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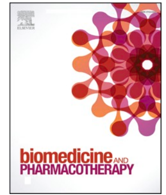

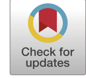

PD-L1 silencing inhibits triple-negative breast cancer development and upregulates T-cell-induced pro-inflammatory cytokines

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A R T I C L E I N F O

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A B S T R A C T

Triple-negative breast cancer (TNBC) is an invasive tumor with a high incidence of distant metastasis and poor prognosis. In TNBC cells, high PD-L1 expression can induce an immunosuppressive tumor microenvironment, repressing the anti-tumoral immune responses. Although FDA-approved agents targeting the PD-1/PD-L1 axis are potent to eliminate tumoral cells, their immune-related adverse events have become worrisome. As the regulator of gene expression, siRNAs can directly target PD-L1 in breast cancer cells. The gene modification of tumoral PD-L1 can reduce our reliance on the current method of targeting the PD-L1/PD-1 axis. We initiated the study with bioinformatics analysis; the results indicated that TNBC and the MDA-MB-231 cells significantly overexpressed PD-L1 compared to other breast cancer subtypes and cell lines. Our results demonstrated that PD-L1 silencing substantially reduced PD-L1 expression at mRNA and protein levels in MDA-MB-231 cells.

Moreover, our results demonstrated that PD-L1 knockdown reduced cancer cell proliferation and induced apoptosis via intrinsic and extrinsic apoptosis pathways. We observed that PD-L1 silencing effectively inhibited the migration of TNBC cells. Further investigation also displayed that silencing of PD-L1 in breast cancer cells induced T-cell cytotoxic function by upregulating the gene expression of pro-inflammatory cytokines, i.e., IL-2, IFN-γ, and TNF-α, and downregulating the gene expression of anti-inflammatory cytokines, i.e., IL-10, and TGF-β, in a co-culture system.

1. Introduction

Breast cancer is the most prevalent cancer among women worldwide [1]. Breast cancer is heterogeneous cancer and can be classified based on molecular patterns [2]. Since triple-negative breast cancer (TNBC) does not express the human epidermal growth factor receptor-2, progesterone receptor, and estrogen receptor, there is no effective targeted therapy for TNBC [3,4]. Thus, there is an urgent need for developing new strategies for treating TNBC patients.

Programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1), which can establish a well-known PD-L1/PD-1 immune checkpoint axis, have crucial roles in modulating the immune responses [5]. This axis has been implicated in regulating T-cells stimulation, facilitating immune evasion of tumor cells, and developing chemoresistance [6].

Many clinical trials have revealed that the blocking of PD-1/PD-L1

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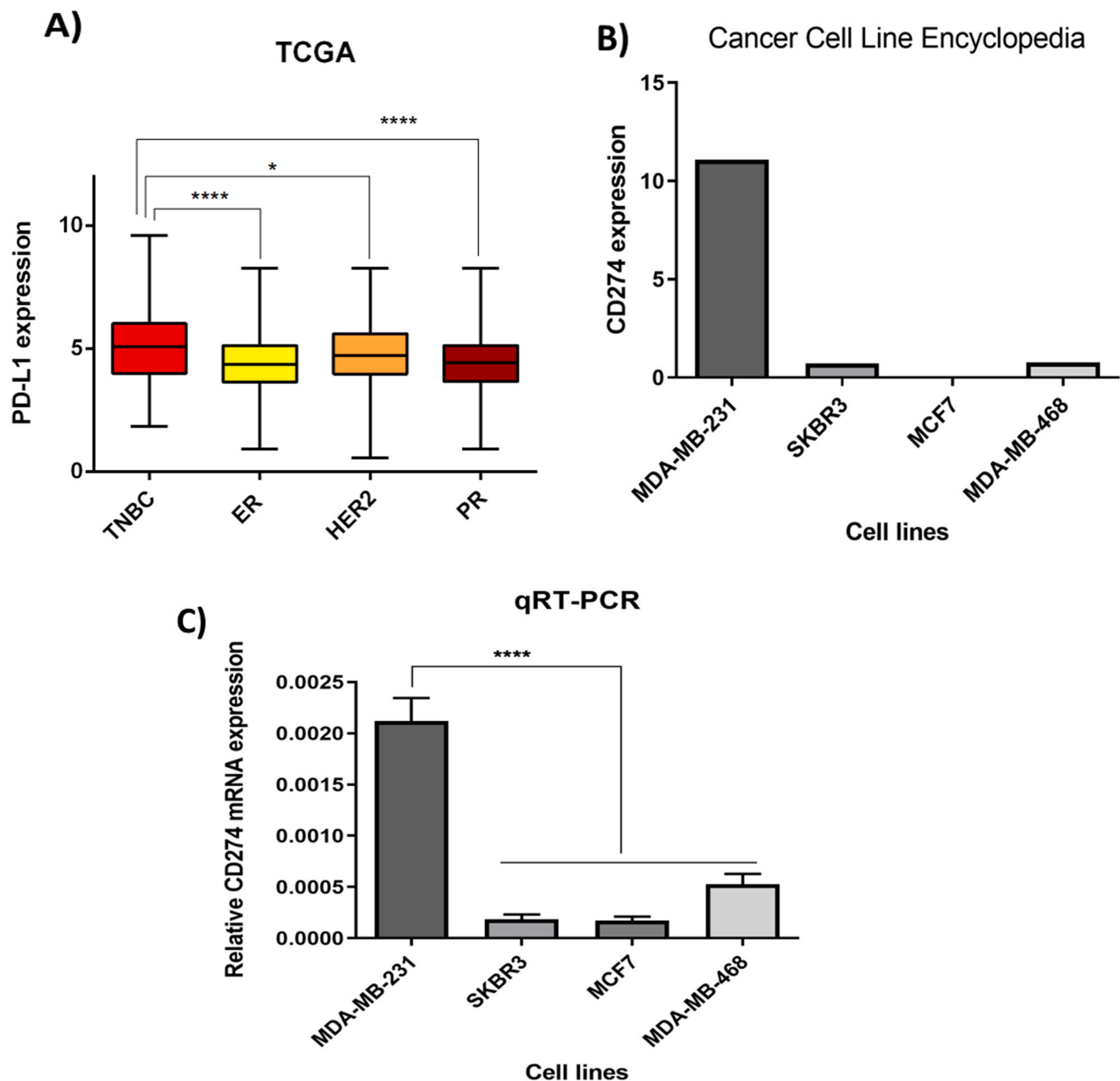

Fig. 1. PD-L1 is highly expressed in triple-negative breast cancers (TNBC) and MDA-MB-231 cells. (A) The Cancer Genome Atlas (TCGA) confirms a high expression level of PD-L1 in the TNBC subtype. (B) Cancer Cell Line Encyclopedia (CCLE) displayed a high expression level of PD-L1 in MDA-MB-231 cells. (C) Real-time PCR analysis showed that PD-L1 was significantly up-regulated in MDA-MB-231 cells compared to SKBR3, MCF7 cell lines; $^{*}P < 0.1$, $^{****}P < 0.0001$.

interactions can significantly enhance anti-tumor activity and improve prognosis in patients with diverse cancers [7–9]. Nevertheless, in many patients, unexpected responses were observed following anti-PD-1/PD-L1 immunotherapy due to the off-target antibody attachment via intravenous infusion, and autoimmune symptoms also were not negligible [10,11]. siRNA-based studies have gained more attention to develop precise and efficient therapies [12–14]. Moreover, using siRNA not only can knockdown PD-L1 expression but also, in some cases, it potentially limits de novo expression of PD-L1, which reduces cancer cell evasion and leads to prolong PD-1/PD-L1 blockade [15]. It has shown that the PD-1/PD-L1 axis is involved in the development of regulatory T-cells (Tregs) [16]. Tregs have a crucial role in tumor progression, and it is demonstrated that their elimination will lead to tumor eradication [17]. Here, we investigated whether the silencing of PD-L1 in a breast cancer cell line (MDA-231 cells) can alter anti-tumor responses of TILs in a co-culture system as an ex vivo model. Our findings indicate that silencing of PD-L1 in breast cancer cells can inhibit tumor development and also could significantly improve T-cell immune response against breast cancer cells and would be utilized as T-cell-based immunotherapy strategy in management cancers.

2. Material and methods

2.1. PD-L1 expression among breast cancer subtypes using TCGA

We investigated the PD-L1 expression among breast cancer subtypes using the Cancer Genome Atlas (TCGA) database. The PD-L1 gene expression data in the breast cancer patient cohort (TCGA-BRCA) were downloaded using Xena Functional Genomics Explorer (https://xena.ucsc.edu/). Furthermore, the PD-L1 expression in breast cancer cell lines, e.g., MDA-MB-231, SKBR3, MCF-7, and MDA-MB-468 cells, was extracted from the Cancer Cell Line Encyclopedia (CCLE) database using

Table 1
List of primer sequences and siRNA.

