Technology in Cancer Research & Treatment
Volume 20: 1–10

DOI: 10.1177/15330338211046432
journals.sagepub.com/home/tct
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Effect of Programmed Death-Ligand 1 in Cancer-Associated Fibroblasts on Advanced Laryngeal Squamous Cell Carcinoma

Zhendong Yang, MD[1,2,*], Jinxin Wang, MD[1,*], Chun Chen, MD[1,*], Peng Sun, MD, PhD[1], and Yafeng Yu, MD, PhD[1]

Abstract

This study aimed to explore the effect of programmed death-ligand 1 (PD-L1) in cancer-associated fibroblasts (CAFs) on advanced laryngeal squamous cell carcinoma (LSCC). The expression of PD-L1 in advanced LSCC tumor tissues was observed in 83 patients with LSCC by immunofluorescence microscopy and compared with that in normal laryngeal mucosa. The CAFs of LSCC and normal fibroblasts (NFs) were isolated, cultured, purified, and examined by fluorescence. The expression of PD-L1 in purified CAFs and NFs was measured by flow cytometry. The expression of PD-L1 in CAFs was downregulated through small interferring RNA (siRNA) transfection. The proliferation and migration capacities of CAFs were observed using proliferation and scratch tests, respectively. The proliferation of HEP-2 cells and T cells was measured after cocultured with CAFs. The secretion of interleukins IL-2 and IL-10 was detected using enzyme-linked immuno sorbent assay (ELISA). PD-L1 was expressed in 62 of 83 cases of the advanced LSCC tumor tissues. Also, CAFs expressed more PD-L1 compared with NFs. The proliferation and migration capacities of CAFs were significantly lower after transfection with PD-L1-siRNA. The proliferation rate of HEP-2 cells cocultured with CAFs decreased in PD-L1-siRNA-transfected cells. However, the proliferation rate of T cells increased in transfected cells. The ELISA results showed that the secretion of IL-2 increased and that of IL-10 decreased in PD-L1-siRNA-transfected cells. The expression of PD-L1 in CAFs of advanced LSCC was higher than that in NFs. The downregulation of PD-L1 reduced the proliferation and migration of CAFs and HEP-2 cells but enhanced the proliferation and pro-inflammatory function of T cells in the coculture experiment.

Keywords

Laryngeal squamous cell carcinoma, programmed death-ligand 1, cancer-associated fibroblast, proliferation, immunosuppression

Abbreviations

BSA, bovine serum albumin; CAFs, cancer-associated fibroblasts; LSCC, laryngeal squamous cell carcinoma; DAPI, 4′,6-diamidino-2′-phenylindole; DMEM, Dulbecco's modification of Eagle's medium; ELISA, enzyme-linked immuno sorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IL, interleukin; NCNN, national comprehensive cancer network; NFs, normal fibroblasts; OCT, optimal cutting temperature compound; PBS, phosphate buffered saline; PD-1, programmed cell death factor-1; PD-L1, programmed death-ligand 1; RT-PCR, reverse transcription-PCR; siRNA, small interferring RNA; TCR, T-cell receptor; TNM, tumor node metastasis; TME, tumor microenvironment; α-SMA, α-smooth muscle actin.

Received: October 19, 2020; Revised: August 21, 2021; Accepted: August 27, 2021.

[1] Department of Otolaryngology, The First Affiliated Hospital of Soochow University, Suzhou, China
[2] Department of Otolaryngology, Suzhou Ninth Hospital affiliated to Soochow University, Suzhou, China

*These authors made equal contribution to this study.

Corresponding Authors:
Yafeng Yu, Department of Otolaryngology, The First Affiliated Hospital of Soochow University, No. 188 Shizi Street, Suzhou, Jiangsu, China.
Email: yfyu1024@163.com
Peng Sun, Department of Otolaryngology, The First Affiliated Hospital of Soochow University, No. 188 Shizi Street, Suzhou, Jiangsu, China.
Email: entsunpeng@126.com

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common head and neck cancer. The prognosis of early-stage (stages I and II) laryngeal cancer is optimistic after standard radiotherapy or surgery with a 5-year survival rate of 80% to 85% according to the National Comprehensive Cancer Network (NCCN) guidelines.[1–3] However, about 60% of laryngeal cancers diagnosed in clinics are already in advanced stages (stages III and IV).[4] The concurrent systemic therapy/radiation therapy or surgery followed by adjuvant therapies was recommended for advanced laryngeal cancer according to the NCCN guidelines.[1] The combined treatments may cause many complications such as aphonia and dysphagia. However, despite using the current methods, the 5-year survival rates of advanced laryngeal cancer are less than 60%.[5] Hence, new treatment strategies need to be urgently developed for patients with advanced LSCC.

In recent years, studies about immune checkpoint inhibitors have gradually become popular and proved to treat many types of cancers effectively. PD-L1 (B7 homolog 1), the third member of the B7 family, was discovered in homology analysis, which is also known as programmed death-ligand 1 (PD-L1).[6] After binding to programmed cell death factor-1 (PD-1) expressed on the surface of activated T cells, PD-L1 can inhibit T-cell receptor (TCR)-mediated T-cell activation and prevent T cell from overactivation.[7] For their undeniable effectiveness in clinics, PD1/PD-L1 inhibitors were recently approved by the FDA for patients with recurrent/metastatic head and neck squamous cell carcinoma.[8] However, how effective PD1/PD-L1 inhibitors are for the advanced LSCC remains to be explored.

An increasing number of studies suggest that the occurrence and metastasis of malignant diseases are closely related to the tumor microenvironment (TME).[9] Cancer-associated fibroblasts (CAFs) are the most abundant cell type within the TME.[10] CAFs regulate TME by secreting various cytokines and pro-inflammatory factors. They not only promote the growth, invasion, and metastasis of the existing cancer cells but also produce carcinogenic factors and affect the normal cells in the adjacent areas.[11] In the present study, the expression of PD-L1 on CAFs isolated from advanced LSCC was measured. Furthermore, the study investigated the role of PD-L1 on CAFs and explored its potential mechanism of immune escape. It shed light on developing new clinical strategies against advanced LSCC.

