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Original Article

Human recombinant soluble PD1 can interference in T cells and Treg cells function in response to MDA-MB-231 cancer cell line

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Abstract: Objectives: PD1/PDL1 pathway targeting using antibodies shows immune related adverse events in patients with tumors. The masking of PD1 ligand by soluble human PD-1 (shPD-1) probably inhibits the PD1/PDL1 interaction between T cells and tumor cells. Accordingly, the goal of this study was to produce human recombinant PD-1-secreting cells and find out how soluble human PD-1 affects T lymphocyte function. Methods: An inducible construct of the human PD-1 secreting gene under hypoxia condition was synthesized. The construct was transfected into the MDA-MB-231 cell line. In six groups exhausted T lymphocytes were co-cultured with transfected or non-transfected MDA-MB-231 cell lines. The effect of shPD-1 on IFNγ production, Treg cell's function, CD107a expression, apoptosis, and proliferation was assessed by ELISA and flow cytometry, respectively. Results: The results of this study showed that shPD-1 inhibits PD-1/PD-L1 interaction and enhances T lymphocyte responses through a significant increase in IFNγ production and CD107a expression. In addition, in the presence of shPD-1, the percentage of Treg cells decreased, while MDA-MB-231 cell apoptosis increased. Conclusions: We concluded that the human PD-1 secreting construct induced under hypoxia condition inhibits the interaction of PD-1/PD-L1 and enhances T lymphocyte responses in tumor environments and chronic infections.

Keywords: Soluble PD1, T lymphocytes, regulatory T cells, MDA-MB-231 cell lines

Introduction

Activation of $CD4^+$ and $CD8^+$ T lymphocytes within cancer is required for an effective immune response against tumor cells [1]. Overexpression of inhibitory immune checkpoint receptors on T cells in tumor microenvironments is a major impediment to immune responses against arising tumor cells and virally infected cells. Following exhaustion of T cells in immune responses, these receptors, such as PD1 (programmed cell death protein 1) and TIM3 (T-cell immunoglobulin domain and mucin domain protein 3), are expressed on the T cell surface [2]. PD1 expressing cells have a diminished capacity to secrete effector cytokines such as IFNγ. Additionally, these cells do not exert an optimal cytotoxic response against tumor cells [3].

The expression of PD1 is mediated by NFAT (nuclear factor of activated T cells) and SPRY2 (sprout homologue 2) upon TCR engagement. In this state, chronic antigenic stimulation leads to sustained expression of PD1 via NFATc1 (NFAT cytoplasmic 1) [4]. The level and duration of this stimulation are the key factors for T cell exhaustion and are related to the severity of T cell dysfunction [5]. Constitutive and induced expression of PD1 ligands, namely PDL1 and PDL2, has been demonstrated in a variety of hematopoietic, non-hematopoietic, and different types of cancer cells [6]. Following PD1/PDL interaction, a signaling pathway begins in

the cells by phosphorylation of the intracellular domain of PD1, which contains an ITIM motif (immune-receptor tyrosine-based inhibitory motif) and an ITSM motif (immune-receptor tyrosine-based switch motif). Accordingly, activation of the PI3K/AKT and RAS signaling pathways suppresses the anti-tumor functions of $CD8^+$ and $CD4^+$ T cells [7]. PD1/PDL interaction also promotes T cell differentiation into $CD4^+CD25^+FOXP3^+$ regulatory T cells by inducing FOXP3 expression [8]. Regulatory T cells are a potent population in the tumor microenvironment that suppresses effector $CD4^+$ and $CD8^+$ T cell responses, resulting in immune evasion and T cell exhaustion [9]. Therefore, T cells exhaustion and the presence of Treg cells provide an immunosuppressive microenvironment that helps tumor cells escape the immune system [10].