PD-L1-siRNA	F	5´-UAUAAAGACAGCAAAUAUCTT-3´
	R	5´-GAUAUUUGCUGUCUUUAUATT-3´
PD-L1	F	5´-TGCCGACTACAAGCGAATTACTG-3´
	R	5´-CTGCTTGTCCAGATGACTTCGG-3´
GAPDH	F	5´-CAAGATCATCAGCAATGCCT-3´
	R	5´-GCCATCACGCCACAGTTTCC-3´
MMP-9	F	5´-TTGACAGCGACAAGAAGTGG-3´
	R	5´-GCCATTCACGTCGTCCTTAT-3´
Caspase3	F	5´- ATGGTTTGAGCCTGAGCAGA -3´
	R	5´- CATCCACACATACCAGTGCGTA -3´
Casape8	F	5´-CTGGTCTGAAGGCTGGTTGTT-3´
	R	5´-GTGACCAACTCAAGGGCTCAG-3´
Caspase9	F	5´-GCAGGCTCTGGATCTCGGC-3´
	R	5´-GCTGCTTGCCTGTTAGTTCGC-3´
BAX	F	5′-GACTCCCCCCGAGAGGTCTT-3′
	R	5′-ACAGGGCCTTGAGCACCAGTT-3′
BCL2	F	5´-CTGTGGATGACTGAGTACCTG-3´
	R	5´-GAGACAGCCAGGAGAAATCA-3
CD44	F	5´-CAAGCCACTCCAGGACAAGG-3´
	R	5´-ATCCAAGTGAGGGACTACAACAG-3´
C-MYC	F	5´-AGGCTCTCCTTGCAGCTGCT-3´
	R	5´-AAGTTCTCCTCCTCGTCGCA-3´
IL-2	F	5´- AAGAATCCCAAACTCACCAG-3´
	R	5´-CGTTGATATTGCTGATTAAGTCC-3´
TNF-α	F	5´-TTCTCCTTCCTGATCGTGGCA-3´
	R	5´-TAGAGAGAGGTCCCTGGGGAA-3´
IFN-γ	F	5´- TCGGTAACTGACTTGAATGTCCA -3´
	R	5´- TCGCTTCCCTGTTTTAGCTGC -3´
TFG-β	F	5´-AACAATTCCTGGCGATACCTC-3´
	R	5´-GTAGTGAACCCGTTGATGTCC-3´
IL-10	F	5´-AACCAAAGGAAGAGAGGGAGC-3´
	R	5´- GATGTCAAACTCACTCATGGCT-3´

Xena browser (Fig. 1).

2.2. Cell culture

Under standardized culture conditions, PD-L1 expression in breast cancer cell lines was investigated. The MDA-MB-231, MCF-7, SKBR3, and MDA-MB-468 cell lines were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% pen/strep (Gibco/Life Technologies, Grand Island, NY). The cultured cells were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO2. After the cells reached 80–90% confluence, the cells were sub-cultured, and PD-L1 expression analysis was done in the log phase of cell growth. The result revealed that the MDA-MB-231 cell line exhibited the highest levels of PD-L1 expression compared to other cell lines (Fig. 1C). Then, the following experiments were performed on this cell line.

2.3. PD-L1 knockdown in MDA-MB-231 cells

MDA-MB-231 cells were transfected with 40, 60, and 80 pmol of PD-L1-siRNA and FITC-labeled siRNA in a 0.4 cm^3 Gene Pulser Cuvette (Bio-Rad). Cells were electroporated at TC = 12.5 ms and Volts = 160 v using a Gene Pulser Electroporation system. Following electroporation, cells were transferred to a 6-well plate containing RPMI-1640 medium with 10% FBS and incubated at 37 °C for 24, 48, and 72 h. Furthermore, real-time quantitative reverse transcription PCR (qRT-PCR) was used to assess transfection efficiency.

The RT-PCR technique was utilized to quantify the mRNA expression of PD-L1 in siRNA-transfected MDA-MB-231 cells. According to the manufacturer's protocol, the total RNA extraction was done by RiboEX reagent (GeneAll Biotechnology, Seoul, Korea). Complementary DNA (cDNA) was synthesized using BioFACT cDNA synthesis kit (Korea). In transfected cells, the relative gene expression of PD-L1 was calculated by comparing it to GAPDH expression as the reference gene. PD-L1-siRNA, PD-L1, and GAPDH primer sequences were provided in Table 1.

2.4. Western blot assay for determining PD-L1 protein expression

According to the manufacturer's instructions, the lysates of whole cells were prepared using RIPA lysis buffer (50 mM NaCl, 50 mM EDTA, 1% Triton X-100). Total protein was separated by SDS-PAGE and transferred to a PVDF membrane, which was blocked with 5% dry milk. Subsequently, the membranes were immersed in diluted primary antibodies specific for PD-L1 and β-actin (1:2000; Santa Cruz Biotechnology) and incubated overnight. Following the incubation, anti-mouse HRP-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was added as the secondary antibody. The protein bands were visualized by western blotting imaging system (Sabz Biomedicals, Iran), and the protein expression of PD-L1 and β-Actin (as control) were semi-quantified using ImageJ software (NIH, Bethesda, MD).

2.5. The analysis of PD-L1 cell surface expression by flow cytometry

The siRNA-transfected MDA-MB-231 cells and the control cells were trypsinized; after being washed three times with FACS buffer, the PD-L1 expression level was evaluated by cell surface-staining with FITC anti-PD-L1 (Biolegend, 329,708). These cells were studied by MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). The results were analyzed using FlowJo™ (software version 10.6).

2.6. The MTT assay following PD-L1 knockdown

The 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to determine the cytotoxicity effect of PD-L1 suppression on MDA-MB-231 cells. PD-L1-siRNA transfected cells and un-transfected cells were seeded in 96-well plates at 1×10^4 cells per well with the culture medium and incubated for 48 h. The cells transfected with scrambled siRNA were considered as a negative control (NC). Then, 50 µl (2 mg/mL) of MTT reagent (Sigma-Aldrich, Saint Louis, MO) was added to each well. The insoluble purple formazan products were dissolved with dimethyl sulfoxide (DMSO). Finally, the absorbance of transfected and un-transfected groups was measured using Tecan microplate reader (Switzerland) at 570 nm.

2.7. The effect of PD-L1 silencing on colony formation

The colony formation assay was performed to investigate the effect of PD-L1-siRNA on the clonogenicity of transfected MDA-MB-231 cells. Therefore, PD-L1-siRNA transfected MDA-MB-231 cells and un-transfected cells were seeded at 5×10^3 cells/well in a 6-well plate. Both groups were incubated at 37 °C with 5% CO_2 for seven days. After the incubation period, cells were washed with PBS (Phosphate-buffered saline) and fixed with 5% paraformaldehyde for 15 min, and stained with 0.5% crystal violet (Sigma, St Louis, MO) in ethanol. Then, the cells were washed with distilled water and visualized by an inverted system microscope.

2.8. The effect of PD-L1 silencing on cell cycle

The flow cytometry was used to evaluate the PD-L1 silencing impact on the cell cycle. The cells were seeded into a 6-well plate at a density of 1×10^6 cells/well and incubated for 48 h. After incubation time, cells were detached, washed with cold PBS, fixed in 70% cold ethanol, and incubated overnight at 4 °C. The next day cells were stained with propidium iodide solution and incubated in a dark place for 30 min. After incubation time, G1, G0/G1, S, and G2 phases in the siRNA-transfected group and control group were assessed by MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell cycle distribution was evaluated by FlowJo™ software.