Materials and Methods

Patients

Eighty-three tumor samples, including 66 cases of stage III and 17 cases of stage IV, were collected from patients with first-onset advanced LSCC at the First Affiliated Hospital of Soochow University, Department of Otorhinolaryngology and Head and Neck Surgery from 2016 to 2019. The age range was from 50 to 81 years (average 64.24 ± 7.51years). Normal laryngeal tissue samples were also taken during surgery from the same patient and used as controls. All studies were approved by the ethics committee of the First Affiliated Hospital of Soochow University.

Immunofluorescence

The tumor sample was washed 3 times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 12 h. The fixed specimens were dehydrated in 10% and 20% sucrose solution for 12 h and dehydrated with 30% sucrose for an additional 24 h. The tissue was embedded with optimal cutting temperature compound (OCT) and sliced with a thickness of 10 μm on a constant cold slicer. The scrolls were directly placed on a glass slide, and the OCT was dissolved in PBS. The slides were air-dried for 30 min and blocked for 40 min at room temperature by using 5% bovine serum albumin (BSA) (containing 0.3% Triton X-100; Sigma-Aldrich). The slides were then incubated at 4 °C overnight with primary antibody (mouse antihuman monoclonal antibody PD-L1; dilution 1:200; BioLenged, RRID:AB_466091), rinsed, and then incubated for 2 h with fluorescein isothiocyanate (FITC) secondary antibody (rabbit antimouse anti-PD-L1; dilution 1:100) (Abcam, RRID:AB_10565185). After rinsing with PBS, the slides were stained with 4′,6-diamidino-2′-phenylindole (DAPI) for 10 min. The sections were then observed and photographed under a fluorescence microscope.

Cell Culture and Purification

The tumor and the control tissues were immersed in PBS (containing 1% penicillin-streptomycin double antibody) for 20 min and cut into 1- to 2 mm pieces before digestion with type IV collagenase (200 U/mL) for 1.5 h. The homogenate was ground, filtered, and centrifuged (1500 rpm, 5 min, 4 °C). The

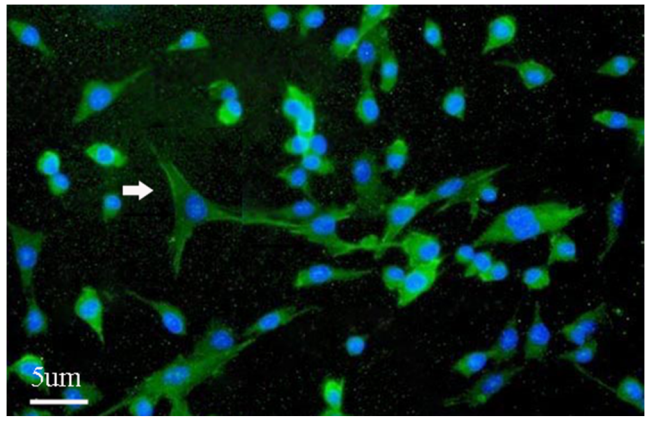

Figure 1. Immunofluorescence staining showed positive expression of programmed death-ligand 1 (PD-L1) (yellow–green fluorescence) in cancer-associated fibroblasts (CAFs) (white arrow).

cell pellet was resuspended in Dulbecco's modification of Eagle's medium (DMEM) (containing 1% cyanide antibiotic and 10% fetal bovine serum (FBS)) and cultured for 2 to 7 days. The CAFs were isolated from the laryngeal cancer cells based on its unique sensitivity to trypsin digestion. After removing the supernatant, the plate was washed 3 times with PBS for 5 min and digested with 1 mL of 0.25% trypsin for 2 to 3 min. Then, an equal volume of 10% FBS with DMEM was added to the plate. The supernatant was collected into a 15 mL centrifuge tube, centrifuged (1500 rpm, 5 min, 4 °C) and washed, and cultured with DMEM containing 10% FBS. This process was repeated 3 times.

Identification of Cancer-Associated Fibroblasts and Normal Fibroblasts

The purified cultured CAFs and normal fibroblasts (NFs) were seeded onto 6-well plates and cultured for 24 h. They were then washed with PBS (5 min, 3 times) and fixed with 4% paraformaldehyde for 15min. They were then blocked for 40 min at room temperature with 5% BSA (10% goat serum containing 0.3% Triton X-100 for membrane rupture) and incubated at 4 °C with mouse antihuman primary antibody (Vimentin, 1:100 [RRID:AB_442141] and α-smooth muscle actin [α-SMA], 1:200 [RRID:AB_442134] from BioLenged,) overnight. After incubating at 37 °C for 1 h, FITC/Cy3 fluorescently labeled rabbit antimouse IgG (rabbit antimouse anti-PD-L1; dilution 1:100) (Abcam, RRID:AB_10737168) was added, and the cells were stained at 37 °C in the dark for 1 h. After washing with PBS (5 min, 3 times), the cells were incubated in the dark and then stained with DAPI for 2 min. They were observed and photographed under an inverted fluorescence microscope.

Identification of Programmed Death-Ligand 1

The purified CAFs and NFs were digested, centrifuged (1500 rpm, 5 min, 4 °C) and resuspended in PBS, and incubated at 4 °C with PD-L1 antibody for 30 min. The suspension was then washed 3 times with PBS before flow cytometry analysis. The data were analyzed using FlowJo 7.6 software.

Cell Passage and Transfection

The purified CAFs were incubated with 100 pmol of small interferring RNA (siRNA) mixed with 5 μL of Lipo2000 in serum-free DMEM for 20 min. Then, the supernatant was replaced with DMEM (10% FBS). After 6 h, the results were determined by reverse transcription-PCR (RT-PCR) after transfection for 48 h and cell passage was again performed.