TIM3, the other immune checkpoint receptor expressed on exhausted T cells, is regulated by the T-bet transcription factor [2]. This molecule could interact with the soluble molecule Gal9. Following this interaction, engagement of the Src family tyrosine kinase Fyn and the p85 PI3K adaptor happens, which reduces cytotoxicity and IFNγ production [11]. TIM3 regulates T cell function negatively and is characterized as a marker of T cell exhaustion, observed in two distinct subsets, including $TIM3^+PD1^+$ and $TIM3^+PD1^-$. In comparison to $TIM3^+PD1^-$ T cells, $TIM3^+PD1^+$ T cells produce fewer pro-inflammatory cytokines, such as IFNγ, and TNFα and more inhibitory cytokines. These subsets of T cells are predominant subsets of exhausted T cells [12, 13].

Blocking the PD1/PDL interaction is used as a therapeutic method in a variety of cancers, and PDL1-expressing tumor cells are the most amenable to anti-PD1/PDL1 therapy and the reinvigoration of exhausted T cells [14]. Anti-PD1/PDL1 blockade could partially reverse T cell exhaustion, improve $CD8^+$ T cytotoxicity and effector cytokine production, inhibit tumor-specific Treg cells, and eradicate tumor cells [15]. Nevertheless, PD1/PDL1 pathway targeting using antibodies shows immune-related adverse events in patients with tumors [16]. As a result, in this study, we used recombinant secretory human PD1 (shPD1) to mask the PD1 ligand and inhibit the PD1/PDL1 interaction on anti-tumor T cells. Then we assessed the function of shPD1 by co-culture of exhausted lymphocytes with the MDA-MB-231 cell line.

Materials and methods

Preparation of shPD1

To prepare shPD1, we used sequences of the extracellular domain of human *PD1* cDNA, IgK chain V-1 region HK101 signal peptide, and green fluorescent protein (*GFP*) as a reporter gene in the construct of s*hPD1* (Genecust, Luxembourg). The construct was cloned in pCDNA3.1 hygro plasmid and then transfected (Polyfect, Qiagen, USA) into human embryonic kidney cells lines (HEK293, ATCC® CRL-1573™) (Pasteur institute, Tehran, Iran) according to the manufacturer's instructions [17].

Cell cultivation and isolation

To non-specific stimulation of T cells by tumor cell lysate, human invasive ductal carcinoma (MDA-MB-231 cells, ATCC® HTB-26™) cell line, the lysate was prepared by repeated rounds (7 times) of freezing and thawing of 10^7 cell.mL^{-1} in -80°C freezer and 37°C water bath. The concentration of protein in lysate supernatant was measured by the Bradford method (2 mg.mL^{-1}).

HEK293 (Pasteur institute, Tehran, Iran) and MDA-MB-231 cells were cultured in DMEM high glucose (Gibco, Gaithersburg, MD, USA) and RPMI 1640 supplemented by 10% FBS and 1% Pen-Strep, respectively (Sigma-Aldrich). Also, PBMCs (peripheral blood mononuclear cells) were isolated by a ficoll-hypaque density gradient from ten human donor venous blood samples and then were cultured in complemented RPMI 1640.

Induction of T cells exhaustion

In order to induce exhaustion in T cells, PBMCs were stimulated by 4 μg/ml conA (Sigma-Aldrich) (as an antigen-independent activator of T cells which could bind to a mannose moiety of cell surface glycoproteins including the TCR) for 3 and 6 days, and over-expression of inhibitory markers, PD-1 and TIM3, was determined to support T cell exhaustion. PD1 and TIM3 expression were assessed on T cells, $CD8^+$ and $CD8^-$ T cells before and after stimulation. PD1 and TIM3 expression on stimulated T

cells was evaluated by flow cytometry using FITC mouse anti-human PD1 antibody (EH12.2H7, Biolegend), PE anti-human TIM3 antibody (F38-2E2 Biolegend), and PerCP/CY5.5 anti-human CD8 antibody (SK1, Biolegend). Matched isotype controls were used to enable correct compensation and to confirm antibody specificity. The frequency of $PD1^+$ and $TIM3^+$ T cells was analyzed by FACS Calibur flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA). Analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Co-culture groups