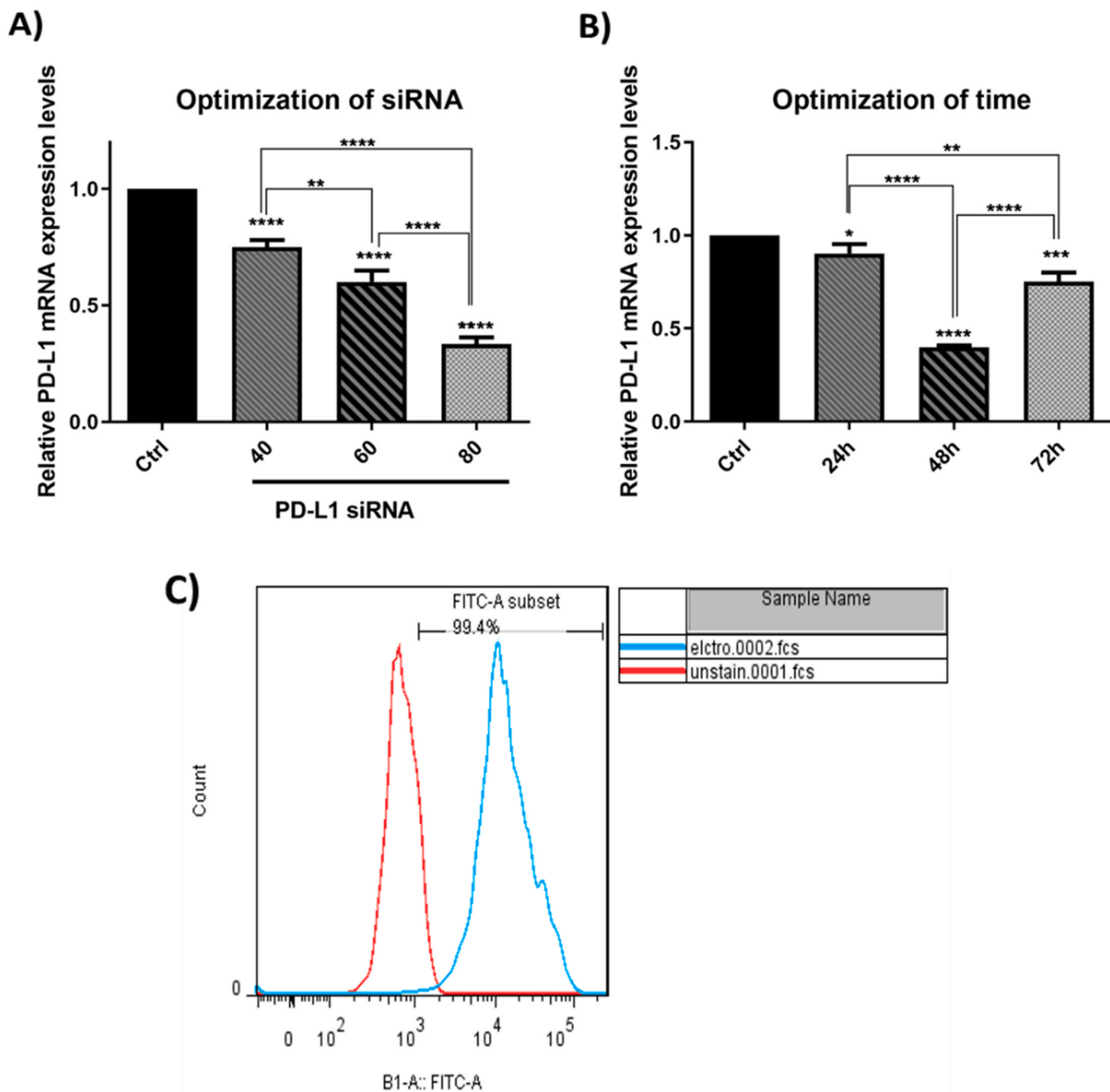

Fig. 2. PD-L1 knockdown in MDA-MB-231 cells. (A) PD-L1-siRNA transfection significantly down-regulated PD-L1 gene expression in a dose-dependent manner compared to other doses of PD-L1-siRNA and un-transfected control cells. (B) PD-L1-siRNA transfection significantly down-regulated PD-L1 gene expression at 48 h of transfection compared to 24 h or 72 h. (C) PD-L1-siRNA was effectively transfected into MDA-MB-231 cells; * $P < 0.\ 1$, *** $P < 0.001$, **** $P < 0.0001$.

2.9. Apoptosis assays

2.9.1. Annexin V/PI assay

After 48 h incubation, transfected and control cells were trypsinized and treated by Annexin-V/PI according to staining kit protocol (BD Biosciences) to determine the rate of PD-L1-siRNA-induced apoptosis. The cells were evaluated by the MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany), and the results were studied by FlowJo™ software.

2.9.2. DAPI staining

The 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to observe the nuclear changes during apoptosis. Subsequently, 1×10^4 cells/well PD-L1-siRNA transfected cells were seeded into 96-well plates and incubated for 48 h. After incubation, the cells were fixed with 4% paraformaldehyde and incubated for 40 min at room temperature. The wells were washed twice with PBS, stained with 100 ng/mL DAPI for 15 min, and washed with PBS. Afterward, chromatin fragmentation was observed via Cytation 5 cell imaging system (Biotek, USA).

2.10. The wound-healing assay

The wound-healing assay was done to evaluate the effect of PD-L1-siRNA on the migration of transfected cells. The transfected and control cells were seeded at 3×10^5 cells per well in 24-well plates. When the cells reached > 90% confluence, a scratch was created with a sterile 100 μl pipette tip. The plates were incubated at 37 °C in 5% CO_2. Photographs were taken using an inverted microscope (Optika, XDS-3, Italy) at 0 h, 24 h, 48 h, and 72 h.

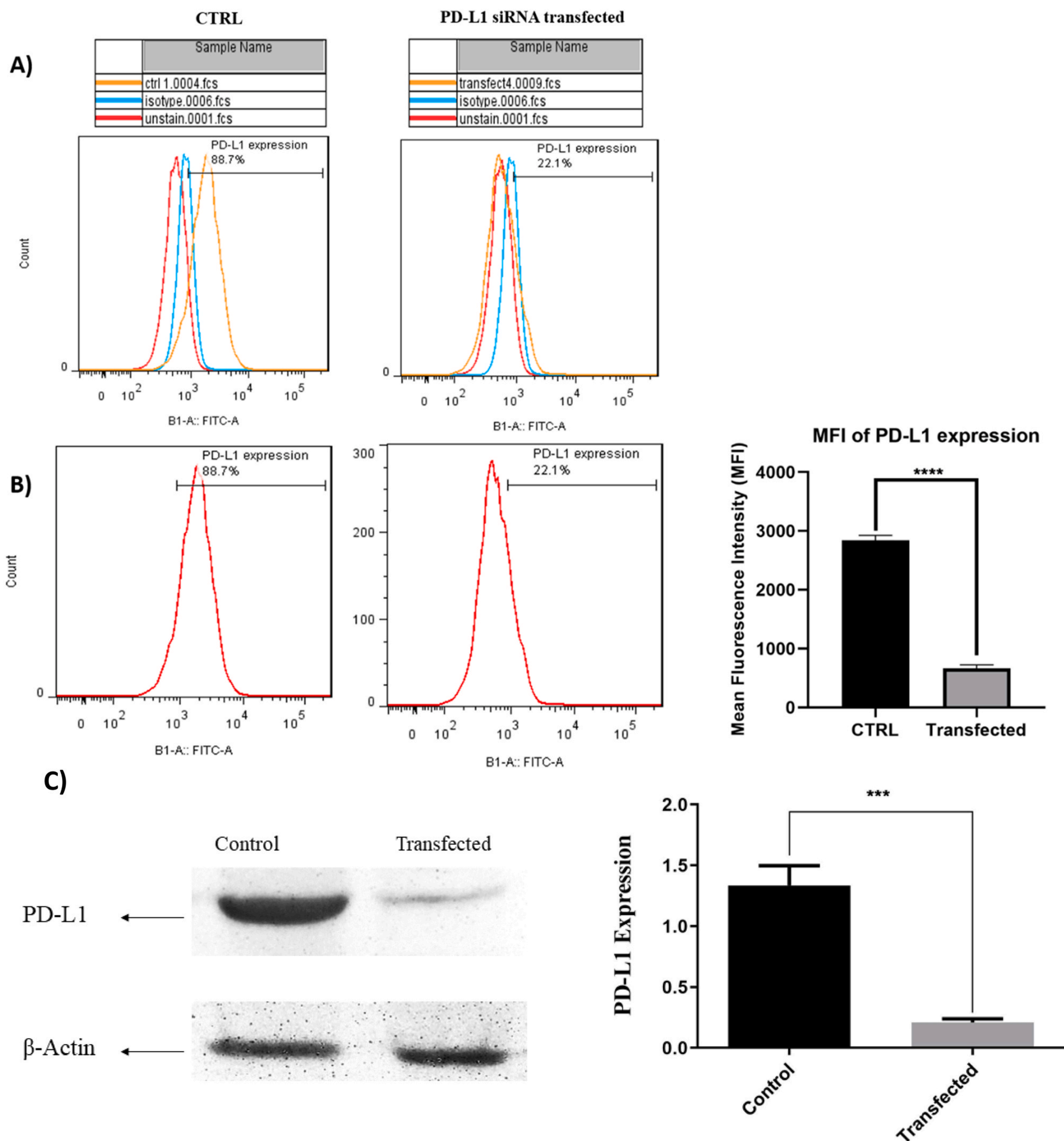

Fig. 3. The PD-L1-siRNA decreased the protein expression of PD-L1. (A). The FACS analysis of cell distribution displayed shifted the peak of fluorescent and reduced cell surface PD-L1 expression on transfected-MDA-MB-231 cells compared to the control group. (B) Mean Fluorescence Intensity (MFI) of PD-L1 expression C) According to the western blot, the protein expression of PD-L1 was decreased significantly following the PD-L1-siRNA transfection at the optimum dose and time. β-actin was used as loading control; *** $P < 0.001$.

