ORIGINAL PAPER

MicroRNA-4458 Regulates PD-L1 Expression to Enhance Anti-tumor Immunity in NSCLC via Targeting STAT3

Weixin Liu[1] · Ruibao Liu[1] · Ronghui Yuan[1] · Xing Wang[1]

Abstract

MicroRNA-4458 (miR-4458) has been reported to be associated with several cancers including non-small-cell lung cancer (NSCLC), while its role in tumor immunity remains unclear. The purpose of the current research was to explore the anti-tumor immunity of miR-4458 in NSCLC. The results showed that the expression level of miR-4458 decreased and STAT3 increased in NSCLC tissues and cells. For in vitro experiments, miR-4458 mimics suppressed cell proliferation and decreased the expression level of PD-L1. Moreover, STAT3 was confirmed as a target gene of miR-4458. Upregulation of STAT3 level ameliorated the inhibitive effects of miR-4458 on cells proliferation and PD-L1 expression in cells. For in vivo studies, overexpression of miR-4458 hindered tumor growth, decreased the proportion of PD-1$^+$ T cells, the expression of PD-L1 and IL-10, upregulated the proportion of CD4$^+$ T, CD8$^+$ T cells as well as the expression of IFN-γ and IL-2, which were all reversed by overexpression of STAT3, and the effects of STAT3 were counteracted after knockdown of PD-L1. MiR-4458 overexpression enhanced anti-tumor immunity via targeting STAT3 to block the PD-L1/PD-1 pathway.

Keywords Non-small-cell lung cancer · Anti-tumor immunity · miR-4458 · STAT3 · PD-L1

Abbreviations

miR-4458	MicroRNA-4458
NSCLC	Non-small-cell lung cancer
PD-1	Programmed cell death-1
PD-L1	Programmed cell death ligand-1
STAT3	Signal transducer and activator of transcription3
HNSCC	Head and neck squamous cell carcinoma
WT	Wild type
MUT	Mutation type
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
P/S	Penicillin and streptomycin
ELISA	Enzyme-linked immunosorbent assay
PVDF	Polyvinylidene fluoride membranes
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
CCK-8	Cell counting kit-8
SD	Standard deviation
ANOVA	One-way analysis of variance

✉ Xing Wang
wangxingliuweixin@163.com

1 Department of Oncology, The People's Hospital of Dongying, No. 317, Nanyi Road, Dongcheng District, Dongying 257091, Shandong, China

Introduction

Lung cancer is one of the most common cancers in the world, non-small-cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases [1]. Although the treatment strategy has been improved, the survival rate of patients with lung cancer, especially those with NSCLC, has not been markedly improved [2] and most patients had a bad prognosis [3]. In recent years, immunotherapy, which suppresses tumor immune escape by blocking immune checkpoints, offers a novel method to treat NSCLC [4].

Tumors can escape from immune detection by utilizing immune checkpoints. Programmed cell death-1 (PD-1)/programmed cell death ligand-1 (PD-L1) is a key immune checkpoint [5]. PD-L1, a transmembrane protein involved in immune system suppression, is the major ligand of PD-1. The binding of PD-L1 expressed by tumor cells and PD-1 produced by T cells initiates an array of inhibitory signals resulting in reduced function and apoptosis of T cells [6]. Recent research found that the suppression of PD-L1 enhanced anti-tumor immunity by inhibiting tumor cells

from evading host immune responses [7]. Signal transducer and activator of transcription 3 (STAT3) belongs to the STAT protein family, it is a transcription factor that can be activated by phosphorylation [8]. Its expression level is increased in many cancers such as pancreatic cancer, HNSCC, and liver cancer and it plays an important role in regulating various genes [9–11]. A study has shown that the activation of STAT3 was significantly associated with PD-L1 level in NK/T-cell lymphoma cells [12]. The level of PD-L1 could be upregulated by fibroblast growth factor receptors via the JAK/STAT3 pathway in human colorectal cancer cells [13]. Another study showed that blocking STAT3 signaling reduced PD-1/PD-L1 in an HNSCC mouse model [10].

MicroRNA (miRNA) is a class of non-coding small RNA with the regulatory function on target mRNA expression, which subsequently acts as a key role in regulating cell proliferation, apoptosis, and tumor progression [14]. The abnormal expression of miRNA in a variety of cancers was discovered [15]. The role of miRNAs on tumor immunity has been studied [16]. For example, miR-142-5p enhanced the anti-tumor immunity by reducing the PD-L1 level in pancreatic cancer mice [17]. MiR-15a/16 deficiency maintained the glioma-infiltrating CD8^{+} T cells' function to inhibit the development of glioma [18]. MiR-4458 has been reported to act as a key regulatory molecule that participates in the development of many cancers, such as colon cancer, liver cancer, melanoma, and NSCLC [19–23]. Studies have shown that miR-4458 blocked the cell cycle and prevented cell proliferation in vitro [24]. Furthermore, Ma et al., found that miR-4458 suppressed migration by targeting HMGA1 in NSCLC cells [23]. The role and potential mechanism of miR-4458 in tumor immunity remain unclear.

The current research aimed to study the role of miR-4458 in tumor immunity and its potential mechanism to offer novel insights into the immunotherapy of NSCLC.

Material and Methods

Samples

A total of 25 pairs of NSCLC tissues and adjacent normal lung tissues samples were obtained from patients ($n = 25$, 14 men and 11 women, age range 54–68 years old) at the People's Hospital of DongYing. Besides, the peripheral blood from 62 NSCLC patients and 50 healthy controls were obtained at the same hospital. The clinical information of 62 NSCLC patients was listed in Table 1. Informed consent was obtained from all patients and their guardians. This study was approved by the Ethics Committee of People's Hospital of DongYing.

Table 1 Clinical characteristics of the NSCLC patients at enrollment

	NSCLC patients, $N = 62$
Age in years, mean ± SD	62.8 ± 8.9
Sex, n (%)	
Male	37 (59.7)
Female	25 (40.3)
Histology, n (%)	
Adenocarcinoma	44 (71.0)
Squamous cell carcinoma	13 (21.0)
Others	5 (8.0)
Smoking status, n (%)	
Never	15 (24.2)
Former/current	47 (75.8)
Stage, n (%)	
I	34 (54.8)
II	15 (24.2)
III	13 (21.0)
Previous surgery, n (%)	
Yes	14 (22.6)
No	48 (77.4)
Adjuvant chemotherapy ± radiotherapy, n (%)	
Yes	4 (6.5)
No	58 (93.5)

Cell Culture

Human NSCLC cell line A549, H1299, SW900, mouse lung cancer cell line LLC, and normal human lung fibroblast cell line 2BS were obtained from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences. The A549 cells were cultured in Ham's F-12K, the SW900 cell line was cultured in Leibovitz's L-15, the H1299 cells were cultured in RPMI-1640 and LLC line was cultured in DMEM medium (Thermo Fisher Scientific, USA) with 10% FBS, 1% penicillin, and streptomycin (P/S) in the atmosphere with 5% CO_2 at 37 °C.