RT-PCR

RT-PCR was performed following the manufacturer's protocol (Bio-Rad). First, 0.8 mL of Trizol solution was added to the plate containing 3×10^5 cells. The solutions were centrifuged

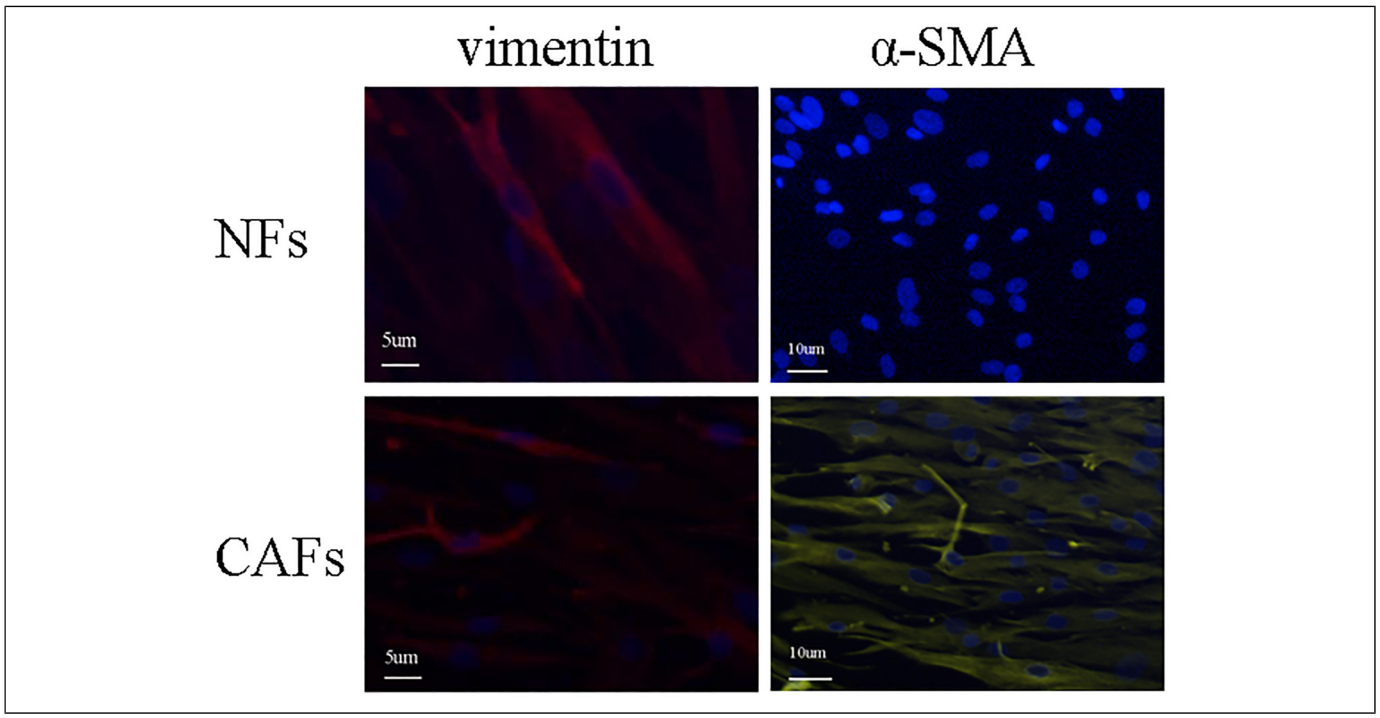

Figure 2. Immunofluorescence staining showed that Vimentin was positive in cancer-associated fibroblasts (CAFs) and normal fibroblasts (NFs) (red fluorescence), while α-smooth muscle actin (α-SMA) was positive only in CAFs (green fluorescence) but not in NFs.

(12 000 rpm, 5 min) and the precipitate was discarded. Then, 160 μL of chloroform was added, left undisturbed for 15 min after mixing, and shaken. The solution was then centrifuged (12 000 *g*, 15 min), and the supernatant was carefully removed before adding 0.4 mL of isopropanol. The solution was then centrifuged (12 000 *g*, 10 min) at 4 °C. After discarding the supernatant, 0.8 mL of 75% ethanol was added. The solution was then centrifuged (8000 *g*, 5 min), and the supernatant was removed. Further, 50 μL of TE buffer was added to the tube to dissolve the RNA sample and incubated at 60 °C for 5 min. The RNA concentration was quantified using an ultraviolet spectrophotometer. A sample of OD value between 1.8 and 2.0 at