2.11. T-cell isolation and the co-culture of T-cells with tumor cells

Peripheral blood mononuclear cells were isolated using density-gradient lymphocyte separating medium-lymphodex (Inno-Train Diagnostic, Germany). T-cell isolation from peripheral blood mononuclear population was done by the negative immunodepletion of $CD3^+$, $CD14^+$, $CD15^+$, $CD16^+$, $CD19^+$, $CD34^+$, $CD36^+$, $CD38^+$, $CD56^+$, $CD123^+$, and $CD235a^+$ using MACs kit (Miltenyi Biotec, Germany). Before co-culture, isolated T-cells were incubated at 37 °C with phytohemagglutinin (PHA; 10 μg/mL; Sigma) for 24 h to stimulate the lymphocytes. After incubation time, transfected MDA-MB-231 cells were co-cultured with activated T-cells for 48 h in a 1:1 ratio. After 48 h incubation, suspended T-cells were harvested. The interleukin (IL)−2, IL-10, transforming growth factor-β (TGF-β), interferon γ (IFN-γ), and tumor necrosis factor (TNF)-α gene expression were evaluated compared to GAPDH expression as the reference gene.

2.12. Flow cytometry for the detection of $CD25^+$ Foxp3+ T-cells

Following 48 h incubation, suspending T-cells were harvested. The intracellular detection of Foxp3 was performed in fixed and permeabilized cells. Briefly, the T-cells were labeled with antibodies for

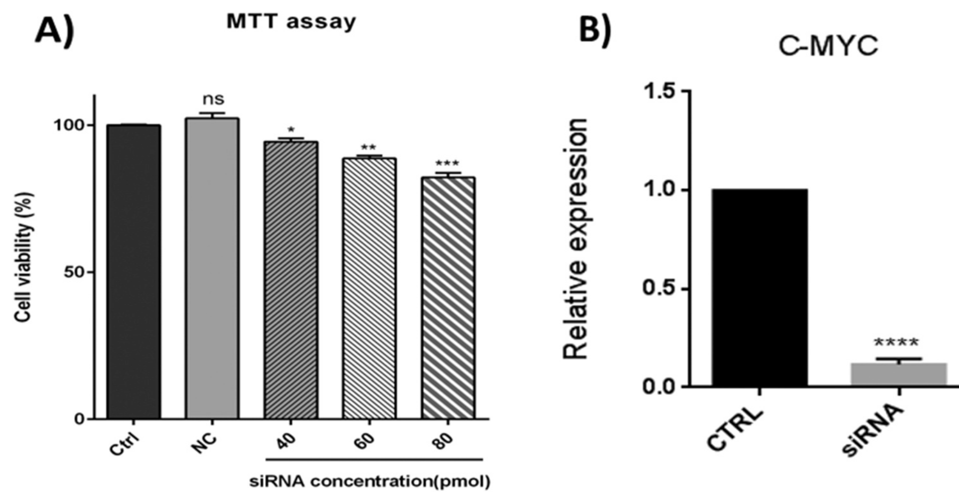

Fig. 4. MTT assay following PD-L1-siRNA transfection in MDA-MB-231 cells. (A) PD-L1-siRNA transfection significantly decreased cell proliferation of MDA-MB-231 cells in a dose-dependent manner. (B) Reduced gene expression level of c-Myc in PD-L1-siRNA transfected MDA-MB-231 cells; *P < 0. 1, **P < 0.01, ***P < 0.001, ****P < 0.0001.

CD25 cell surface molecule by APC anti-human CD25 (biolegend, 302,609). Next, the cells were fixed, permeabilized, and stained with Brilliant Violet 421™ anti-human FOXP3 (biolegend, 320,123) according to the manufacturer's instructions. Flow cytometry assay was done using FACSCalibur (Becton Dickinson, San Jose, CA, USA). The results were analyzed using FlowJo™ software.

2.13. Statistical analysis

All statistical analyses were done by GraphPad Prism version 8.0 software (GraphPad Software, Inc., San Diego, CA, USA). All results are presented as the mean± standard error. The paired student's *t*-test was performed to compare the statistical difference between the two groups. One-way analysis of variance (ANOVA) test was done to determine the statistical significance between more than two groups. P values < 0.05 were considered statistically significant. (* P < 0.1, **P < 0.05, *** P < 0.001, **** P < 0.0001).

3. Results

3.1. MDA-MB-231 cell line had the highest level of PD-L1 expression

Using TCGA database, we analyzed the PD-L1 expression in breast cancer subtypes. As presented in (Fig. 1A), the TNBC subtype displayed a significant increase in PD-L1 expression than that of ER^{+}, HER2, and PR^{+} subtypes. Based on the furthermore bioinformatics analysis, there was a significant increase in the PD-L1 expression of the MDA-MB-231 cell line compared to other cell lines (Fig. 1B). In the experimental analysis of different breast cancer cell lines, the PD-L1 expression of MDA-MB-231 cells was significantly higher than the other breast cancer cell lines (Fig. 1C, P < 0.0001).

3.2. Down-regulation of PD-L1 mRNA in MDA-MB-231 cells following siRNA transfection

Compared to the control group, the transfection of PD-L1-siRNA significantly reduced PD-L1 gene expression in a dose-dependent manner at 40, 60, 80 pmol of siRNA (Fig. 2A, P < 0.0001, P < 0.0001, and P < 0.0001, respectively). Furthermore, PD-L1-siRNA was transfected at 24 h, 48 h, and 72 h of incubation time to identify the optimum time for transfection of PD-L1-siRNA in MDA-MB-231 cells. Compared to the control group, PD-L1-siRNA was more effective in the knockdown of PD-L1 expression at 48 h of transfection time (Fig. 2A, P < 0.0001). Therefore, the following tests were adjusted at 80 pmol as the optimum dose and 48 h as the optimum time.

The FITC-labeled siRNA was also incubated with MDA-MB-231 cells to confirm the PD-L1-siRNA uptake. Flow cytometry results showed the efficiency of entered-siRNA (Fig. 2C).

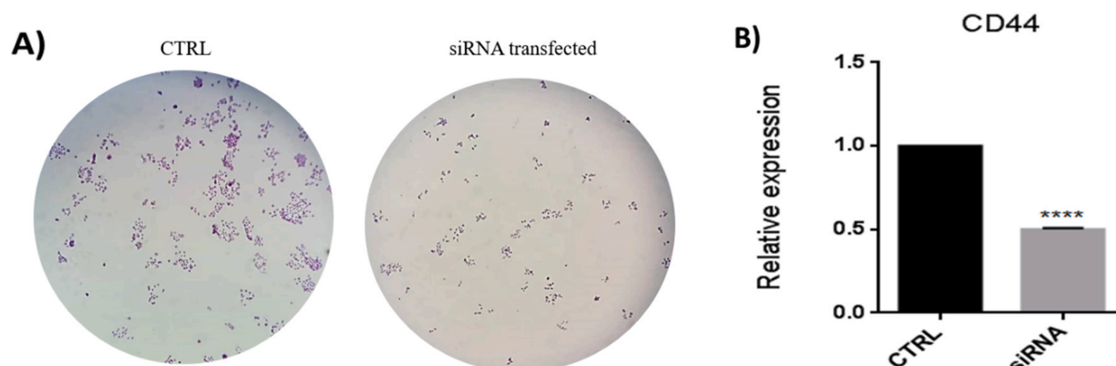

Fig. 5. The PD-L1 knockdown can decrease the clonogenicity and CD44 mRNA expression in MDA-MB-231 cells. (A) The transfection of PD-L1-siRNA decreased the clonogenicity of MDA-MB-231 cells. (B) CD44 mRNA expression displayed a significant reduction in the PD-L1-siRNA transfected MDA-MB-231 cells compared to the control group; ****P < 0.0001.