The experimental designs of co-culture groups were performed according to the following groups: 1. Control group: co-culture of exhausted T cells with MDA-MB-231 cells for 24 hours (effector to target (E:T) ratio was 5:1). 2. shPD-1 group: co-culture of exhausted T cells with MDA-MB-231 cells (E:T, 5:1) and shPD-1 for 24 hours. shPD-1 was added to MDA-MB-231 cells one hour before co-culture with exhausted T cells. 3. Transfected cell line group: co-culture of exhausted T cells with transfected MDA-MB-231 cells (E:T, 5:1) in media containing 100 μM CoCL2 for 24 hours. 4. Lysate group: co-culture of exhausted T cells and MDA-MB-231 cells lysate with MDA-MB-231 cells (E:T, 5:1) for 24 hours. Cell lysate (10 $\mu g.ml^{-1}$) was added to exhausted cells after 3 days of stimulation with conA. 5. Lysate/shPD-1 group: co-culture of exhausted T cells/MDA-MB-231 cells lysate with MDA-MB-231 cells (E:T, 5:1) and shPD-1 for 24 hours. shPD-1 was added to MDA-MB-231 cells one hour before co-culture with exhausted T cells. 6. Lysate/transfected cell line group: co-culture of exhausted T cells and MDA-MB-231 cell lysate (10 $\mu g.ml^{-1}$) with transfected MDA-MB-231 cells in the media containing 100 μM CoCL2. All experiments were performed in triplicate.

The effect of shPD1 on PD1 and FOXP3 expression

To assess FOXP3 expression and Treg frequency, suspended cells from all co-culture groups were collected and stained with FITC conjugated anti-human CD4 antibody (RPA-T4, Biolegend) and PE/CY7 conjugated anti-human CD25 antibody (BC96, Biolegend) one day after co-culture. The cells were then fixed and permeabilized with the FOXP3 Fix/Perm buffer set (BD Company) at room temperature, followed by intracellular staining with PE conjugated anti-human FOXP3 antibody (259D/C7, BD Biosciences, USA). The frequency of $CD25^+$ $FOXP3^+CD4^+$ Treg cells was analyzed by FACS Calibur flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA). Analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Measurement of IFNγ

To determine the concentration of IFNγ, supernatant of cell cultures in all co-culture groups were collected 24 h after co-culture. IFNγ concentration was measured by an ELISA kit according to the manufacturer's protocol (Biolegend, San Diego, CA). The limit of sensitivity was 0 and 500 pg ml^{-1} for IFNγ assay.

Evaluation of CD107a expression

CD107a expression was measured using flow cytometry to assess the cytotoxic activity of exhausted T lymphocytes. One hour after co-culture of exhausted T cells with MDA-MB-231 cell lines, PE conjugated anti-human CD107a antibody (eBioH4A3, Biolegend) and monensin (Biolegend) were added to 96 round well cell culture plates. After 6 hours of incubation at 37°C, T cells from all co-cultured groups were collected and surface staining with PerCP/CY5.5 conjugated anti-human CD8 (SK1, Biolegend) antibody was performed. The frequency of CD107a+ T cells was analyzed by FACS Calibur flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA). Analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

Evaluation of MDA-MB-231 cell lines' proliferation

Proliferation assay of co-cultured MDA-MB-231 cell lines was performed by CFSE staining assay, and MTT assay. The day before co-culture, MDA-MB-231 cell line was detached by 1× trypsin/EDTA. Cell pellets were suspended into 1 mL PBS containing 5 μM CFSE (Biolegend) solutions for 20 minutes at room temperature under dark conditions. 2×10^4 cells were seeded in a 96 wells cell culture plate and incubated for 24 hours at 37°C. Exhausted T cells were added to each well and incubated at

37°C. After 24 hours, suspended cells were removed and adherent MDA-MB-231 cell lines were detached by 1×0.25% trypsin/EDTA (Sigma-Aldrich). Then, cell pellets were suspended in PBS buffer and analyzed by flow cytometer.

For MTT assay, one day after co-culture of exhausted T cells with transfected MDA-MB-231 cell lines in a 96 well cell culture plate, suspended cells were removed and adherent cells washed with PBS buffer. Buffer was removed and 10 μL MTT solution was added to each well and incubated for 4 hours at 37°C. Then, 50 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich) solution was added to each well and incubated for 2 hours in dark condition. Finally, the absorbance of each well was read at 570 nm.