ELISA

The levels of cytokines IFN-γ, IL-2, and IL-10 were measured by ELISA kit (ExCell Bio, China). The experimental detection wavelength was 450 nm.

qRT-PCR

Total RNA was separated by the Total RNA Kit (Tiangen, China). Subsequently, 1 μg RNA was reverse transcribed into cDNA and qRT-PCR was performed with FastKing

One-Step RT-qPCR kit (SYBR Green) (Tiangen, China) using Applied Biosystem 7300 (Applied Biosystems, USA). The sequences of primers were shown in Table 2. The relative changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Proteins were extracted from tumor tissues or cells using RIPA (Beyotime, China). BCA Assay Reagent Kit (Beyotime, China) was applied to detect protein concentration. Proteins were separated using 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). After being blocked, the membranes were incubated with specific primary antibodies: p-Tyr705-STAT3 (CST, 1:1200), STAT3 (CST, 1:1200), PD-L1 (CST, 1:800), GADPH (CST, 1:1000) overnight at 4 °C. Then, the membranes were incubated with a peroxidase-conjugated secondary antibody at room temperature for 1 h. Image J was applied to analyze the expression levels of proteins.

Cell Transfection

miRNA-mimics are double-stranded miRNA-like RNA molecules that can enhance the function of endogenous miRNA duplexes. And miR-NC, pcDNA3.1-NC, and siRNA-NC in this study were used as the corresponding negative control. The miR-4458 mimics, miR-NC mimics, pcDNA3.1-STAT3, pcDNA3.1-NC, siRNA-PD-L1, siRNA-NC plasmids were obtained from GenePharma Co., Ltd. (Shanghai, China). The A549 and LLC cells (5×10^5 cells/well, respectively) were seeded into 6-well plates and cultured overnight and then transfected with indicated molecules including miR-4458 mimics or miR-NC mimics, pcDNA3.1-STAT3 or pcDNA3.1-NC, and siRNA-PD-L1 or siRNA-NC by Lipofectamine 2000 (Invitrogen, USA). The transfected cells were selected using 800 µg/ml of G418 48 h after transfection. All the assays were performed using G418-resistant cells.

CCK-8 Assay

CCKit-8 (Sigma, Japan) was applied to analyze the proliferation of cells. After transfection, cells (5×10^3 cells/well) were seeded into 96-well plates and cultured in 5% CO_2 at 37 °C and incubated with CCK-8 reagent (10 µL) for 2 h.

The absorbance was assessed at 450 nm by a microplate reader (Thermo, USA) at various time points.

Animal Experiments

Female C57BL/6 mice aged 6–8 weeks were obtained from the Shanghai Experimental Animal Center and raised at 22–24 °C with free access to food. A total of 45 mice were subcutaneously injected with 1×10^6 LLC cells in 100 µl serum-free DMEM in corresponding groups (Blank, miR-NC, miR-4458, miR-NC+pcDNA-NC, miR-NC+pcDNA-STAT3, miR-4458+pcDNA-NC, miR-4458 mimics+pcDNA-STAT3, miR-4458 mimics+pcDNA-STAT3+siNC, miR-4458 mimics+pcDNA-STAT3+siPD-L1) (5 mice/group) into the right flank. The mean tumor size (mm^3) and tumor weight (g) were detected, and the tumor volumes were assessed: tumor volume = (length × width^2)/2. All animal experiments were undertaken according to the NIH Guide for Care and Use of Laboratory Animals.

Flow Cytometry

Tumor tissues were minced and filtered through a 300 mesh to make a single-cell suspension 15 days after injection. Cells were stained with the following antibodies: CD3-FITC, CD4-APC, CD8-APC, PD-1-PE (Biolegend, USA). Cells were fixed and permeabilized with BD Cytofix/Cytoperm (Biolegend, USA) for intracellular staining. Flow cytometry was performed on BD AccuriC6 (BD Biosciences, USA) and Flow Jo was selected to analyze the data.

Luciferase Activity Experiment

The WT or MUT of the human STAT3 3′ UTR containing miR-4458 binding site was cloned into the luciferase reporter plasmid vector (Promega). The plasmid containing MUT or WT of STAT3 3'UTR and miR-4458 mimics or negative control were transfected into cells, and Dual-Luciferase Reporter Assay System (Promega, USA) was used to detect luciferase activities after transfection 48 h.

Statistical Analysis

All trials were conducted at least three times and all the data were shown as mean ± SD. Statistical analysis was

Table 2 Forward and reverse primers sequences of each gene

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
miR-4458	AGAGGTAGGTGTGGAAGAA	GCGAGCACAGAATTAATACGAC
STAT3	ACCTGCAGCAATACCATTGAC	AAGGTGAGGGACTCAAACTGC
GAPDH	CCATCTTCCAGGAGCGAGAT	TGCTGATGATCTTGAGGCTG
U6	CTCGCTTCGGCAGCACATATA	ACGCTTCACGAATTTGCGTGT

used GraphPad Prism 8.0 (USA). The data were analyzed by ANOVA or t-test. P value < 0.05 was considered statistically significant.

Results

The Expression Level of miR-4458 was Downregulated and STAT3 was Upregulated in NSCLC Tissues and Cells

We first measured the levels of lymphocyte subsets and the levels of PD-L1 and PD-1 in peripheral blood of NSCLC patients and healthy subjects (normal). In NSCLC samples,

the levels of CD3$^+$ T, CD4$^+$ T, CD8$^+$ T, and NK cells were slightly lower (Table 3), while the expression levels of PD-L1 and PD-1 were remarkably increased (Table 4).

The expression levels of miR-4458 and STAT3 were assessed by qRT-PCR. The results demonstrated that the expression of miR-4458 was downregulated (Fig. 1A, $P < 0.01$) and STAT3 was upregulated in NSCLC tumor tissues (Fig. 1B, $P < 0.01$). Pearson's correlation analysis was applied to analyze the potential correlation between the expression levels of miR-4458 and STAT3. Figure 1C showed that the level of miR-4458 ($F = -0.201$, $P < 0.05$) was negatively correlated with the STAT3 level. Besides, the levels of miR-4458 in H1299, A549, and SW900 cells were lower than that in normal human embryonic lung

Table 3 The detection of lymphocyte subsets in peripheral blood of patients in each group (%, $\overline{\chi} \pm s$)

	Case	CD3$^+$ T	CD4$^+$ T	CD8$^+$ T	NK
Healthy	50	71.09 ± 10.12	39.11 ± 11.95	34.69 ± 9.21	23.67 ± 9.87
NSCLC	62	67.89 ± 11.02	35.10 ± 10.32	30.11 ± 8.31	21.02 ± 10.03

Table 4 The detection of PD-L1, PD-1 in peripheral blood of patients in each group (%, $\overline{\chi} \pm s$)