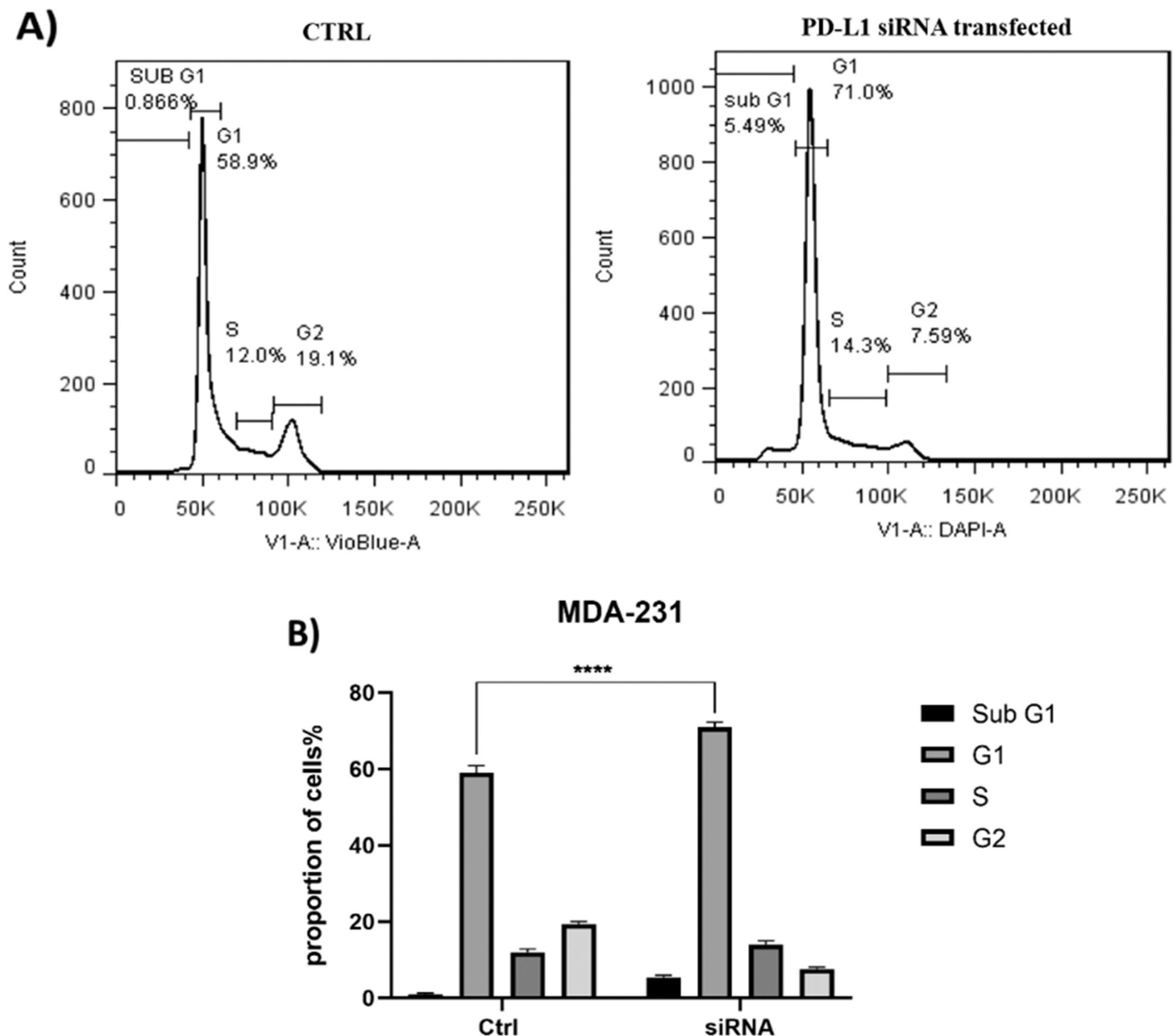

Fig. 6. Effect of PD-L1 siRNA on cell proliferation. (A). PD-L1 siRNA induced G1 cell cycle arrest in MDA-MB-231 cells. (B). Graph representing cell cycle status; ****$P < 0.0001$.

3.3. Down-regulated PD-L1 protein expression at the cell surface

Compared to the control group, flow cytometry results showed that PD-L1-siRNA transfection could significantly decrease the accumulation of MDA-MB-231 cells in the G1 phase (Fig. 3A). Following the transfection of PD-L1-siRNA, the peak of fluorescent shifted to the lower intensity part, with the mean fluorescent intensity decreased from 2847 to 693 (Fig. 3B). Compared to the control group, western blot results revealed that PD-L1-siRNA transfection at the optimum dose and time could significantly decrease the PD-L1 protein expression of MDA-MB-231 cells (Fig. 3B, $P < 0.001$).

3.4. The effect of PD-L1 silencing on cell viability

The MTT assay was done to estimate the cytotoxicity effect of PD-L1 knockdown on transfected cells. Compared to the control and negative groups, the results indicated that PD-L1-siRNA statistically decreases cell viability in PD-L1-si-RNA transfected cells after 48 h of transfection in a dose-dependent manner, i.e., 40, 60, and 80 pmol (Fig. 4A, $P < 0.1$, $P < 0.01$, and $P < 0.001$, respectively).

We also studied c-Myc gene expression as a crucial marker of cell proliferation. Compared to the control group, the results showed that PD-L1-siRNA transfection significantly decreased c-Myc mRNA expression in the transfected MDA-MB-231 cells (Fig. 4B, $P < 0.0001$).

3.5. PD-L1 knockdown significantly reduced clonogenicity of MDA-MB-231 cells

Compared to the control group, the PD-L1-siRNA transfection showed a significant decrease in clonogenicity in the PD-L1-siRNA transfected MDA-MB-231 cells (Fig. 5A). Furthermore, CD44 mRNA expression displayed a significant decrease in the PD-L1-siRNA transfected MDA-MB-231 cells compared to the control group (Fig. 5B, $P < 0.0001$).

3.6. The transfection of PD-L1-siRNA arrested MDA-MB-231 cells at the G1 phase

Flow cytometry analysis was performed to determine the effect of PD-L1 gene knockdown on the cell cycle of breast cancer cells. Knockdown of PD-L1 gene induced G1 cell cycle arrest and prevented cells from entering the mitosis phase (M phase). Additionally, cell cycle arrest