Statistical analysis

Statistical analysis was performed using SPSS 20 software. The statistical difference between groups was analyzed using an independent T test and one-way ANOVA. Data are shown as mean ±_SEM. Significant *P* values were marked as follows; P<0.05.

Results

Concanavalin A (ConA) stimulation induces T cell exhaustion

To evaluate the effect of shPD1 on the blockade of PD1/PDL1, it was necessary to prepare highly expressed PD1 cells. In tumor microenvironments and chronic viral infections, these cells exist as exhausted T cells. In preliminary studies, 89% of con.A-stimulated PBMCs were $CD3^+$ T cells.

In further experiments, our data indicated low expression of PD1 on T cells, $CD8^+$ T cells, and $CD8^-$ T cells before con.A stimulation. Con.A stimulation after 6 days significantly increased (P<0.05) the expression of PD1 on T cells, $CD8^+$ T and $CD8^-$ T cells (**Figure 1A** and **1B**).

Also, in an additional series of experiments, our data indicated low expression of Tim3 on T cells, $CD8^+$ T and $CD8^-$ T cells before con.A stimulation, but after 6 days of stimulation, Tim3 expression was significantly (P<0.05) increased on T cells and $CD8^-$ T cells, while its increased expression on $CD8^+$ T cells was not significant (**Figure 1C** and **1D**).

Moreover, in assessment of the co-expression of Tim-3 and PD1 on the T cells, $CD8^+$ and $CD8^-$ T cells, we found that con.A stimulation significantly (P<0.05) increased the percentage of exhausted phenotype, $PD1^+TIM3^+$ and $PD1^+TIM3^-$ in T cells and $CD8^-$ T cells populations (P<0.05) (**Figure 1E** and **1F**). Although $PD1^+TIM3^+CD8^+$ T cells and $PD1^+TIM3^-CD8^+$ T cells were enhanced after stimulation, the enhancement of the exhausted phenotype in $CD8^+$ T cells was not significant (**Figure 1G**).