	Case	CD4$^+$PD-L1	CD8$^+$PD-L1	CD4$^+$PD-1	CD8$^+$PD-1
Healthy	50	5.09 ± 1.12	9.11 ± 1.95	4.62 ± 1.67	1.04 ± 0.45
NSCLC	62	19.89 ± 7.06	25.10 ± 8.37	30.11 ± 9.04	21.06 ± 5.54
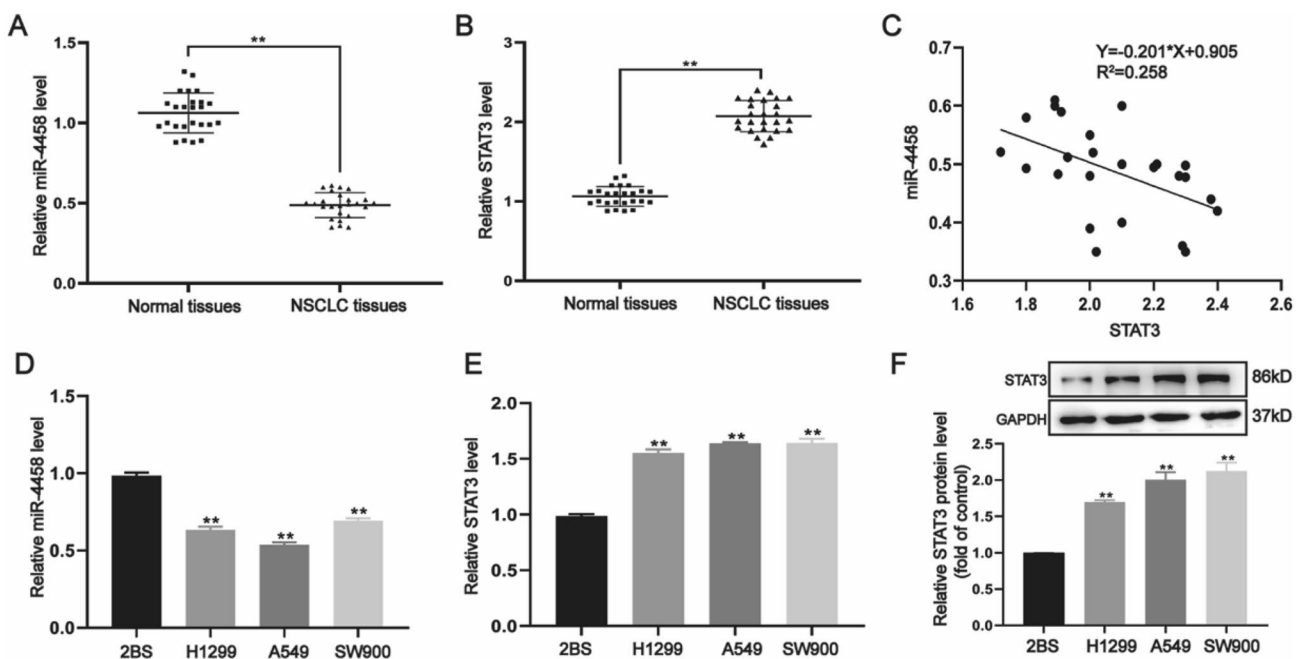
Fig. 1 The expression of miR-4458 and STAT3 in NSCLC tissues and NSCLC cells. **A** and **B** qRT-PCR was used to detect the expression of miR-4458 and STAT3 in NSCLC lung cancer tissues and tumor-adjacent normal tissues. **C** The correlation analysis between the expression levels of miR-4458 and STAT3. **D** and **E** qRT-PCR was used to detect the expression of miR-4458 and STAT3 in human normal lung cell (2BS) and NSCLC cell lines (A549, H1299, SW900). **F** The protein levels of STAT3 in A549, H1299, SW900, and 2BS were detected by western blot. The data were expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$

fibroblasts 2BS, while the expression level of STAT3 was markedly upregulated in lung cancer cells (Fig. 1D, E, $P < 0.01$). The result of the western blot on the detection of STAT3 expression was consistent with that of qRT-PCR (Fig. 1F, $P < 0.01$). The above results indicated that the level of miR-4458 was reduced and the STAT3 level was raised in NSCLC tissues and cells.

Overexpression of miR-4458 Inhibited Cell Proliferation, Tumor Growth, and Enhanced Anti-tumor Immunity

To analyze the role of miR-4458 on cell proliferation, miR-4458 or miR-NC mimics were transfected into A549 and LLC cells. Figure 2A showed that the level of miR-4458 was significantly higher in the miR-4458 mimics group ($P < 0.01$). The result of the CCK-8 assay showed that the proliferation of A549 and LLC cells was remarkably suppressed in the miR-4458 mimics group at 72 and 96 h (Fig. 2B, $P < 0.01$).

We then explored the effect of miR-4458 on tumor immunity using the xenograft LLC mice model. We observed that the tumor volume was decreased in the miR-4458 mimics group on day 12 and 15 ($P < 0.01$). Tumor weight was also significantly reduced in the miR-4458 mimics group (Fig. 2C, $P < 0.05$). Immune cells were extracted from tumors and stained for flow cytometry analysis. The proportion of $CD4^+$ T and $CD8^+$ T cells were dramatically upregulated in the miR-4458 mimics group (Fig. 2D, $P < 0.05$). Besides, the serum IFN-γ and IL-2 levels were markedly upregulated in the miR-4458 mimics group, while the IL-10 level was reduced (Fig. 2E, $P < 0.05$). The above outcomes demonstrated that overexpression of miR-4458 could inhibit cell proliferation, tumor growth, and enhance anti-tumor immunity.

MiR-4458 Directly Targeted STAT3

Bioinformatics analysis (TargetScan 6.2 database) predicted that STAT3 was a target of miR-4458 (Fig. 3A). The luciferase reporter experiment was conducted to confirm this prediction. Figure 3B implicated that the relative luciferase activity was remarkably reduced by co-transfection with STAT3 wild-type (WT) and miR-4458 mimics compared with co-transfection with STAT3 mutation (MUT) and miR-4458 mimics ($P < 0.01$). To further confirm the influence of miR-4458 on STAT3 expression, we transfected miR-4458 mimics into A549 and LLC cells and discovered that miR-4458 abated expression of STAT3, along with decreased phosphorylation level of Tyr705-STAT3 (Fig. 3C, D, $P < 0.05$). These findings illustrated that STAT3 was a direct target gene of miR-4458.

MiR-4458 Inhibited Cell Proliferation by Downregulating STAT3

To verify whether miR-4458 inhibits cell proliferation by regulating STAT3, miR-4458 mimics, and pcDNA-STAT3 or negative control were transfected into cells. The results indicated that the mRNA expression of STAT3 was inhibited by miR-4458 mimics, which was reversed by STAT3 overexpression in both A549 and LLC cells (Fig. 4A, $P < 0.05$). Moreover, the inhibitive effects of miR-4458 mimics on the proliferation of A549 and LLC cells were markedly recovered after the overexpression of STAT3 (Fig. 4B, $P < 0.01$). The results reported that STAT3 was involved in the inhibitive effect of miR-4458 on cell proliferation.

MiR-4458 Enhanced Anti-tumor Immunity by Inhibiting PD-L1 Expression via Regulating STAT3

PD-L1 is a critical molecule involved in tumor cell immune escape, so we next explored whether miR-4458 could regulate PD-L1 expression. The result of the western blot showed that miR-4458 mimics markedly downregulated the expression of PD-L1, which was reversed by STAT3 overexpression in both A549 and LLC cells (Fig. 5A, $P < 0.05$). The changes in the level of PD-L1 in xenograft LLC mice tumor tissues were consistent with that in LLC cells (Fig. 5B, $P < 0.05$). The above results demonstrated that miR-4458 could inhibit the expression of PD-L1 through regulating STAT3.