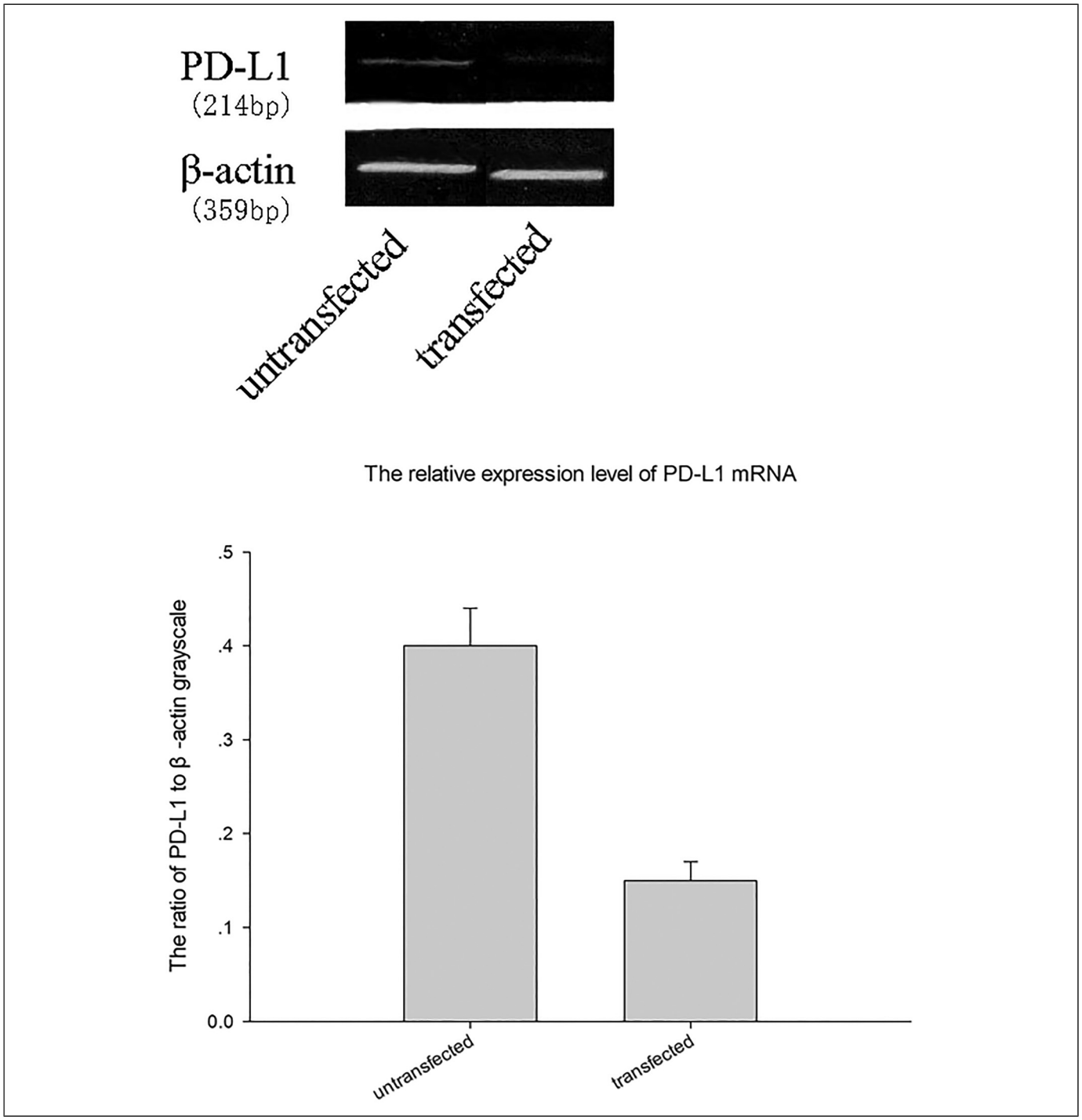

Figure 3. RT-PCR showed that the relative expression level of programmed death-ligand 1 (PD-L1) mRNA of PD-L1-siRNA-transfected cells was significantly lower.

A260 nm/A280 nm was used for the next experiment. The procedure was performed using an RT-PCR one-step kit (Bio-Rad). RT-PCR was conducted in a 50 μL reaction mixture containing 5 μg RNA sample, 1.5 μL of AMV reverse transcriptase, and 0.5 μL of RNase inhibitor. β-actin was used as an endogenous control for PCR quantification. The PCR cycling conditions were set as follows: 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min, extension at 72 °C for 10 min, and preservation at 4 °C. The result was obtained with a 1.5% agarose gel electrophoresis test. The gray value of the electrophoresis bands was analyzed using BandScan gel image analysis software, and the relative expression level of PD-L1 mRNA was expressed by the ratio of PD-L1 to β-actin grayscale. The DNA sequences of primers (forward and reverse) were as follows: PD-L1, 5′-GCCGACT ACAAGCGAATTAC-3′ and 5′-TCTCAGTGTGCTGGTCAC AT-3′; β-actin, 5′-GCTCGTCGTCGACAACGGCTC-3′ and 5′-CAAACATGATCTGGGTCATCTTCTC-3′.

Cell Proliferation Assay

Purified CAFs and PD-L1-siRNA-transfected CAFs were seeded onto 96-well plates (containing 0.2 mL of DMEM) and cultured for 6 days. On the second, fourth, and sixth days of the culture, 20 μL of CCK-8 was added. The absorbance at 450 nm was measured after culturing for 3 h in 5% CO_2 incubator at 37 °C.

Scratch Test

Purified CAFs and PD-L1-siRNA-transfected CAFs were seeded onto plates. When the cells grew close to confluence, scratches were applied in the center of the plate using a 100 μL pipette tip. The tip was maintained perpendicular to the bottom of the plate to ensure the same width of the scratch. DMEM was then removed and washed 3 times with PBS. Subsequently, fresh DMEM was added. The width of the scratches was recorded using a phase-contrast microscope after 12, 24, and 48 h. The result was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics).

Cell Coculture

Purified CAFs and PD-L1-siRNA-transfected CAFs were cultured in 96-well plates containing 0.2 mL of DMEM at a density of 2×10^4 cells/well. After 48 h, the supernatant was replaced with a medium containing mitomycin C (20 mg/L). The cells were then stabilized for 30 min before adding 2×10^5 HEP-2 cells or 2 mg/L PHA and 2×10^5 T cells. According to the time point of adding HEP-2 cells as 0 o'clock, on the first, second, and third days, 20 μL of CCK-8 solution (1:10 dilution) was added to the wells. According to the time point of adding T cells as 0 o'clock, on the second, fourth and sixth days, 20 μL of CCK-8 solution (1:10 dilution) was added to the wells. The absorbance at 450 nm was