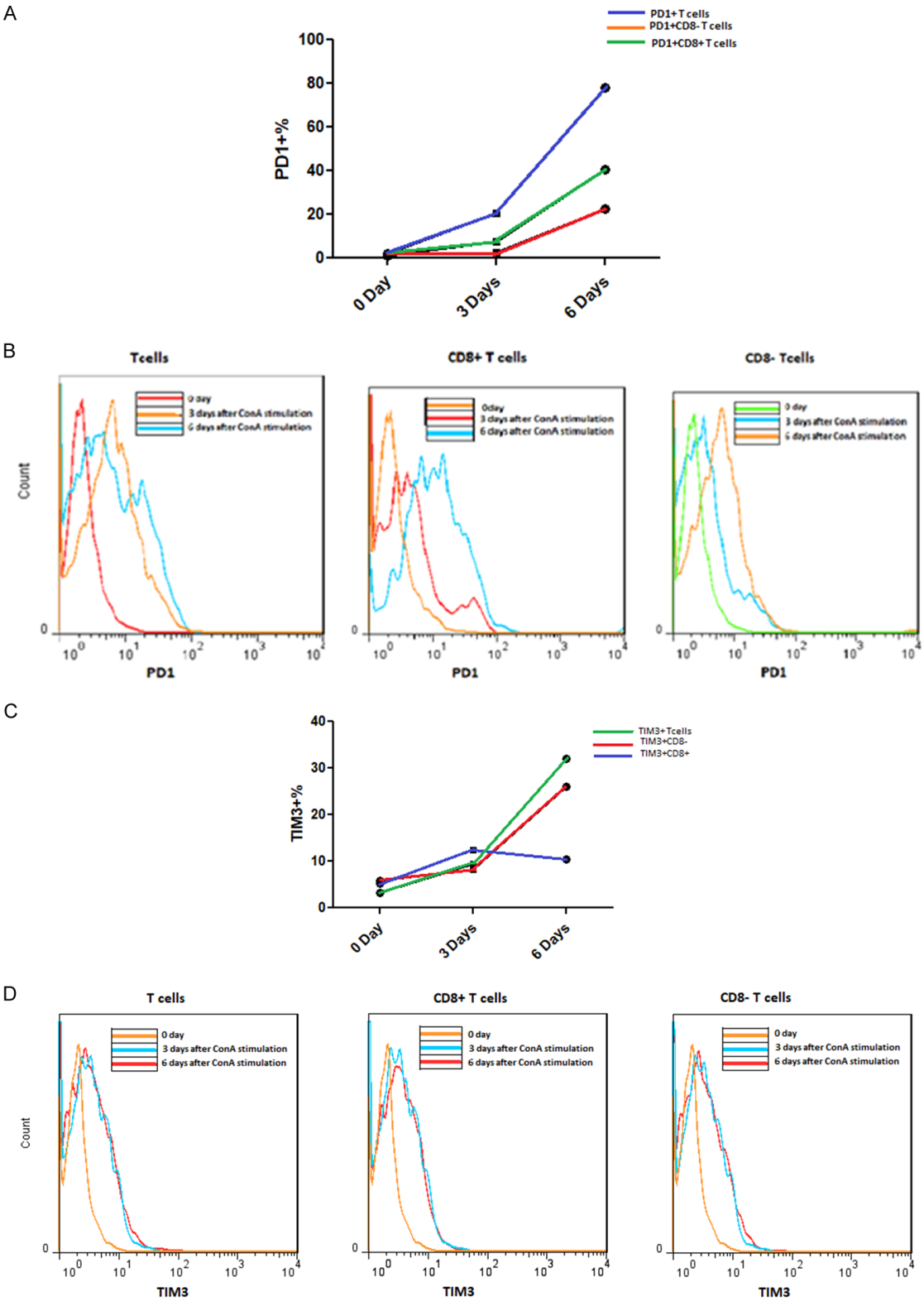
shPD1 reduced PD1 expression

To investigate whether shPD1 can block the PD1/PDL pathway, we co-cultured exhausted T cells with MDA-MB-231 cell lines. In co-culture of exhausted T cells with MDA-MB-231 cell lines in the absence of shPD1, the expression of PD1 on T cells was lower than exhausted T cells (after 6 days of stimulation with con.A), but it was not significant (**Figure 2A**). However, PD1 expression on $CD8^+$ and $CD8^-$ T cells was significantly lower than exhausted T cells (P<0.05). The percentages of $PD1^+$ T cells, $PD1^+CD8^+$ and $PD1^+CD8^-$ T cells were decreased significantly (P<0.05) in the presence of shPD1 compared with co-culture groups and exhausted groups (**Figure 2A**).

Also, in the presence of shPD, the mean fluorescence intensities (MFI) of PD1 expression on T cells (**Figure 2B**) and $CD8^-$ T cells in co-culture with MDA-231 cells were significantly (P<0.05) lower than the exhausted T cells (6 days stimulation with con.A) and the co-culture group. This is because shPD makes the T cells more active. However, we found a significant increase in MFI of PD1 expression on $CD8^+$ T cells co-culture in the presence of shPD1 compared with exhausted group and also co-culture group (**Figure 2B**).

shPD1 increased IFNγ secretion

Exhausted T cells are identified by their reduced ability to secrete IFNγ. Therefore, to determine whether shPD-1 has any effect on IFNγ secretion by exhausted T cells, IFNγ concentration was measured in 6 groups of co-cultured exhausted cells with MDA-MB-231 cell lines. IFNγ concentration was increased significantly