For further detecting whether miR-4458/STAT3 enhanced anti-tumor immunity by regulating PD-L1, miR-4458, pcDNA-STAT3, siPD-L1, and their negative control were co-transfected into the LLC cells and LLC cells were injected into mice for constructing LLC xenograft mice. Tumor growth was detected in xenograft LLC mice and the results indicated that the overexpression of STAT3 stimulated tumor growth and neutralized the inhibitory influence of miR-4458 overexpression on tumor growth, while knockdown of PD-L1 reversed the effect of STAT3 (Fig. 5C, $P < 0.01$). The result of flow cytometry demonstrated that the percentage of $CD4^+$ T, $CD8^+$ T cells were downregulated, $PD-1^+$ T cells were upregulated in the miR-NC+pcDNA-STAT3 group and miR-4458+pcDNA-STAT3 group, while the percentage of $CD4^+$ T, $CD8^+$ T cells were upregulated, the proportion of $PD-1^+$ T cells was decreased in the miR-4458+pcDNA-STAT3+siPD-L1 group (Fig. 6A, $P < 0.01$). Figure 6B indicated that overexpression of STAT3 canceled the effect of miR-4458 on the production of cytokines, reducing the expression of IFN-γ and IL-2, promoting IL-10 expression, while inhibition of PD-L1 reversed the effect of STAT3 ($P < 0.05$). The above results demonstrated that miR-4458 enhanced anti-tumor immunity by downregulating PD-L1 expression via targeting STAT3.
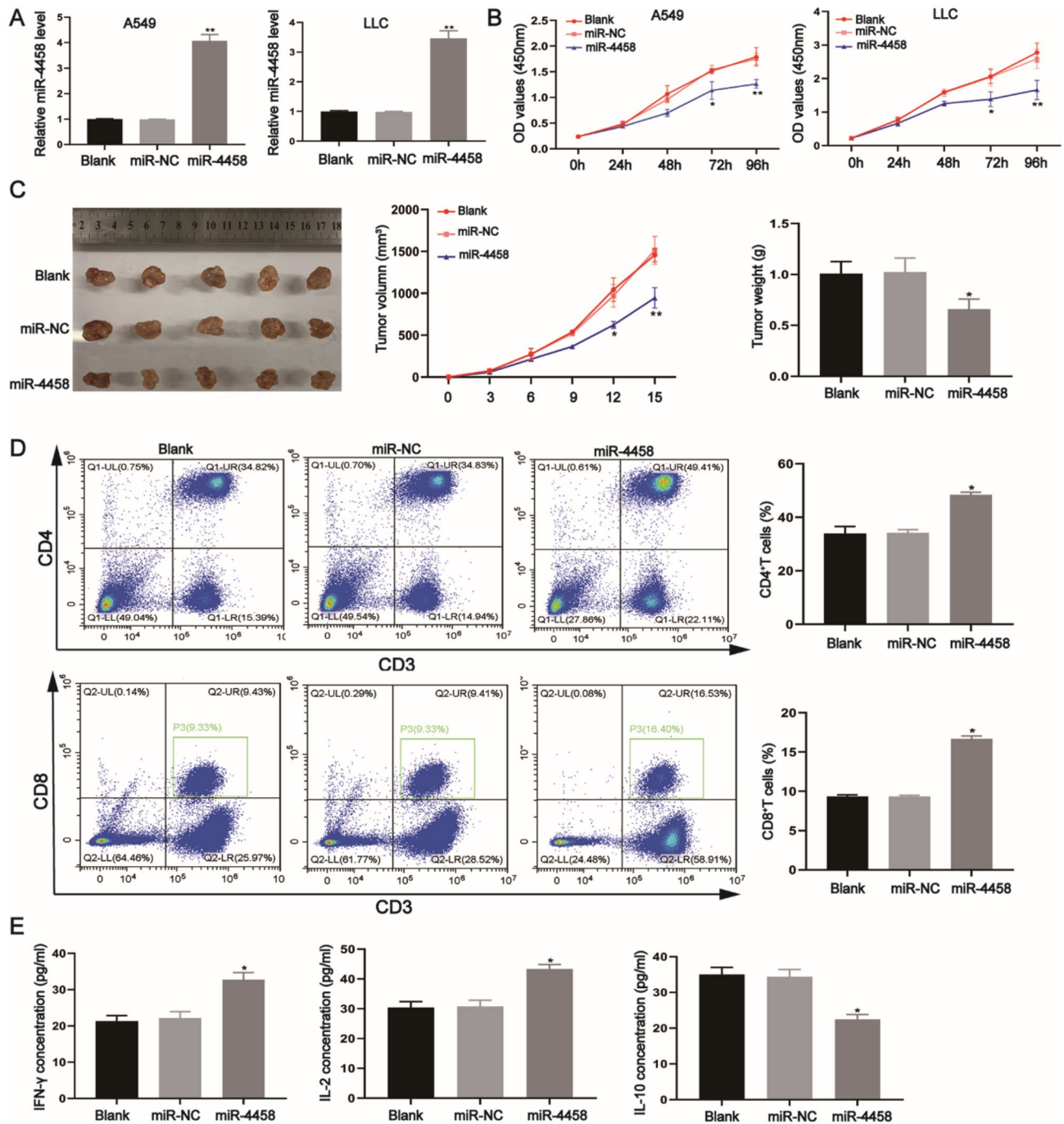
Fig. 2 MiR-4458 inhibited cell proliferation, tumor growth, and enhanced anti-tumor immunity. **A** qRT-PCR was used to detect the expression of miR-4458 in A549 and LLC cells. **B** CCK-8 assay detected the effect of miR-4458 on the proliferation of A549 and LLC cells. **C** The growth curve and weight of the lung tumor of xenograft LLC mice. **D** Flow cytometry was used to detect the proportion of CD4$^+$ and CD8$^+$ T cells in tumor tissues of xenograft LLC mice. **E** The expression of cytokines IFN-γ, IL-2, IL-10 in serum of xenograft LLC mice were detected by ELISA. The data were expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$

Discussion

NSCLC is an aggressive disease with one of the poorer prognoses among cancers [25]. The effectiveness of the existing radiotherapy, chemotherapy, and targeted therapy on the