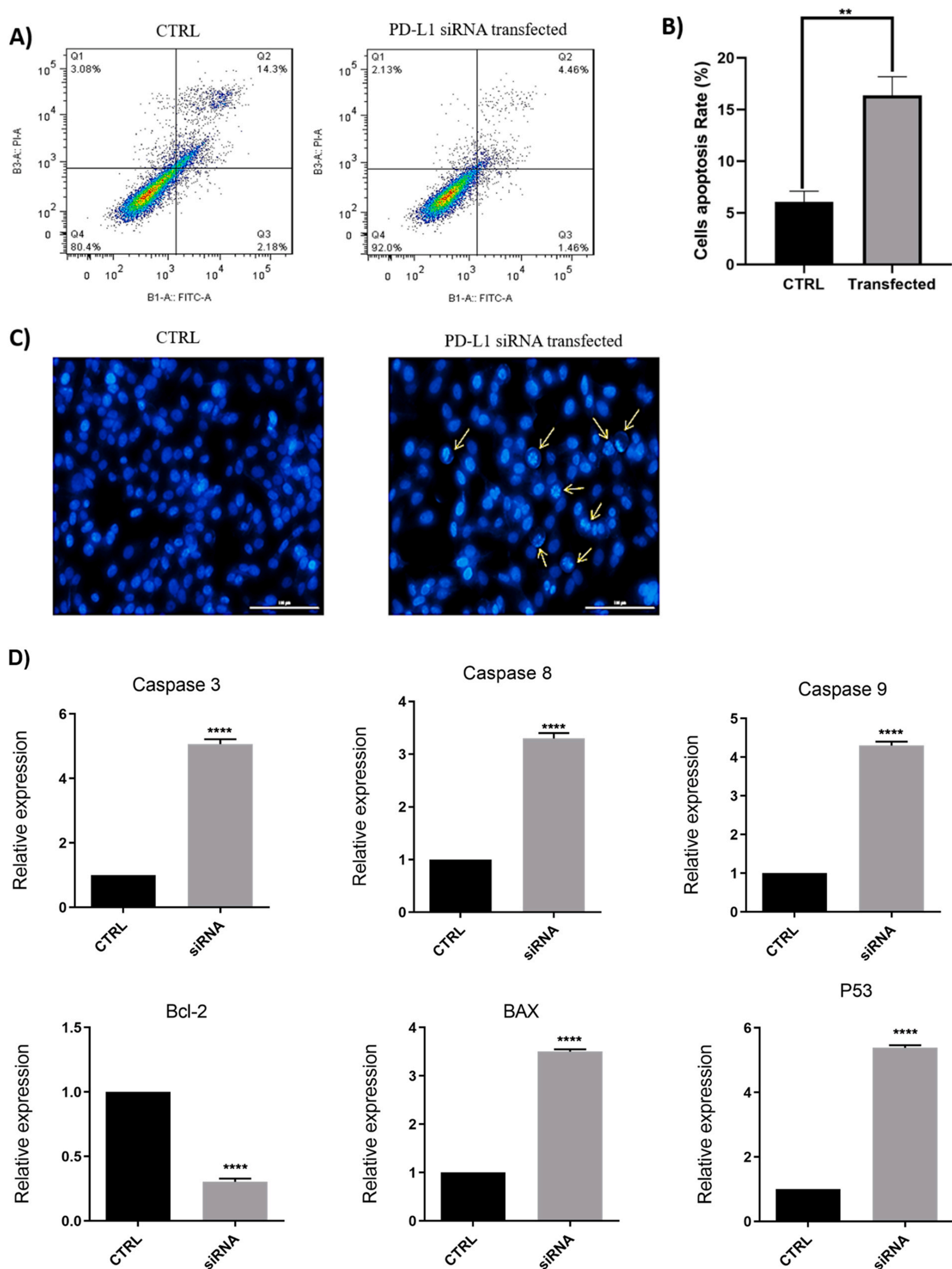

Fig. 7. Silencing of PD-L1 expression promoted cell apoptosis in the MDA-MB-231 cell line. (A) Flow cytometry analysis displayed that there were 14.3% apoptotic cells in PD-L1-siRNA transfected cells in compared to 4.46% in the control group. (B) Early and late apoptosis of control and PD-L1-siRNA transfected cells were shown in column statistic **P < 0.01 (P-value = 0.0021). (C) The result of DAPI staining showed that PD-L1-siRNA induced chromatin condensation in transfected MDA-MB-231 cells in comparison to the control group. (D) PD-L1-siRNA induced apoptosis through both extrinsic and intrinsic apoptosis pathways, qRT-PCR results displayed increased Bax, P53, caspase-3, caspase-8, and caspase-9 gene expression in the transfected MDA-MB-231 cell line. ****P < 0.0001.

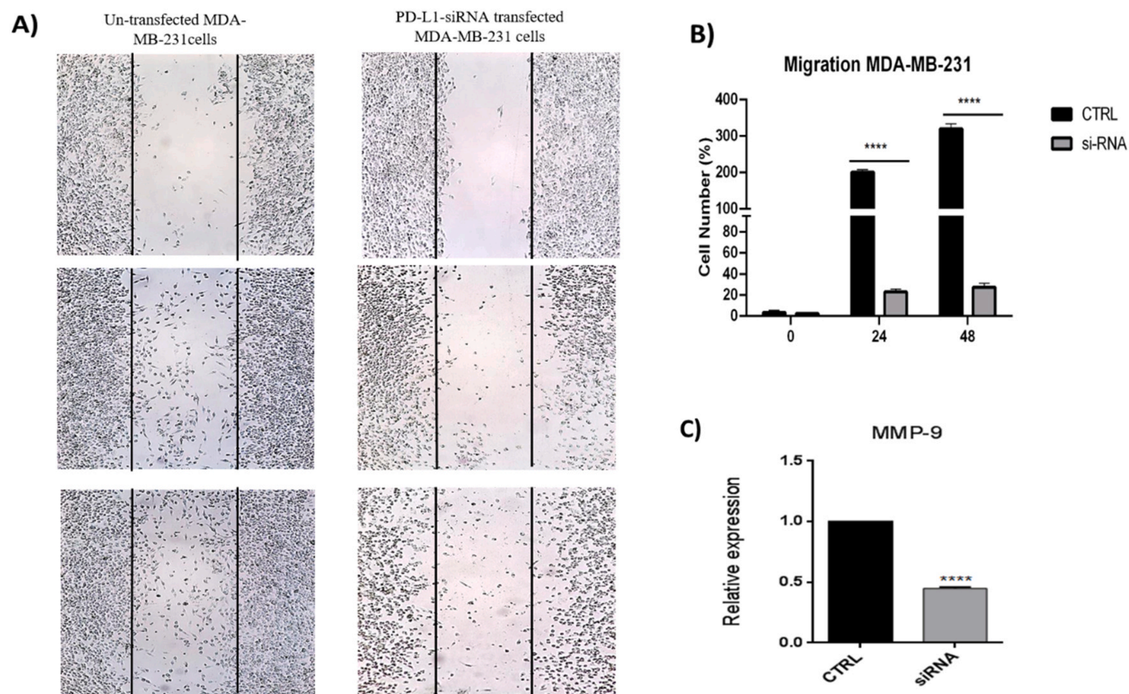

Fig. 8. PD-L1 siRNA decreased MDA-MB-231 cell migration. (A) Cell migration was evaluated using a wound healing model after 0, 24 h, and 48 h of culture. (B) Relative migration rate in PD-L1 siRNA transfected cells at 24 h and 48 h were significantly less than the control group. (C) MMP-9 as a key regulator of cancer cells metastasis showed reduced gene expression level in siRNA transfected cells in compared to the control group; ****$P < 0.0001$.

was also determined in the sub G1 area in transfected cells, confirming apoptosis induction results. As demonstrated in (Fig. 6), PD-L1 siRNA induced arrest in the G1 phase in transfected cells in comparison with the control group.

3.7. PD-L1-siRNA transfection induced apoptosis and upregulated the caspase 3/caspase 9 in the MDA-MB-231 cells

Compared to the control cells, the flow cytometry results showed significant induction in apoptosis of PD-L1-siRNA transfected MDA-MB-231 cells ($P < 0.001$). PD-L1-siRNA could significantly increase apoptosis rates in transfected cells to 16.48%, in the control group apoptosis rate was 5.92% (Fig. 7A). Consistent with the flow cytometry assay of apoptosis, DAPI staining also confirmed apoptosis induction in siRNA transfected cells. Fragmentation of DNA indicated the role of PD-L1-siRNA in the activation of apoptosis mechanism in breast cancer cell line (Fig. 7C). To investigate cell death-related mechanisms as pointed out by the cell cycle assay in siRNA-transfected MDA-MB-231 cells, we analyzed the effect of PD-L1 knockdown on apoptosis-related gene alternation. We found that PD-L1 knockdown significantly induced high expression of caspase-3, caspase-9, caspase-8, P53, and Bax genes, but in contrast, the expression of Bcl-2 decreased in comparison to the control group (Fig. 7D).

3.8. Silencing of PD-L1 expression inhibited the migration of breast cancer cell line

To evaluate the association of PD-L1 silencing effect on MDA-MB-231 cell migration, we further performed wound healing (scratch) test. The results displayed that the migration potency of siRNA-transfected cells was significantly lower than that in the control group (un-transfected cells). As displayed in (Fig. 8A and B), silencing of PD-L1 can reduce the cell migration of breast cancer cells in the wound healing assay. Further investigation showed that MMP-9 gene expression level as an important regulator of tumor cell metastasis reduced in siRNA-transfected cells in compared to the control group (Fig. 8C).

3.9. Analysis of cytokine profile of T-cells in co-culture system following PD-L1 knockdown

In order to evaluate the effect of PD-L1 knockdown on cytokine profile of T-cells in co-culture system, we analyzed cytokine gene expression in T-cells co-cultured with siRNA-transfected MDA-MB-231 cells. The results indicated that IL-2, IFN-γ, and TNF-α cytokine gene expression were significantly increased, but IL-10 and TGF-β expression significantly were decreased compared to control cells ($P < 0.001$) (Fig. 9).

3.10. PD-L1 knockdown reduced CD25+ Foxp3+ Tregs population

To investigate the effect of PD-L1 knockdown on Tregs population in co-culture system, flow cytometry results in three groups displayed that CD25+ Foxp3+ Tregs population in non-activated T-cells group (T cells with no PHA) were 3.20%, in the activated T-cells group (T cells activated with PHA) were 1.27%, in T-cells co-cultured with un-transfected cells, were 24.6% and T-cells with PD-L1-siRNA-transfected cells were 2.31% (Fig. 10).

4. Discussion

Despite the effectiveness of adjuvant and neoadjuvant chemotherapies in TNBC, chemoresistance is an unavoidable phenomenon in TNBC patients [18]. Although therapeutic anti-PD-1 and anti-PD-L1 antibodies play an important role in improved clinical outcome in TNBC patients [19], immune-related adverse events induced by these therapies should not be ignored [20,21], thus exploring an efficient therapeutic strategy to inhibit the PD-1/PD-L1 pathway in patients with TNBC is essential [22].