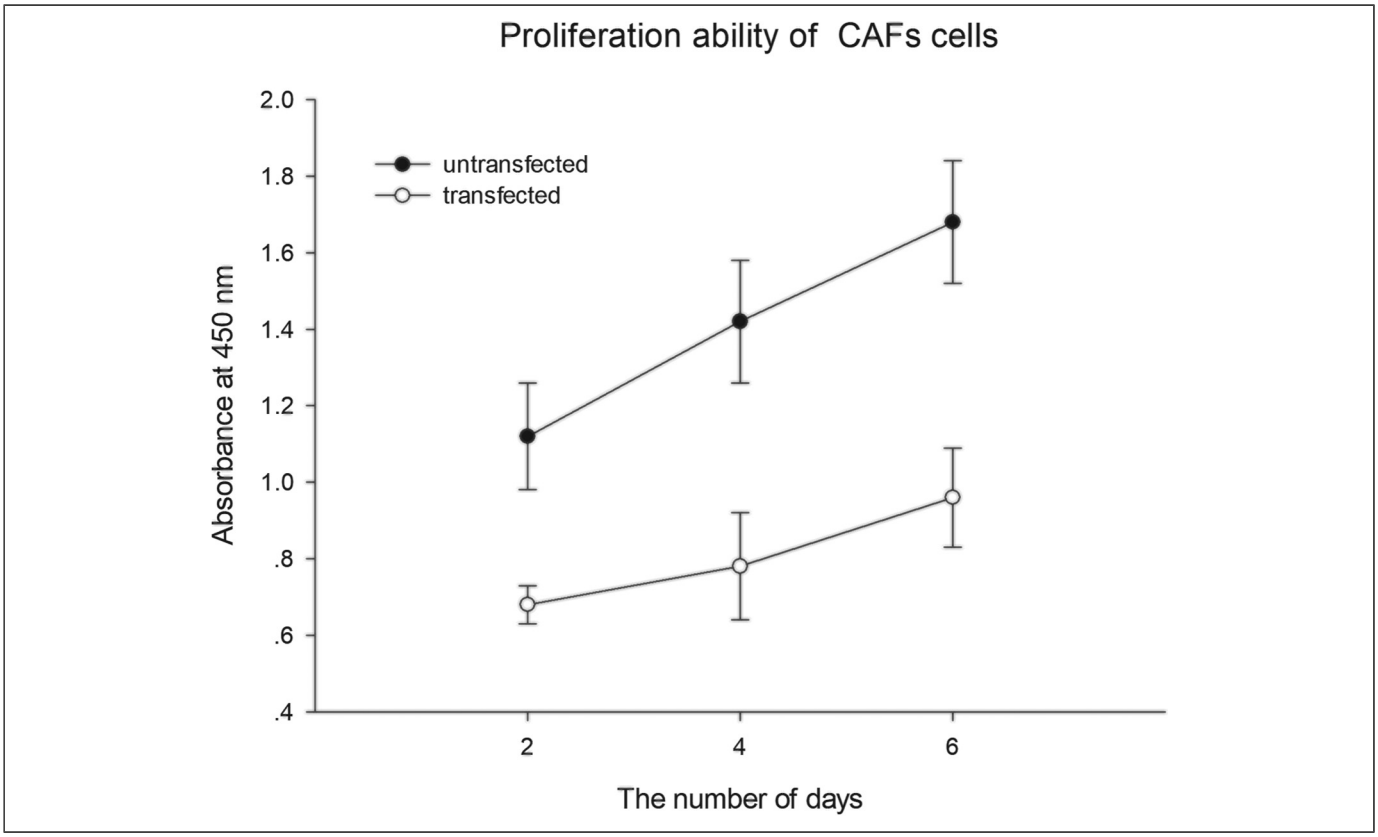

Figure 4. The proliferation capacity of programmed death-ligand 1 (PD-L1)-siRNA-transfected cells was significantly impaired compared to controls at the second, fourth, and sixth days of the culture ($P < .05$).