prognosis of lung cancer patients is not satisfied. One of the outstanding characteristics of NSCLC is the low immunogenicity and the induction of immune tolerance [26]. Immunotherapy targeted PD-1 or PD-L1 has been applied to the treatment of NSCLC [27].
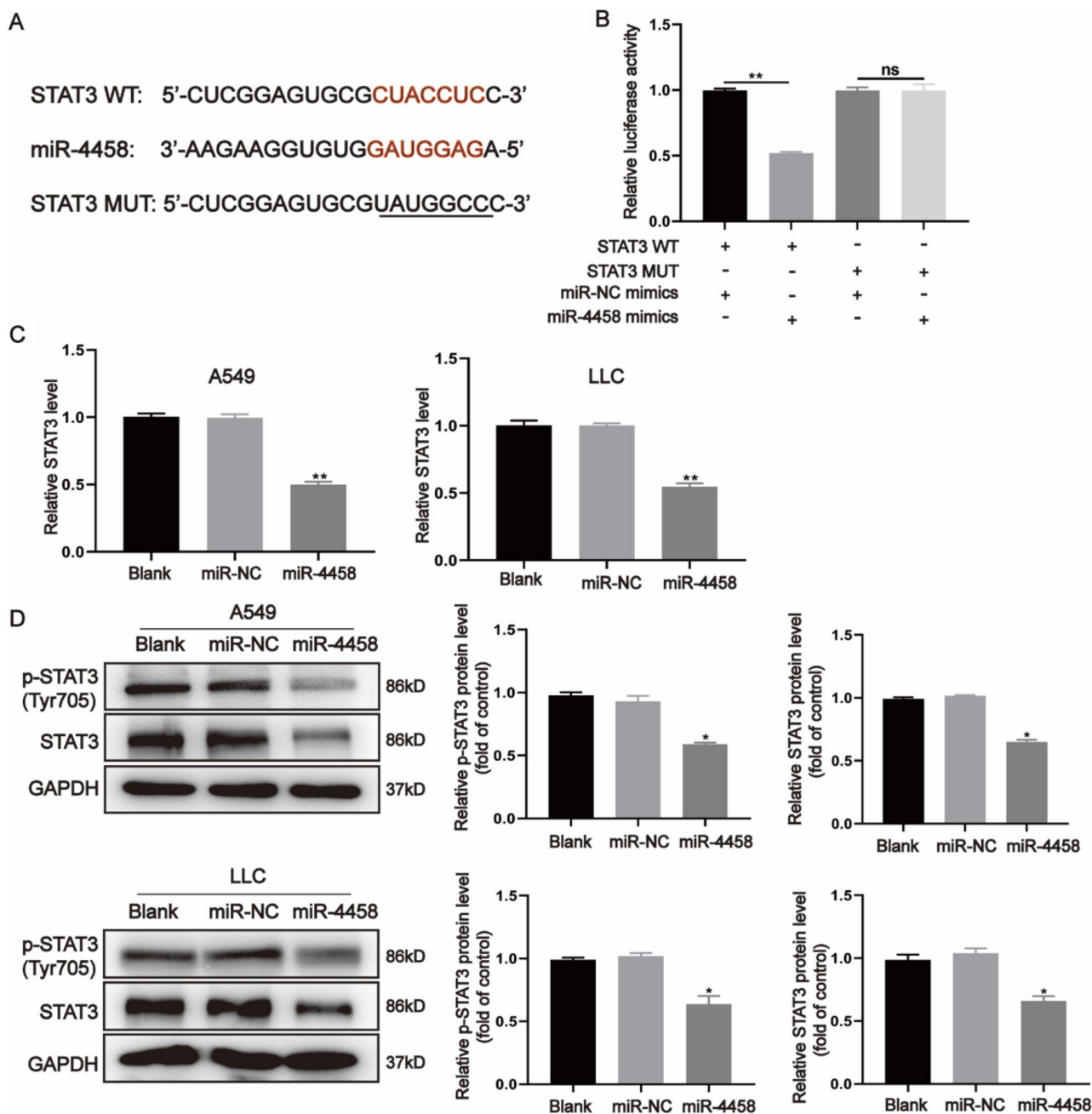
Fig. 3 STAT3 was regulated by miR-4458. **A** The miR-4458 binding sites in the 3'-UTR of PD-L1. **B** Luciferase activity in miR-NC or miR-4458 and STAT3-WT or STAT3-MUT co-transfected cells was detected by dual-luciferase reporter assay. **C** The mRNA expression of STAT3 was detected by qRT-PCR in A549 and LLC cells. **D** Western blot was used to detect the expression level of total STAT3 and phosphorylation level of Tyr705-STAT3 in A549 and LLC cells. The data were expressed as mean $\pm$ SD of three independent experiments. *$P < 0.05$, **$P < 0.01$

The PD-1/PD-L1 pathway is an essential channel of tumor immune evasion [26, 28, 29]. PD-L1 engages with PD-1 to suppress the proliferation of T cells and the production of cytokines [30]. The method to regulate PD-L1 expression is essential for exploring the new treatment strategies to improve the blocking effect of tumor immune

checkpoints. Our research found that the levels of PD-L1 and PD-1 were remarkably increased in peripheral blood of NSCLC patients, which was showing no difference from the findings of Meniawy et al. They showed that the percentages of PD-L1(+) CD3(+) T cells were higher in NSCLC patients [31]. MiRNAs have also been found to act as key

Fig. 4 MiR-4458 inhibited cell proliferation by down-regulating STAT3. **A** qRT-PCR was used to detect the expression of STAT3 in A549 and LLC cells. **B** CCK-8 assay was used to detect the proliferation of A549 and LLC cells. The data were expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$
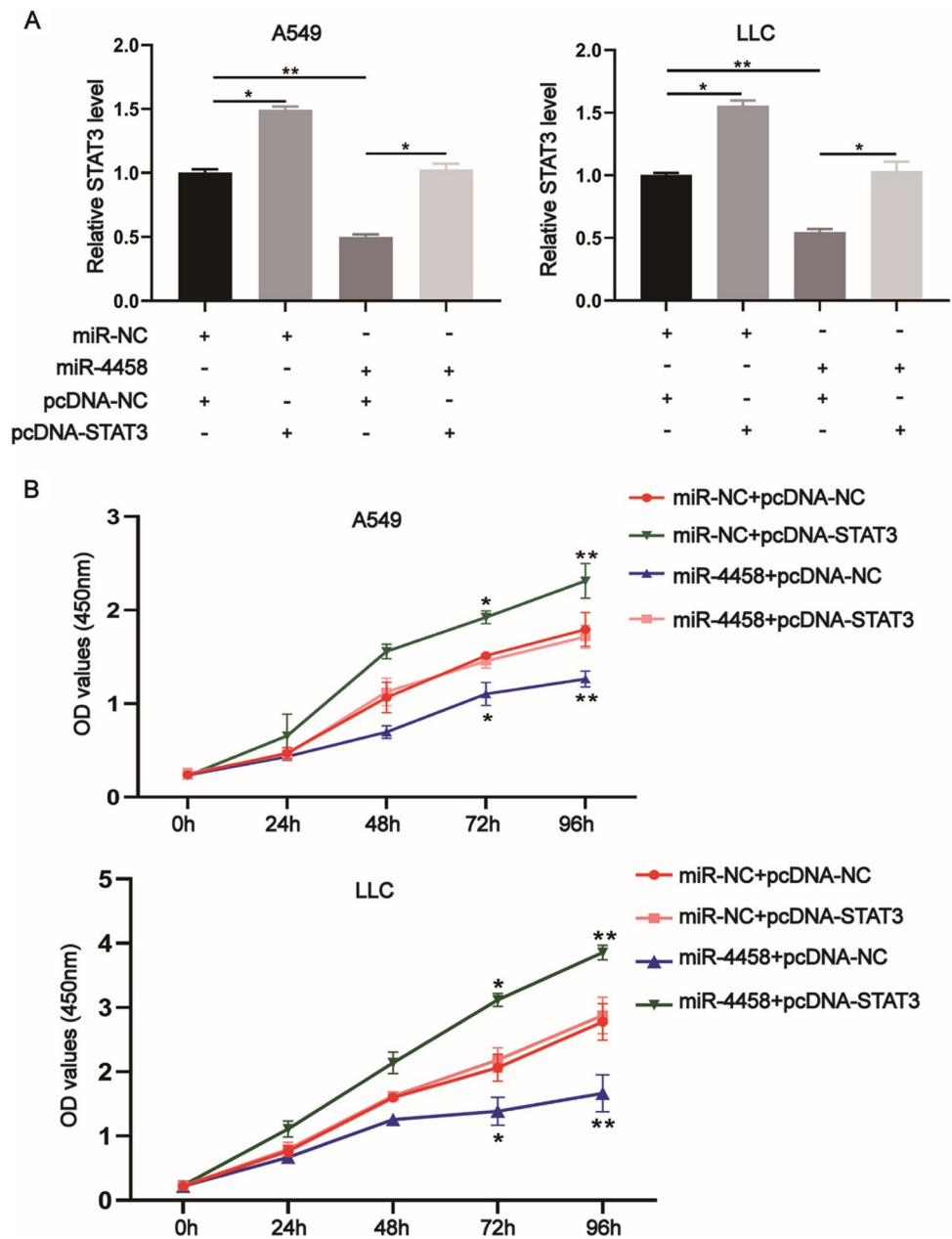
factors to regulate PD-L1 expression in cancer cells. MiR-424 suppressed PD-L1 and CD80 expression by directly binding to their 3'-UTR and reduced the chemoresistance of ovarian cancer [32]. Another study found that overexpression of miR-195 alleviated immune escape of DLBCL via regulating PD-L1 [33]. In recent years, miR-4458 was found to act as a critical regulatory molecule that participates in cellular processes in many cancers [21, 34, 35]. The action mechanisms of miR-4458 on tumor immunity have not been explored before. In the present research, we reported that the level of miR-4458 decreased significantly in NSCLC tissues and cells, its abnormal expression level might be involved in the initiation of NSCLC. Besides, miR-4458

