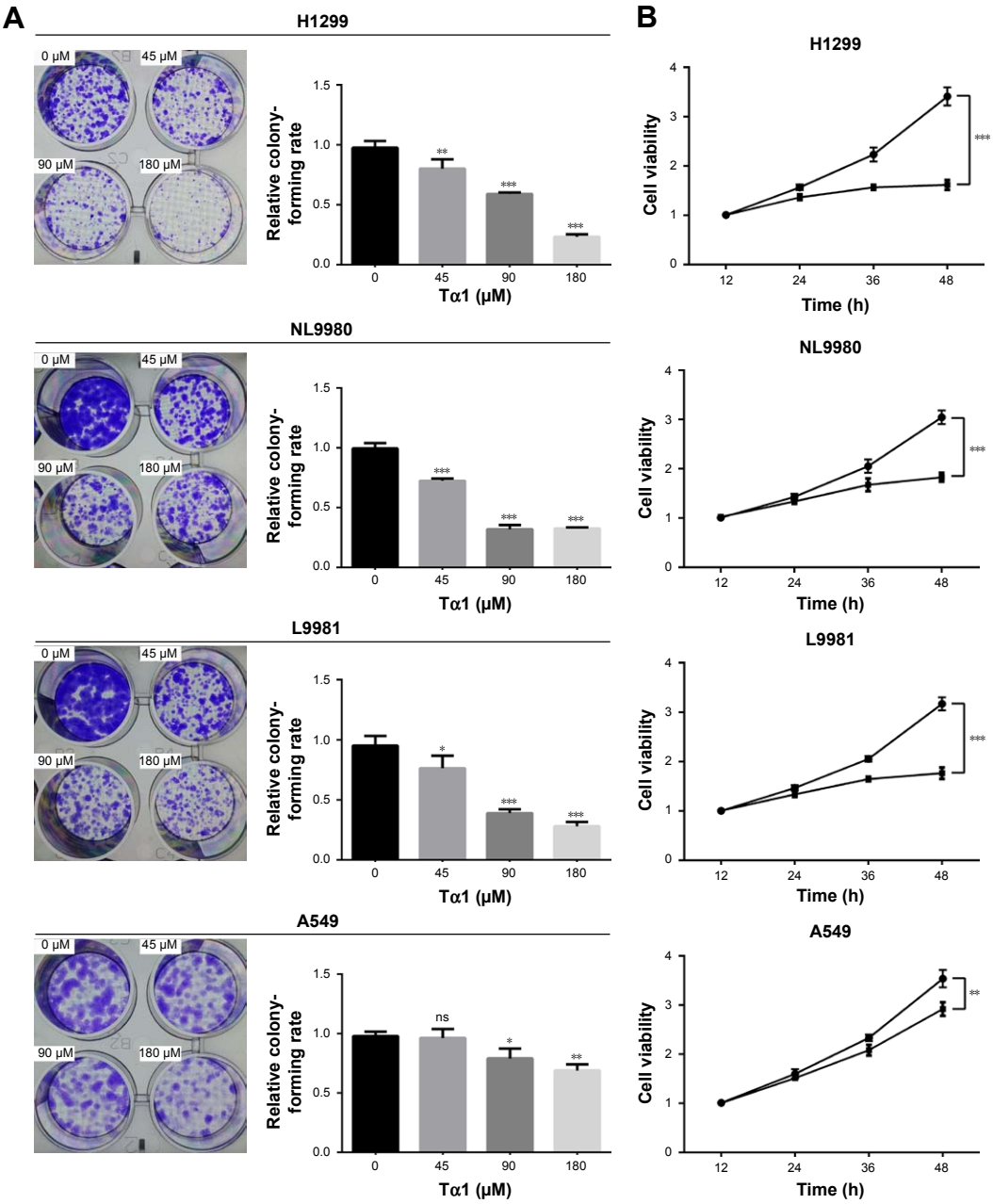

Figure S1 (*Continued*)