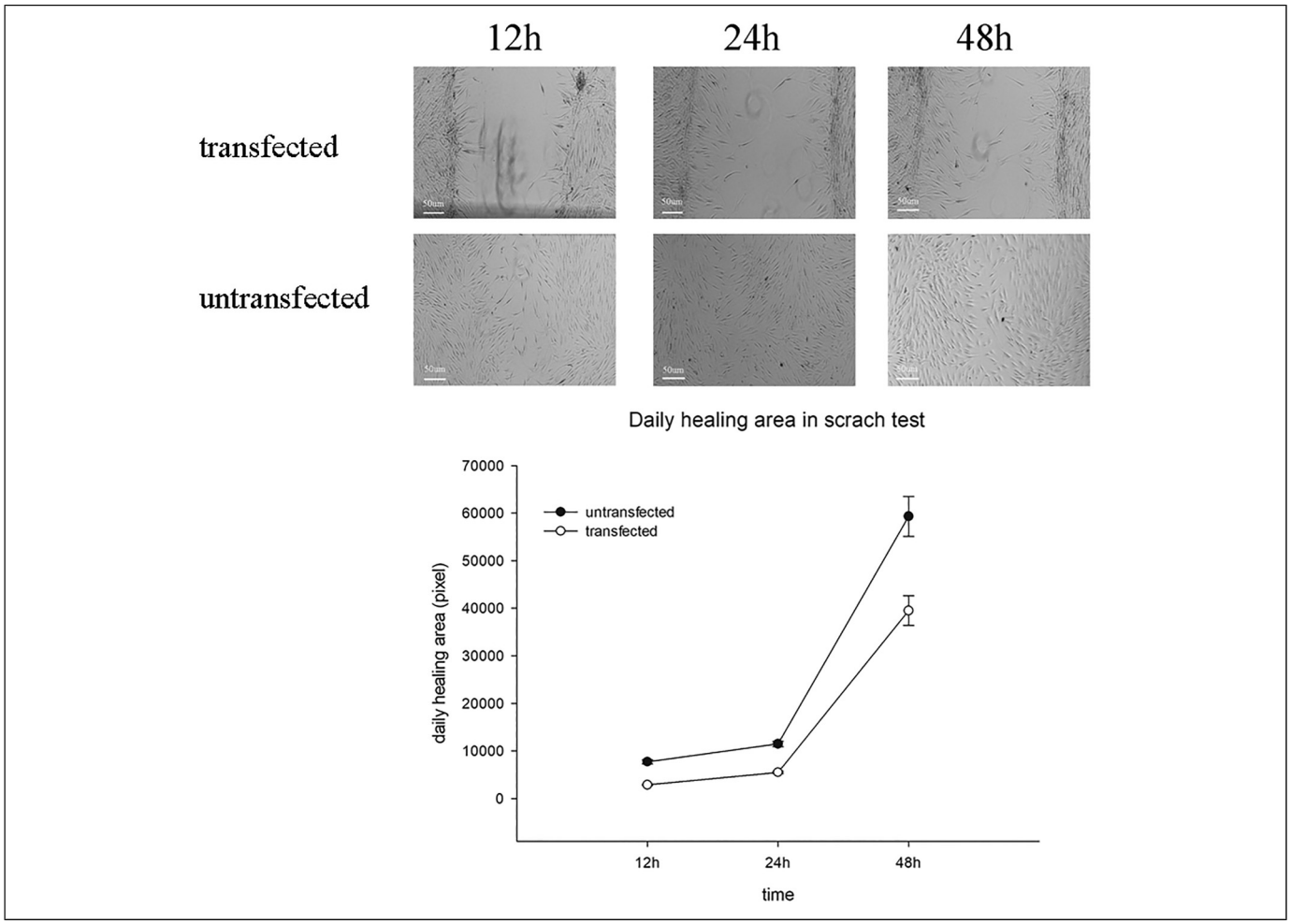

Figure 5. Scratch test of programmed death-ligand 1 (PD-L1)-siRNA-transfected cells and controls at 12 h, 24 h, and 48 h. The migration capacity of the PD-L1-siRNA-transfected cells was significantly reduced compared to controls at 12, 24, and 48 h of the culture ($P < .05$).

measured. In the T-cell coculture experiment, the secretion of IL-2 and IL-10 after 72 h was determined using enzyme-linked immuno sorbent assay (ELISA).

Statistical Analysis

The sample size selected in this study meets the requirements of statistics. All statistical analyses were performed using SPSS software (SPSS), version 22.0. A paired-sample *t*-test was used for comparison between the two groups. A P value <.05 indicated a statistically significant difference.

Results

Programmed Death-Ligand 1 Expressed in Cancer-Associated Fibroblasts

PD-L1 expression (yellow–green fluorescence) was identified in CAFs in 62 of 83 cases of advanced LSCC tumor tissue by immunofluorescence staining (Figure 1).

Cancer-Associated Fibroblasts Are Validated by α-Smooth Muscle Actin

The CAFs were isolated and purified after 3 passages of the mixed cells. NFs were purified at the second passages. The most common marker of CAFs is α-SMA,[12] which is one of the many positive markers. Immunofluorescence staining showed that Vimentin was positive in CAFs and NFs, while α-SMA was positive only in CAFs but not in NFs (Figure 2).

The Expression of Programmed Death-Ligand 1 in Cancer-Associated Fibroblasts Was Higher Than That in Normal Fibroblasts

The flow cytometry data showed that the expression of PD-L1 in CAFs $(99.30 \pm 0.56)\%$ was higher in CAFs than in NFs $(83.10 \pm 1.73)\%$ ($P < .05$).

The Expression of Programmed Death-Ligand 1 Was Decreased by RT-PCR

The relative expression level of PD-L1 mRNA was significantly lower in PD-L1-siRNA-transfected cells compared with the control ($P < .05$) (Figure 3).

The Proliferation Ability of Cancer-Associated Fibroblasts Was Reduced After Transfection

The expression of PD-L1 in CAFs was downregulated through siRNA transfection. The proliferation capacity of PD-L1-siRNA-transfected cells was significantly impaired compared with that of controls on the second, fourth, and sixth days of the culture ($P < .05$, Figure 4).

The Migration Capacity of Cancer-Associated Fibroblasts Was Reduced After Transfection

The migration capacity of cells was determined using the scratch test. The data showed that the migration capacity of the PD-L1-siRNA-transfected cells was significantly reduced compared with that of controls after 12, 24, and 48 h of the culture ($P < .05$) (Figure 5).

Cell Coculture

The proliferation rate of HEP-2 cells cultured with PD-L1-siRNA-transfected cells was significantly reduced compared with that of controls on the first, second, and third days of the culture ($P < .05$) (Figure 6). Interestingly, the proliferation rate of T cells cultured with PD-L1-siRNA-transfected cells significantly increased compared with that of controls on the second, fourth, and sixth days of the culture ($P < .05$) (Figure 7). The results of ELISA suggested that the secretion of IL-10 was significantly lower after coculture with transfected cells while the secretion of IL-2 was significantly enhanced ($P < .05$) (Tables 1 and 2).

Discussion

PD-L1 can facilitate tumor metastasis and recurrence by evading the host immune surveillance system.[13] The members of the B7 family are costimulatory molecules that help transmit signals to T cells from antigen-presenting cells.[14] During T-cell activation, the members of the B7 family from antigen-presenting cells bind to the CD28 receptors expressed on T cells. Among these, B7-1 and B7-2 have been shown to be able to stimulate and maintain T-cell pro-inflammatory function. For instance, they can increase the production of cytokines such as IFN-γ and IL-2, and induce the expression of