markedly suppressed the proliferation of NSCLC cells and tumor growth. At the same time, it significantly upregulated the proportion of CD4$^+$ T, CD8$^+$ T cells and promoted the expression of cytokines IFN-γ, IL-2, indicating that miR-4458 had anti-tumor immune effects.

STAT3 regulates the expression of genes associated with the cell cycle, survival, and immune response associated with the development of a series of cancers [36]. Research has shown that STAT3 could regulate PD-L1 expression [37]. The inhibition of STAT3 expression suppressed the expression of PD-L1 [38]. We found that miR-4458 directly targeted the STAT3 and inhibited its expression. Overexpression of STAT3 reduced the inhibitory effects of miR-4458
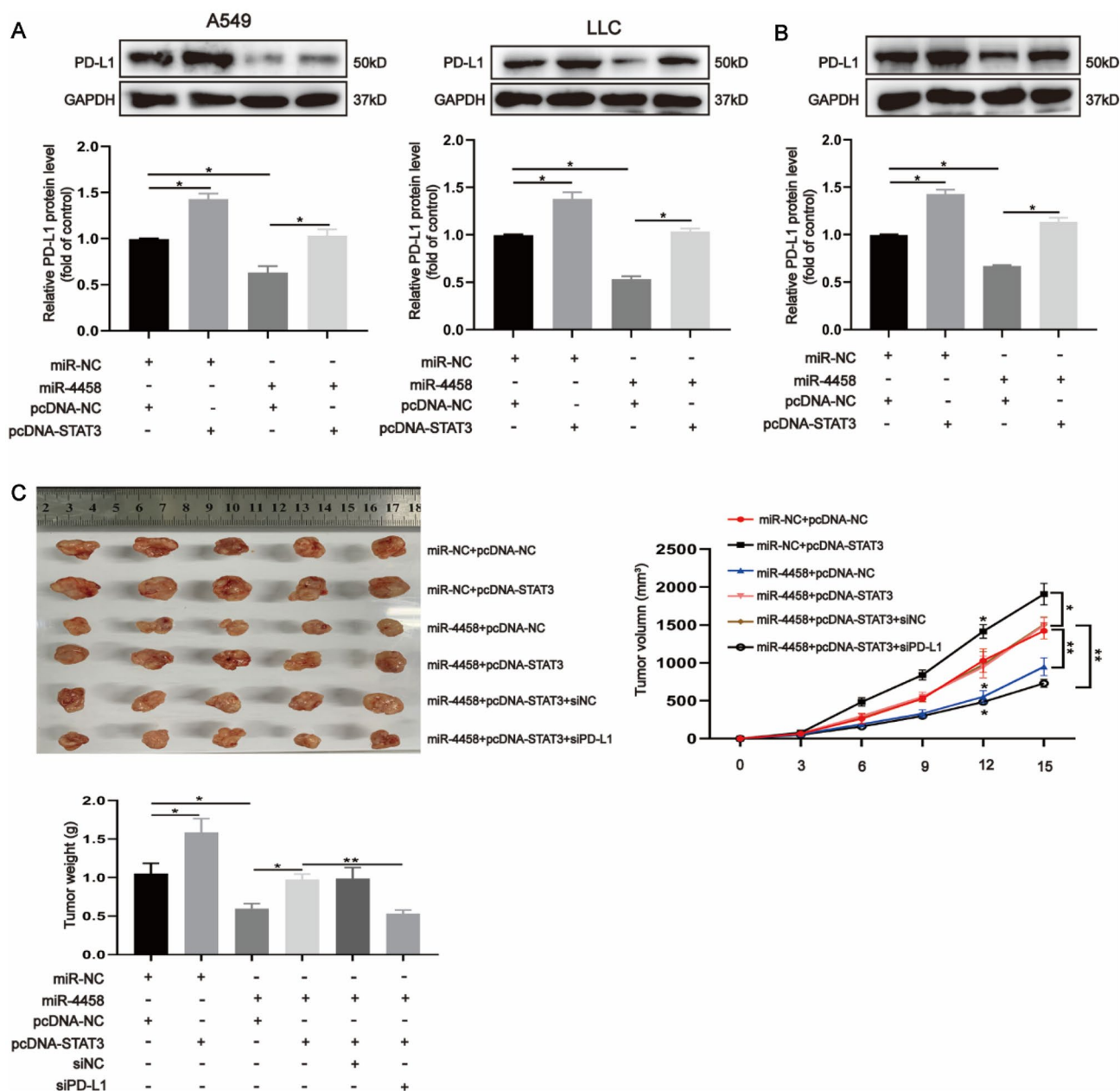
Fig. 5 MiR-4458 inhibited tumor growth by down-regulating PD-L1 via STAT3. **A** Western blot was used to detect the expression of PD-L1 in A549 and LLC cells. **B** Western blot was used to detect the expression of PD-L1 in tumors of xenograft LLC mice. **C** The growth curve and weight of the lung tumor of xenograft LLC mice. The data were expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$

on lung cancer cell proliferation, tumor growth, PD-L1 and PD-1 expression, and partially reversed the upregulation of CD4^{+} T, CD8^{+} T cell proportions and cytokine IFN-γ, IL-2 productions which were induced by miR-4458. Based on these results, we inferred that miR-4458 inhibited tumor immunity by targeting STAT3 and exerted an anti-tumor role. In addition, knockdown of PD-L1 reversed the effect of STAT3 on tumor escape, suggesting that miR-4458 played an anti-tumor immune role by reducing the expression of

PD-L1 via targeting STAT3. The finding of the present study could facilitate the understanding of immune modulation mechanisms of miR-4458, and the effects of a miR-4458 agonist with PD-1/PD-L1 immune checkpoint inhibitor in NSCLC cancer could be carried out to investigate in future work.