It is demonstrated that non-coding RNA molecules such as microRNAs (miRNAs) and short interfering RNA (siRNA) are effective tools in cancer treatment because of their potency to directly target the cancer-related genes and they also up/downregulate signaling pathways that are involved in the control of cancer cells function [23,24]. siRNA-based

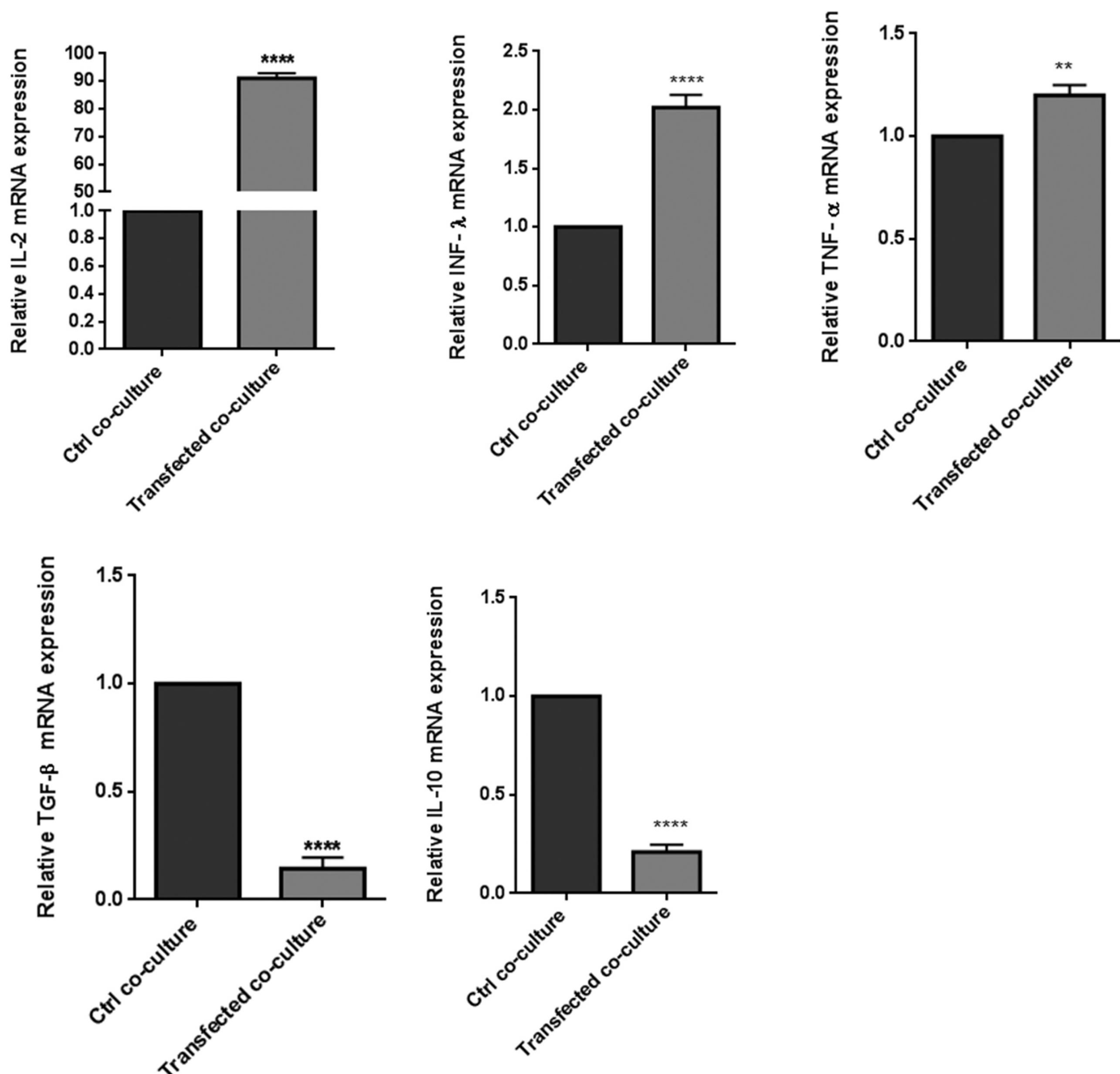

Fig. 9. Cytokines gene expression profile following co-culture of T-cells with PD-L1 siRNA transfected MDA-MB-231 cells**.** Gene expression results displayed significantly enhanced effector cytokine responses, such as IL-2, IFN-γ, and TNF-α in contrast to lower IL-10 and TGF-β gene expression. ($P < 0.001$).

therapies have been promising for treating cancers, and also this strategy can reduce the risk of developing immune-related adverse events of immune checkpoint inhibitors [25–27].

This study has highlighted the efficacy of PD-L1-siRNA in restraining tumor proliferation, arresting the cell cycle, suppressing tumor CD44, stimulating tumor apoptosis, suppressing tumor migration, and inhibiting tumor clonogenicity in TNBC cells. Furthermore, this study has indicated that PD-L1-siRNA can effectively upregulate T-cell-induced pro-inflammatory cytokines and inhibit the Treg.

Proliferation and progression of cancer are mainly associated with the ability of anti-tumor immunity evasion of cancer cells [28]. Ghebeh et al. have indicated that tumoral PD-L1 expression might be associated with breast cancer development [29]. It has been demonstrated that serum PD-1/PD-L1 levels in cats are elevated in cats bearing HER2-positive and triple-negative normal-like carcinomas. Thus, the importance of this inhibitory axis in promoting breast cancer development has also been established in HER2-positive cancer [30]. Besides the elevated serum PD-1/PD-L1 levels in cats bearing breast cancer, the serum level of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), as another well-established immune checkpoint, has been considerably elevated in cats bearing mammary carcinoma [31]. In the current study, TCGA data have indicated that PD-L1 expression is elevated in TNBC cases compared to the estrogen receptor-positive and progestogen receptor-positive breast cancer patients. [32]. Based on CCLE data, PD-L1 expression in MDA-MB-231 cell lines has been higher than other cell lines. Therefore, these results indicate that TNBC cells might be more sensitive to PD-L1 target therapy.

In the current study, the results of qRT-PCR and western blot analyses have displayed that PD-L1-siRNA transfection can substantially reduce the mRNA and protein expression of PD-L1. Moreover, PD-L1-siRNA can suppress the proliferation of TNBC cells, suggesting that PD-L1 upregulation in TNBC may have an essential role in the growth of TNBC cells. Furthermore, our results have shown that PD-L1-siRNA transfection can induce cell cycle arrest at the G1 phase. To investigate the underlying mechanism of PD-L1-induced cell growth and proliferation, we have measured the c-Myc gene expression level, which