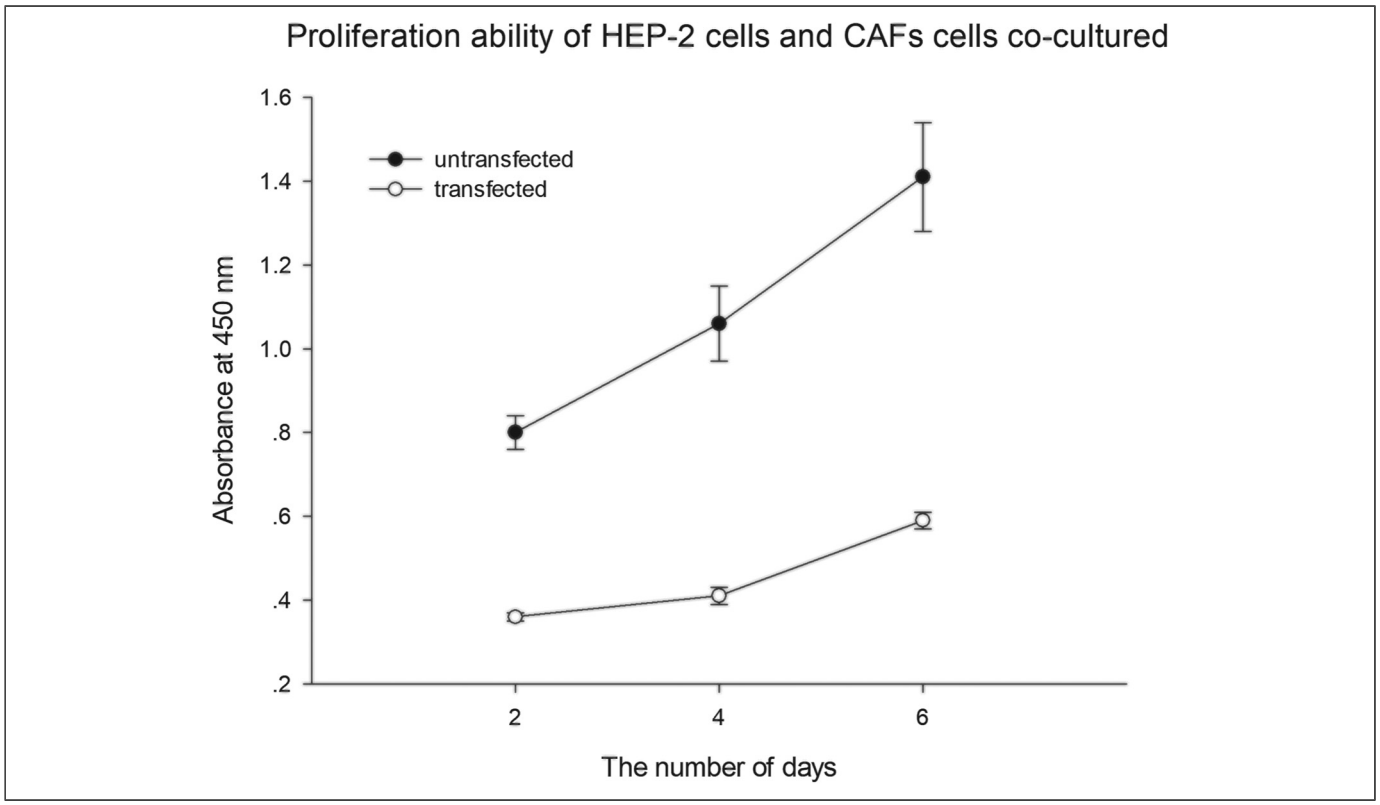

Figure 6. The proliferation rate of HEP-2 cells when culturing with programmed death-ligand 1 (PD-L1)-siRNA-transfected cells was significantly reduced compared to the control ($P < .05$).