Collectively, we found that miR-4458 enhances anti-tumor immunity via targeting STAT3 to inhibit PD-L1 expression in NSCLC. Our research demonstrated the role of
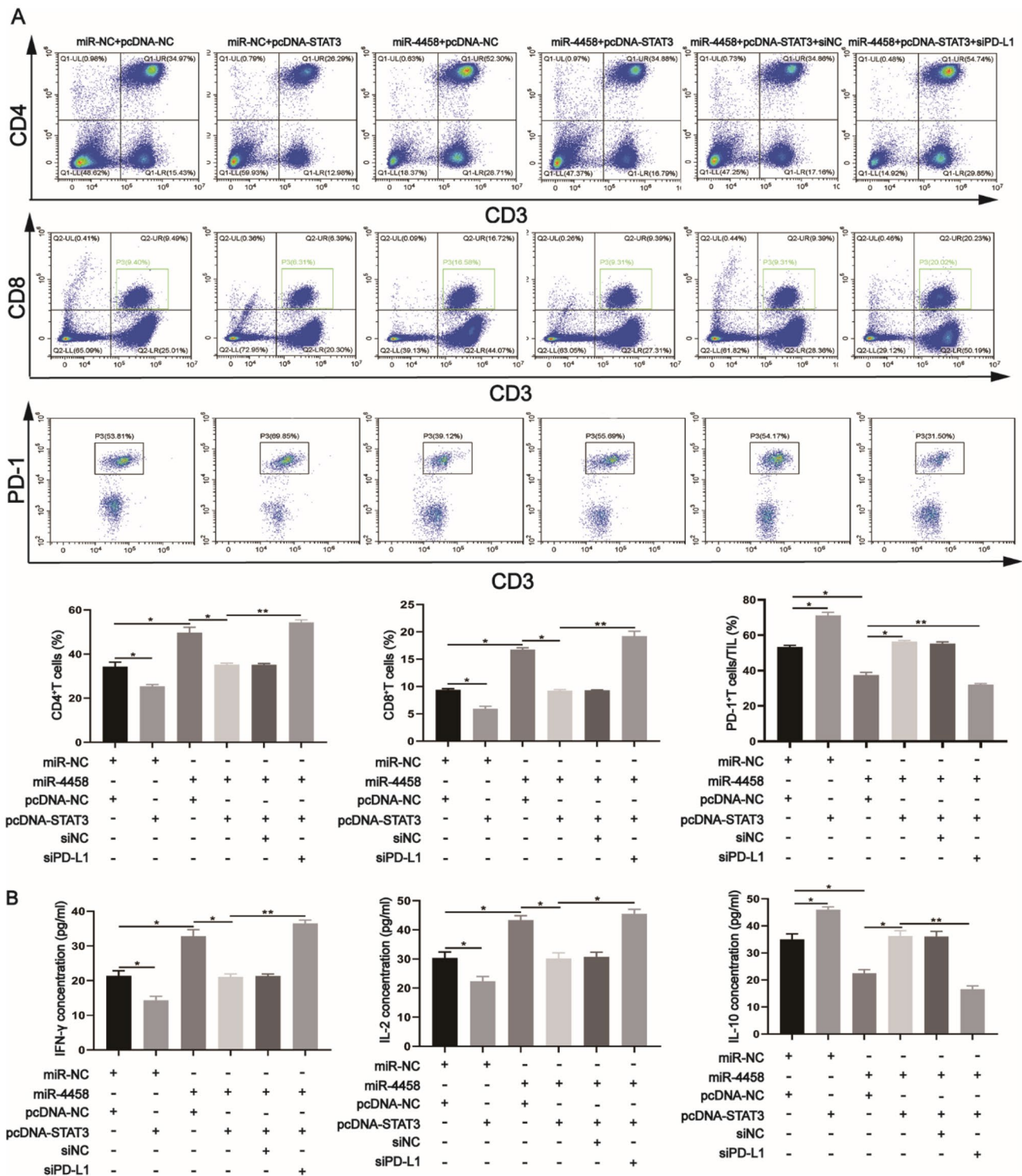
Fig. 6 MiR-4458 enhanced anti-tumor immunity by down-regulating PD-L1 via STAT3. **A** Flow cytometry was used to detect the proportion of CD4+ T, CD8+ T, and PD-1+ T cells in tumor tissues of xenograft LLC mice. **B** The expression of cytokines IFN-γ, IL-2, IL-10 in the serum of xenograft LLC mice were detected by ELISA. The data were expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$

miR-4458 in tumor immunity and discussed its mechanism in NSCLC for the first time.

Declarations

References

1. Tian, L., Wu, Y., Wang, D., Zhou, Z., Xue, S., Zhang, D., Wei, Y., & Liu, W. (2019). Upregulation of long noncoding RNA (lncRNA) X-inactive specific transcript (XIST) is associated with cisplatin resistance in non-small cell lung cancer (NSCLC) by downregulating MicroRNA-144-3p. *Medical Science Monitor, 25*, 8095–8104.

2. Gazdar, A., Bunn, P., & Minna, J. (2017). Small-cell lung cancer: What we know, what we need to know and the path forward. *Nature Reviews. Cancer, 17*, 765.

3. Miller, K. D., Nogueira, L., Mariotto, A. B., Rowland, J. H., Yabroff, K. R., Alfano, C. M., Jemal, A., Kramer, J. L., & Siegel, R. L. (2019). Cancer treatment and survivorship statistics, 2019. *CA: A Cancer Journal for Clinicians, 69*, 363–385.

4. Brody, R., Zhang, Y., Ballas, M., Siddiqui, M., Gupta, P., Barker, C., Midha, A., & Walker, J. (2017). PD-L1 expression in advanced NSCLC: Insights into risk stratification and treatment selection from a systematic literature review. *Lung Cancer (Amsterdam, Netherlands), 112*, 200–215.

5. Valverde, A., Sinnett-Smith, J., Van Lint, J., & Rozengurt, E. (1994). Molecular cloning and characterization of protein kinase D: A target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proceedings of the National Academy of Sciences of the United States of America, 91*, 8572–8576.

6. Kuol, N., Stojanovska, L., Nurgali, K., & Apostolopoulos, V. (2017). PD-1/PD-L1 in disease. *Immunotherapy, 10*, 149.

7. Mu, C., Huang, J., Chen, Y., Chen, C., & Zhang, X. (2011). High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. *Medical Oncology (Northwood, London, England), 28*, 682–688.

8. Arora, L., Kumar, A. P., Arfuso, F., Chng, W. J., & Sethi, G. (2018). The role of signal transducer and activator of transcription 3 (STAT3) and its targeted inhibition in hematological malignancies. *Cancers*. https://doi.org/10.3390/cancers10090327

9. Toyonaga, T., Nakano, K., Nagano, M., Zhao, G., Yamaguchi, K., Kuroki, S., Eguchi, T., Chijiiwa, K., Tsuneyoshi, M., & Tanaka, M. (2003). Blockade of constitutively activated Janus kinase/signal transducer and activator of transcription-3 pathway inhibits growth of human pancreatic cancer. *Cancer Letters, 201*, 107–116.

10. Bu, L. L., Yu, G. T., Wu, L., Mao, L., Deng, W. W., Liu, J. F., Kulkarni, A. B., Zhang, W. F., Zhang, L., & Sun, Z. J. (2017). STAT3 induces immunosuppression by upregulating PD-1/PD-L1 in HNSCC. *Journal of Dental Research*. https://doi.org/10.1177/0022034517712435

11. He, G., & Karin, M. (2011). NF-κB and STAT3—Key players in liver inflammation and cancer. *Cell Research, 21*, 159–168.

12. Song, T., Nairismagi, M., Lim, J., Nagarajan, S., Pang, J. W., et al. (2017). Oncogenic activation of stat3 pathway drives PD-L1 expression in natural killer/T cell lymphoma. *Hematological Oncology, 35*, 163–164.

13. Piao, L., Tingting, H., Qi, Z., Dian, L., Yihua, W., et al. (2019). FGFR2 Promotes expression of PD-L1 in colorectal cancer via the JAK/STAT3 signaling pathway. *Journal of Immunology., 202*, 3065–3075.