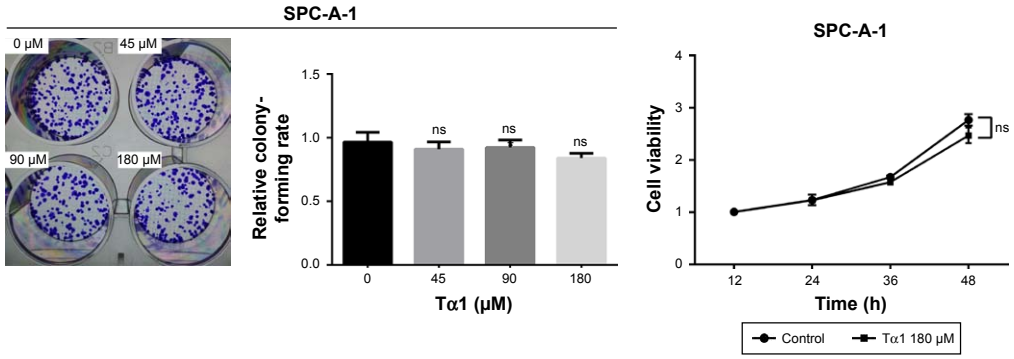

Figure S1 Tα1 had a tendency to suppress the proliferation more potently in PD-L1 high-expressing NSCLC cells compared with PD-L1 low-expressing NSCLC cells.
Notes: (**A**) For colony formation assay, the colony-forming abilities of H1299, NL9980, and L9981 were significantly suppressed by 45, 90, and 180 μM Tα1 treatment for 48 h, while the colony-forming ability of A549 was significantly suppressed only by 90 and 180 μM Tα1 after 48 h treatment, and the colony formation rate of SPC-A-1 did not change significantly. (**B**) For CCK-8 assay, the cell viabilities were suppressed more potently in H1299, NL9980, and L9981 cells compared with A549 cells, while the cell viability of SPC-A-1 did not change significantly after 180 μM Tα1 treatment for 48 h. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs control group.
Abbreviations: CCK-8, cell counting kit-8; NSCLC, non-small-cell lung cancer; PD-L1, programmed cell death ligand 1; Tα1, thymosin α1; ns, not statistically significant.

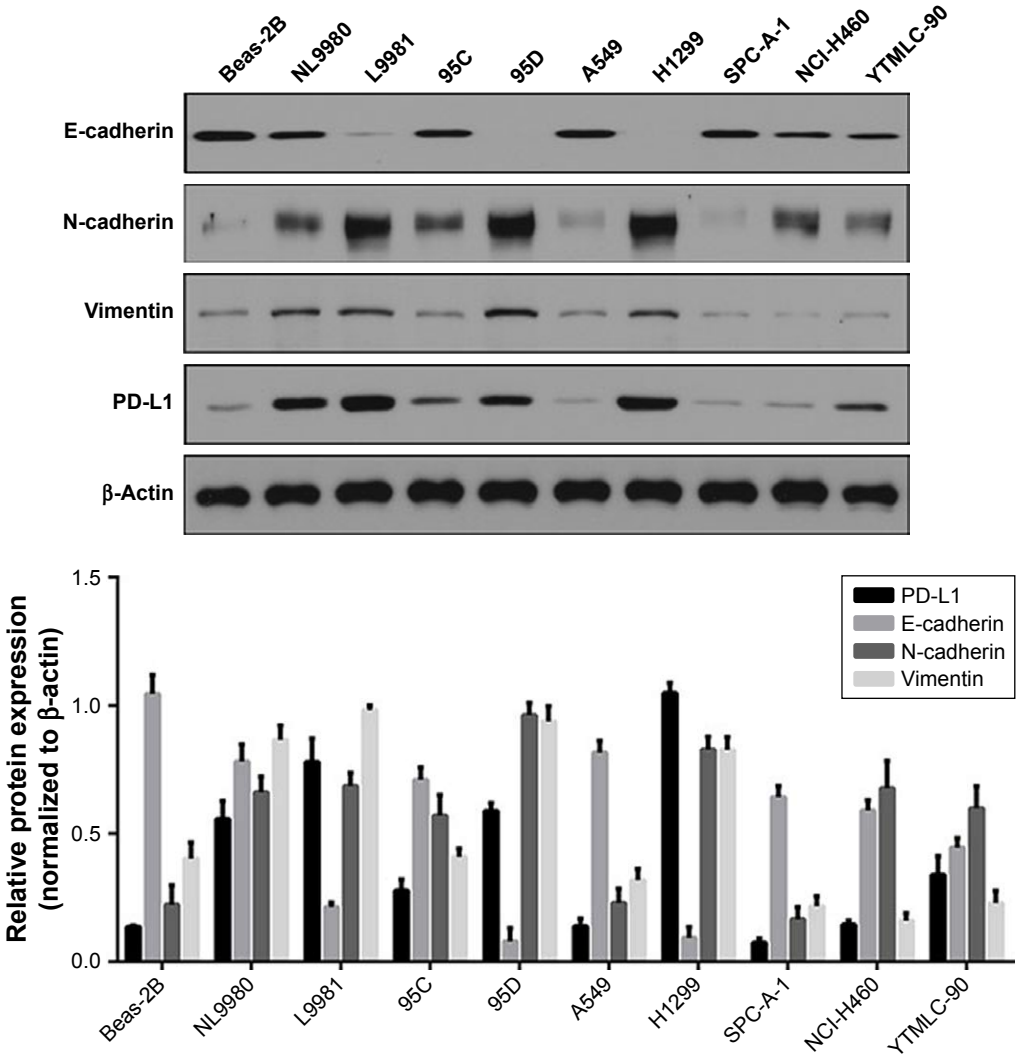

Figure S2 The different protein expression of epithelial–mesenchymal transition-associated marker and PD-L1 in human bronchial epithelial cells and a series of NSCLC cell lines.
Note: The protein level of E-cadherin was negatively correlated with PD-L1, while the protein levels of N-cadherin and vimentin were positively correlated with PD-L1 across the cell lines.
Abbreviations: NSCLC, non-small-cell lung cancer; PD-L1, programmed cell death ligand 1.