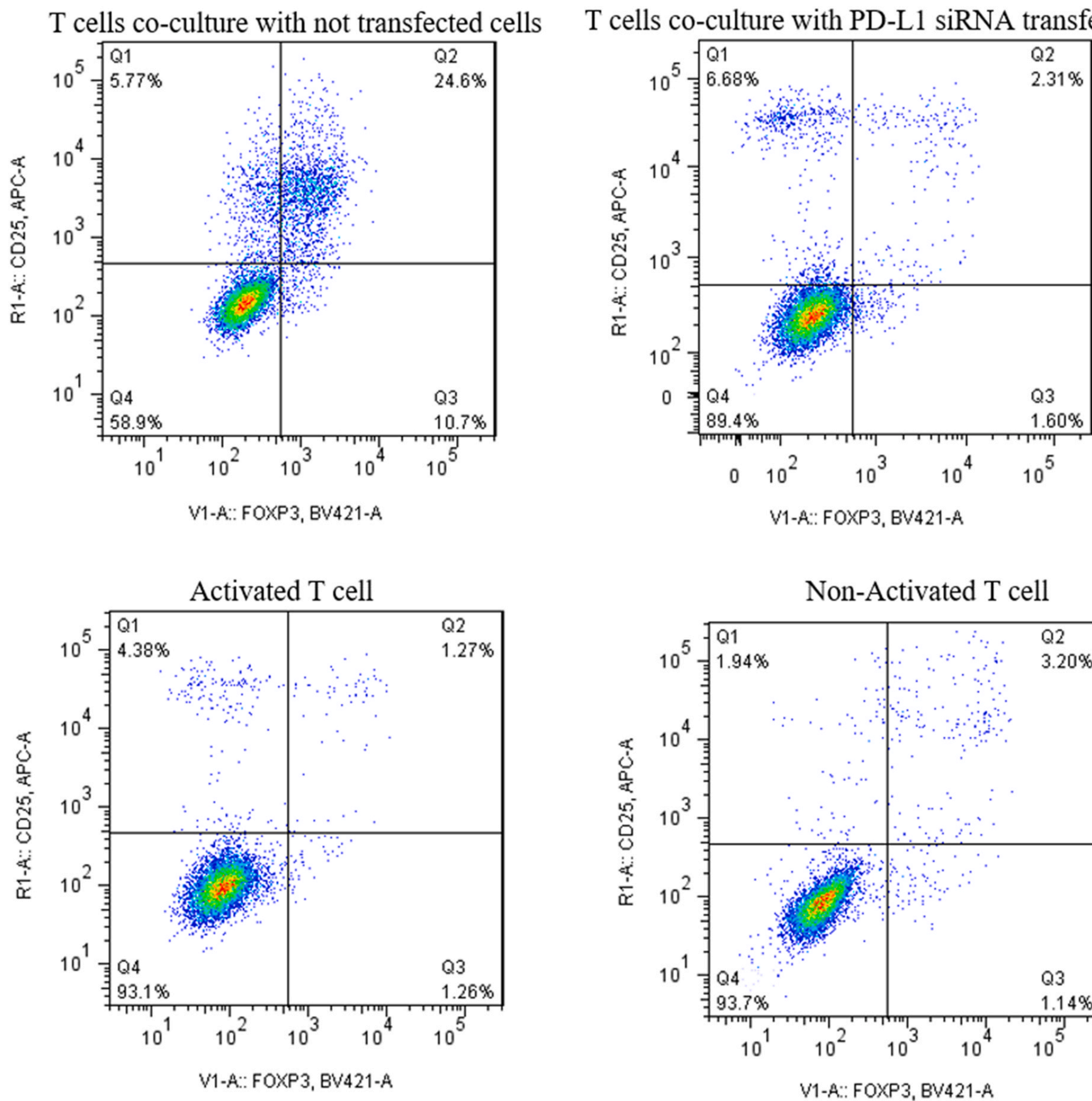

Fig. 10. The percentages of CD4+CD25+Foxp3+ Treg cells within the CD4+ T cell population were determined by FACS. Knockdown of PD-L1 expression may involve in Treg reduction in T-cells population co-cultured with PD-L1 siRNA transfected MDA-MB-231 cells.

contributes to cell cycle progression and cell proliferation in breast cancer cells [33]. Our results have demonstrated that c-Myc gene expression can be reduced following PD-L1-siRNA transfection in TNBC cells. Consistent with these, Kim et al. shed light on the association between cMyc and PD-L1 in lung cancer patients [34]. In TNBC patients, MUC-1, via inducing c-Myc, has been implicated in PD-L1 expression [35]. Intrinsic and extrinsic apoptosis pathways are two arms of programmed cell death. In the intrinsic pathway, the activation of the signaling cascade of caspases releases cytochrome C and second mitochondria-derived activator of caspases (SMAC), which ultimately can lead to cell death. In the extrinsic pathway, death receptor ligation on the cell surface induces caspases activation [36,37]. Our results have indicated that PD-L1-siRNA transfection can induce apoptosis via the intrinsic and extrinsic apoptosis pathways in TNBC cells. Indeed, PD-L1-siRNA transfection stimulates the pro-apoptotic gene expression, i.e., caspase-3, caspase-8, and caspase-9. Moreover, PD-L1-siRNA transfection can down-regulate the expression of Bcl-2 as an oncogene and upregulate the expression of Bax, which can stimulate apoptosis [38]. Based on these results, probably PD-L1 via upregulation of other apoptosis-related genes or pathways is involved in promoting apoptosis and further studies are needed to find this association.

Moreover, PD-L1, which promotes stem cell-like features of melanoma cells, inhibits the apoptosis of tumoral cells [39]. Furthermore, PD-L1 is critical to promote stem cell-like properties in breast cancer [40]. CD44, as a cancer stem cell marker, has been implicated in tumorigenesis and tumor invasion [41]. Michael O et al. have shown that TNBC patients with increased $CD44^{+}/CD24^{-/low}$ breast cancer stem cells exhibited a higher incidence of relapse and distant metastasis [41, 42]. Therefore, we have investigated the CD44 expression, and clonogenicity of PD-L1-siRNA transfected TNBC cells. Our results have demonstrated a substantial decrease in the level of CD44 expression and clonogenicity of PD-L1-siRNA transfected TNBC cells. Thus, PD-L1 silencing might help the elimination of cancer stemness properties in TNBC cells.

Metastasis is the infiltration of cancer cells to the basement layer and the development of malignant colonies in distant tissues. MMPs, which are the family of zinc-dependent proteases, degrade the extracellular matrix by proteolytic activity; thus, they have a critical role in

metastasis. MMP-9 has been associated migration and metastasis of breast cancer cells. In metastatic breast cancer, MMP-1 and MMP-9 are induced by extracellular matrix, contributing to cancer cell migration [43,44]. Our results have indicated that PD-L1-siRNA transfection can effectively reduce the gene expression of the MMP-9. In line with this, the wound-healing assay has confirmed the inhibitory effect of PD-L1-siRNA on the migration of TNBC cells. Therefore, PD-L1 silencing might decrease the migration of TNBC cells.

The PD-1/PD-L1 axis is one of the culprits in the development of Tregs and promotes the conversion of Th1 cells into Tregs [45,46]. Therefore, we have investigated the effect of PD-L1-siRNA transfection on the Treg cells co-cultured with transfected TNBC cells. Indeed, we have designed a co-culture system to evaluate whether PD-L1 silencing can affect Treg differentiation. After 48 h of co-culture, the cells were examined for the presence of $CD25^{+}$, $Foxp3^{+}$ Tregs and also for evaluation of pro-inflammatory and anti-inflammatory cytokine gene expression. In the present study, we showed that PD-L1 overexpression in TNBC cells could induce Treg expansion. In line with this, Francisco et al. have displayed that PD-L1-coated beads can induce Tregs development in vitro. Indeed tumoral PD-L1 can facilitate FoxP3 expression and pave the way for Treg development. Furthermore, PD-L1 can induce differentiation of $CD4^{+}$ naïve T-cells to Tregs via inhibition of the Akt/mTOR /ERK2 pathway and upregulation of PTEN [16,47]. Tregs are immunosuppressive T-cells in the tumor microenvironment and contribute to tumor development. Increased into the tumor microenvironment has been associated with poor prognosis of cancers [48,49]. Moreover, our results have demonstrated that silencing of PD-L1 silencing can substantially reduce $CD25^{+}$, $Foxp3^{+}$ Tregs population in a co-culture system. As mentioned above, the Akt/mTOR/ERK2 pathway suppression and the PTEN upregulation are critical factors in Treg development via the PD-1/PD-L1 axis [16]. Yvon et al. have reported that Notch ligand, Jagged-1, and DLL are also involved in Treg development [50]. PD-L1, via the phosphatase activity of the SHP1/2 signaling pathway downstream of PD-1, downregulates STAT in T-cells and induces Treg differentiation [51]. To investigate T-cells responses to PD-L1-silencing, we have also analyzed the cytokine gene expression after 48 h of co-culture. Our results indicate a remarkable increase in IL-2, IFN-γ, TNF-α, and a substantial decrease in IL-10 and TGF-β cytokine gene expression. The most significant upregulation has been the increase in IL-2 gene expression in T-cells co-cultured with PD-L1-silenced breast cancer cells. IL-2 is an important cytokine in NK cells and helper T-cells proliferation and development, and it has been employed as anti-cancer agent [52,53]. Consistent with our results, Wu et al.have shown that the co-culture of PD-1 silenced T-cells with PD-L1 silenced MCF-7 cells can increase the expression of IFN-γ was significantly increased [24]. IFN-γ and TNF-α are associated with anti-proliferative and anti-tumor mechanisms. It has been demonstrated that IFN-γ can induce necrosis and tumor vessel destruction and it can increase infiltration of monocyte in to tumor microenvironment. TNF-α induce apoptosis by binding to tumor cell surface receptors; it also induces effector T-lymphocytes activation by blocking T-reg cells [54–56].

Moreover, we observed reduced IL-10 and TGF-β cytokines genes expression. IL-10 and TFG-β are immunosuppressive cytokines that are produced by T-regs, and they have an inhibitory effect on anti-tumor function of effector T-cells [57,58]. Moreover, it is demonstrated that IL-10 is involved in M2 macrophage polarization. On the other hand, IL-10 secretion is elevated in TNBC [56], so exerting a method to diminish anti-inflammatory cytokines will improve anti-tumor response. Therefore, the increased gene expression of IL-2, IFN-γ and TNF-α gene and decreased gen expression of IL-10 and TGF-β might indicate increased effector function of T-cells, co-cultured with PD-L1-silenced breast cancer cells.

5. Conclusion

With the rising concerns of administrating immune checkpoint inhibitors, access to the gene modification of PD-L1 has become more appealing for TNBC treatment. This study has shown that PD-L1-siRNA transfection can substantially decrease tumoral PD-L1 expression, improve co-cultured T-cells function. PD-L1 silencing can suppress Tregs development and abrogate the immunosuppressive feature of the tumor microenvironment. Therefore, PD-L1-siRNA-based therapy might be a novel therapeutic method for targeting the PD-1/PD-L1 axis.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Author Contributions

P.L. performed the majority of experiments and data analysis, T.K. and S.S. contributed to cellular assays and data analysis, P.L. also wrote the manuscript, M.A, E.R.A, M.A.S revised the manuscript and interrupted the results, A.D. and E.B. contributed to data analysis, S.S, and F. J.N. contributed to English editing, B.B and N.S. supervised the manuscript.

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