14. Zhou, Y., Shi, H., Du, Y., Zhao, G., & Huang, Y. (2019). lncRNA DLEU2 modulates cell proliferation and invasion of non-small cell lung cancer by regulating miR-30c-5p/SOX9 axis. *Aging, 11*, 7386–7401.

15. Zhao, Y., Liu, H., Li, Y., Wu, J., Greenlee, A. R., Yang, C., & Jiang, Y. (2011). The role of miR-506 in transformed 16HBE cells induced by anti-benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide. *Toxicology Letters, 205*, 320–326.

16. Sahraei, M., Chaube, B., Liu, Y., Sun, J., Kaplan, A., Price, N., Ding, W., Oyaghire, S., García-Milian, R., Mehta, S., Reshetnyak, Y., Bahal, R., Fiorina, P., Glazer, P., Rimm, D., Fernández-Hernando, C., & Suárez, Y. (2019). Suppressing miR-21 activity in tumor-associated macrophages promotes an antitumor immune response. *The Journal of Clinical Investigation, 129*, 5518–5536.

17. Jia, L., Xi, Q., Wang, H., Zhang, Z., Liu, H., Cheng, Y., Guo, X., Zhang, J., Zhang, Q., Zhang, L., Xue, Z., Li, Y., Da, Y., Zhao, P., & Zhang, R. (2017). miR-142-5p regulates tumor cell PD-L1 expression and enhances anti-tumor immunity. *Biochemical and Biophysical Research Communications, 488*, 425–431.

18. Yang, J., Liu, R., Deng, Y., Qian, J., Lu, Z., Wang, Y., Zhang, D., Luo, F., & Chu, Y. (2017). MiR-15a/16 deficiency enhances anti-tumor immunity of glioma-infiltrating CD8+ T cells through targeting mTOR. *International Journal of Cancer, 141*, 2082–2092.

19. Wu, J., Miao, J., Ding, Y., Zhang, Y., Huang, X., Zhou, X., & Tang, R. (2019). miR-4458 inhibits breast cancer cell growth, migration, and invasion by targeting CPSF4. *Biochimie et Biologie Cellulaire [Biochemistry and Cell Biology], 97*, 722–730.

20. Qin, Y., Cheng, C., Lu, H., & Wang, Y. (2016). miR-4458 suppresses glycolysis and lactate production by directly targeting hexokinase2 in colon cancer cells. *Biochemical and Biophysical Research Communications, 469*, 37–43.

21. Tang, D., Sun, B., Yu, H., Yang, Z., & Zhu, L. (2015). Tumor-suppressing effect of MiR-4458 on human hepatocellular carcinoma. *Cellular Physiology and Biochemistry, 35*, 1797–1807.

22. Zhou, H., Rao, Y., Sun, Q., Liu, Y., & Chen, J. (2020). MiR-4458/human antigen R (HuR) modulates PBX3 mRNA stability in melanoma tumorigenesis. *Archives for Dermatological Research, 312*, 663–675.

23. Ma, Y., Li, X. N., Chen, S., Du, B. L., & Li, Y. M. (2019). Micro-RNA-4458 suppresses migration and epithelial–mesenchymal

transition via targeting HMGA1 in non-small-cell lung cancer cells. *Cancer Management and Research, 11*, 637–649.

24. Bao, L., Wang, L., Wei, G., Wang, Y., Wuyun, G., & Bo, A. (2016). Role of microRNA-4458 in patients with non-small-cell lung cancer. *Oncology Letters*. https://doi.org/10.3892/ol.2016.5176

25. Di Paolo, A., Del Re, M., Petrini, I., Altavilla, G., & Danesi, R. (2016). Recent advances in epigenomics in NSCLC: Real-time detection and therapeutic implications. *Epigenomics, 8*, 1151–1167.

26. Gardiner, R., Jahangeer, S., Forde, P., Ariffin, A., Bird, B., Soden, D., & Hinchion, J. (2015). Low immunogenicity in non-small cell lung cancer; do new developments and novel treatments have a role? *Cancer Metastasis Reviews, 34*, 129–144.

27. Naylor, E. C., Desani, J. K., & Chung, P. K. (2016). Targeted therapy and immunotherapy for lung cancer. *Surgical Oncology Clinics of North America, 25*, 601–609.

28. Dermani, F. K., Samadi, P., Rahmani, G., Kohlan, A. K., & Najafi, R. (2019). PD-1/PD-L1 immune checkpoint: Potential target for cancer therapy. *Journal of Cellular Physiology, 234*, 1313–1325.

29. Iwai, Y., Terawaki, S., & Honjo, T. (2005). PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *International Immunology, 17*, 133–144.

30. Freeman, G., Long, A., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L., Malenkovich, N., Okazaki, T., Byrne, M., Horton, H., Fouser, L., Carter, L., Ling, V., Bowman, M., Carreno, B., Collins, M., Wood, C., & Honjo, T. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *The Journal of Experimental Medicine, 192*, 1027–1034.

31. Meniawy, T., Lake, R., McDonnell, A., Millward, M., & Nowak, A. (2016). PD-L1 on peripheral blood T lymphocytes is prognostic in patients with non-small cell lung cancer (NSCLC) treated with EGFR inhibitors. *Lung Cancer (Amsterdam, Netherlands), 93*, 9–16.

32. Xu, S., Tao, Z., Hai, B., Liang, H., Shi, Y., Wang, T., Song, W., Chen, Y., OuYang, J., Chen, J., Kong, F., Dong, Y., Jiang, S., Li, W., Wang, P., Yuan, Z., Wan, X., Wang, C., Li, W., et al. (2016). miR-424(322) reverses chemoresistance via T-cell immune response activation by blocking the PD-L1 immune checkpoint. *Nature Communications, 7*, 11406.

33. He, B., Yan, F., & Wu, C. (2018). Overexpressed miR-195 attenuated immune escape of diffuse large B-cell lymphoma by targeting PD-L1. *Biomedicine & Pharmacotherapy [Biomedecine & Pharmacotherapie], 98*, 95–101.

34. Wu, J., Miao, J., Ding, Y., Zhang, Y., Huang, X., Zhou, X., & Tang, R. (2019). MiR-4458 inhibits breast cancer cell growth, migration, and invasiveness by targeting CPSF4. *Biochemistry and Cell Biology [Biochimie et Biologie Cellulaire], 97*, 722–730.

35. Wu, M., Tang, Y., Hu, G., Yang, C., Ye, K., & Liu, X. (2020). miR-4458 directly targets IGF1R to inhibit cell proliferation and promote apoptosis in hemangioma. *Experimental and Therapeutic Medicine, 19*, 3017–3023.

36. Furtek, S. L., Backos, D. S., Matheson, C. J., & Reigan, P. (2016). Strategies and approaches of targeting STAT3 for cancer treatment. *Acs Chemical Biology, 11*, 308.

37. Marzec, M., Zhang, Q., Goradia, A., Raghunath, P., Liu, X., Paessler, M., Wang, H., Wysocka, M., Cheng, M., Ruggeri, B., & Wasik, M. (2008). Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7–H1). *Proceedings of the National Academy of Sciences of the United States of America, 105*, 20852–20857.

38. Ding, L., Chen, X., Xu, X., Qian, Y., Liang, G., Yao, F., Yao, Z., Wu, H., Zhang, J., & He, Q. (2018). PARP1 suppresses the transcription of PD-L1 by poly(ADP-ribosyl)ating STAT3. *Cancer Immunology Research, 7*, 136–149.