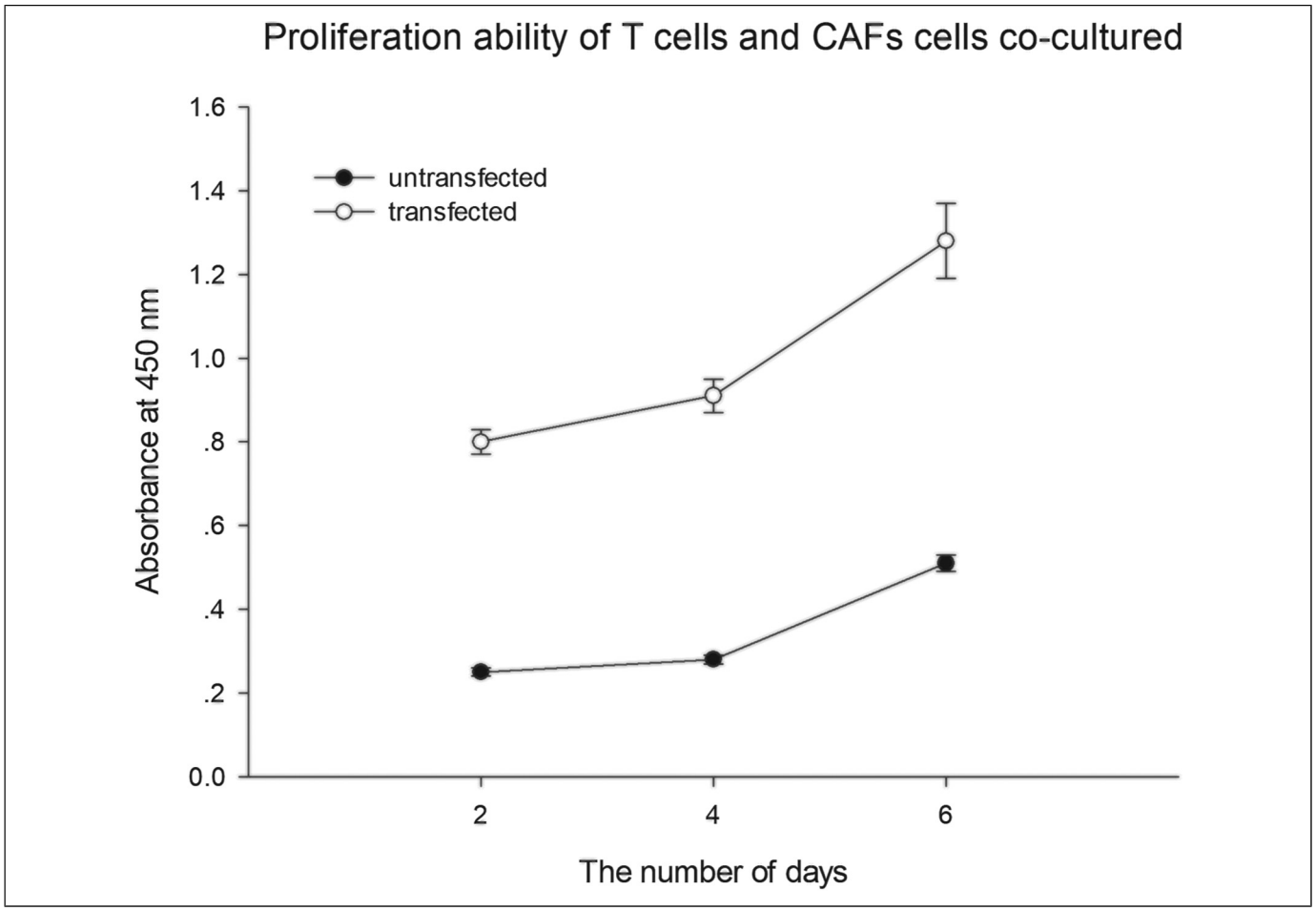

Figure 7. The proliferation rate of T cells when culturing with programmed death-ligand 1 (PD-L1)-siRNA-transfected cells was significantly increased compared to the control ($P < .05$).

Table 1. The Main Clinical Characteristics of Our Cohort.

	N
Sex	
Male	80
Female	3
Age	Mean
Male	64.48
Female	58
Localization	
Supraglottic	13
Glottic	59
Transglottic	8
Subglottic	3
TNM stage	
III	66
T3N0M0	58
T3N1M0	8
IV	17
T3N2bM0	2
T3N2cM0	1
T4aN0M0	12
T4aN1M0	1
T4aN2bM0	1

TNM: tumor node metastasis.

antiapoptotic protein Bcl-XL.[15] PD-L1, as one of the B7 family members, binds to PD-1, an important member in the CD28 family, and plays an important role in restraining the immune response.[16] PD-1/PD-L1 inhibits the proliferation of T cells and negatively regulates the CD28/B7-2 pathway.[17,18]

The data suggested that the expression of PD-L1 was found in most advanced LSCCs, which was consistent with previous reports.[19] A high PD-L1(PD-L1) level was associated with a better prognosis in laryngeal squamous cell cancer.[20] TME plays an important role in the invasion and spread of tumor cells.[21] The number of CAFs, the main component of TME, was inversely related to the number of T lymphocytes in the tumor.[22] Studies suggested that the PD-L1-expressing CAFs were immunosuppressive and participated in evading the

Table 2. Secretion of IL-10 and IL-2 After Coculture of T Cells and Cancer-Associated Fibroblast Cells for 72 h (ng/L, Mean ± SEM).

	Untransfected	PDL1-siRNA transfected
IL-10	290.833 ± 17.151	149.167 ± 20.351
IL-2	780.833 ± 54.444	1505.00 ± 148.896

immune surveillance.[23] The data found that the expression of PD-L1 on the surface of CAFs was significantly higher than that on NFs.

The CAFs were transfected with PD-L1 siRNA to further confirm the role of PD-L1 in regulating CAF cellular function. The proliferation and migration capacity of CAFs were impaired after the transfection. Furthermore, the proliferation rate of HEP-2 cells also decreased after coculture with transfected CAFs. Interestingly, the proliferation rate of T cells significantly increased after coculture with the transfected CAFs. Also, the pro-inflammatory function of T cells was detected by testing IL-2 and IL-10. IL-2 and IL-10 are important cytokines secreted by T cells upon activation. IL-2 is important in activating NK cells and inducing differentiation of regulatory T cells and apoptosis of self-reactive T cells.[24] IL-10 is generally immune suppressive and participates in tumor immune evasion by suppressing tumor-specific T-cell responses, inhibiting dendritic cell maturation, and promoting regulatory T-cell function.[25] The data suggested that IL-2 levels significantly increased but IL-10 levels significantly decreased after downregulation of PD-L1. These results supported the hypothesis that the expression of PD-L1 on CAFs was important in facilitating tumor cells evading the immune system. Therefore, PD1/PD-L1 inhibitors blocking PD-L1 on CAFs may help stop the tumor progression and improve the prognosis of advanced LSCC.

Of course, there are still some insufficient considerations and limitations in the experimental design, such as the positive labeling of CAF. In this study, selecting only α-SMA and Vimentin as the proteins for identifying CAF and NFS is not strict enough. It is not rigorous enough to select only α-SMA and Vimentin proteins as the proteins to identify CAFs and NFS in this study. If other positive markers of CAFs can be selected, the results will be more reliable. Second, no animal experiments were performed to verify the results of *in vitro* experiments. Finally, the upstream regulatory mechanism of PDF-L1 expression increase in CAFs and the molecular mechanism that promotes the proliferation and migration of CAFs will be further studied in future work.

Conclusions

PD-L1 was expressed in most advanced LSCC tumor tissues. Moreover, the expression of PD-L1 in CAF was higher than that of NFs in advanced LSCC. Downregulation of PD-L1 reduced the proliferation and migration of CAF and HEP-2 cells but enhanced the proliferation and pro-inflammatory functions of T cells.

Acknowledgments

The authors are very thankful to Professor Guang-bo Zhang of Institute of Clinical Immunology of Soochow University for giving experimental guidance.

Declaration of Conflicting Interests

The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate

The present study was approved by the ethical review committee of the first affiliated hospital of Soochow University. Written informed consent was obtained from all enrolled patients.

Funding

This work was supported by grants from the Jiangsu Provincial Commission of Health and Family Planning (No. H2017061), the Science and Technology Bureau of Suzhou (No. SS201861), the Science and Technology Bureau of Suzhou (No. SYS2018040), the Science and Technology Bureau of Suzhou (No. SLT202007), and the Technology Bureau of Wujiang (No. wwk201905).

ORCID iD

Yafeng Yu https://orcid.org/0000-0003-3558-0263

Supplemental Material

Supplemental material for this article is available online.

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