A
PD1+T cells
PD1+CD8- T cells
PD1+CD8+ T cells
PD1+%
0 Day
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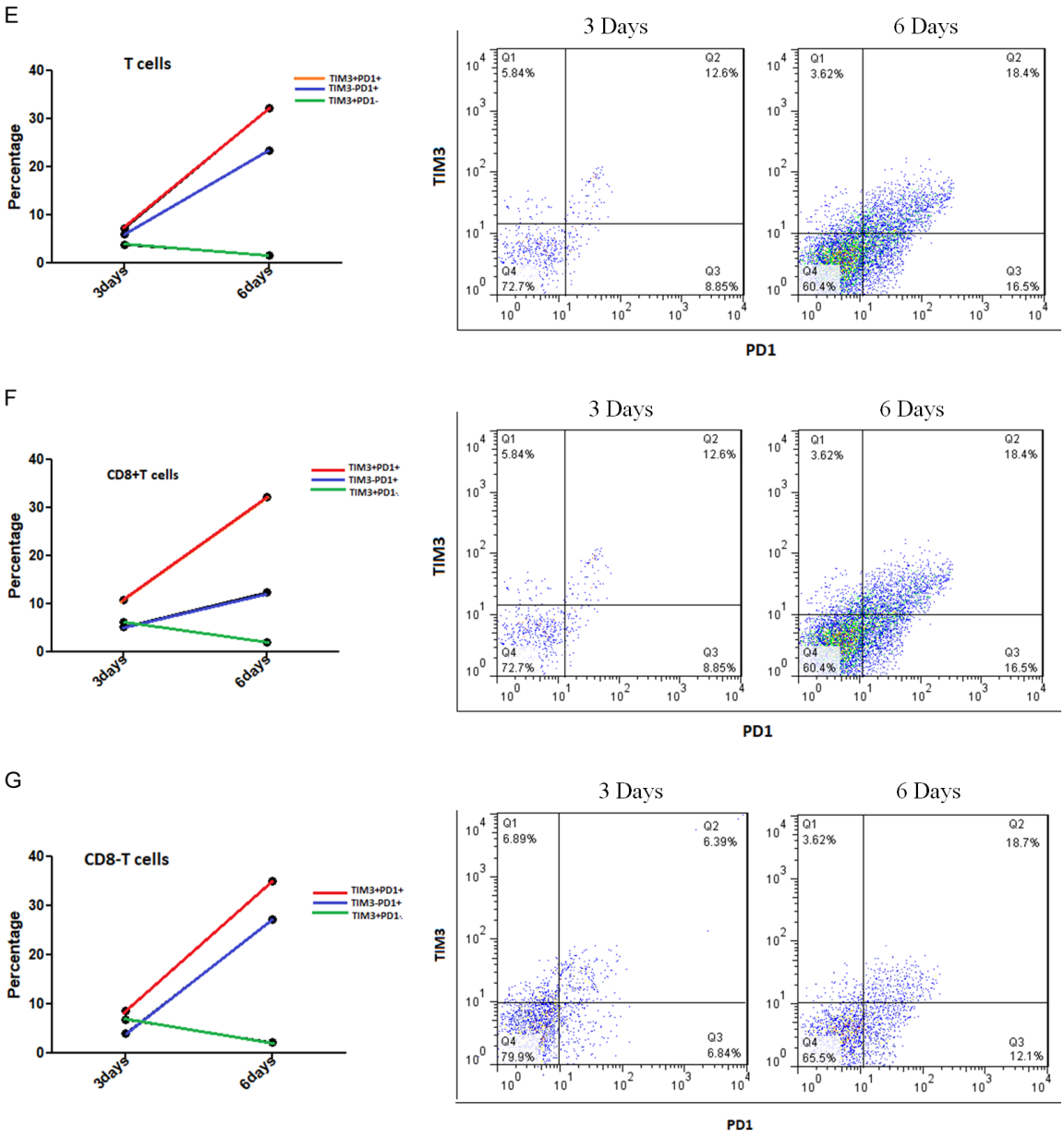

Figure 1. Con.A stimulation induced T cells exhaustion markers. A. $PD1^+$ T cells, $PD1^+CD8^+$ T cells, $PD1^+CD8^-$ T cells were increased significantly ($P<0.05$) after stimulation with con.A (4 $\mu g.ml^{-1}$), after 3 and 6 days. B. Flow cytometry histogram of variation of PD1 expression on T cells, $CD8^+$ T cells, and $CD8^-$ T cells after 3 days and 6 days of con.A stimulation. C. TIM3 expression was increased significantly after stimulation with con.A (4 $\mu g.ml^{-1}$) on T cells, $CD8^-$ T cells (3 and 6 days, $P<0.05$) and $CD8^+$ T cells (3 days). D. Flow cytometry histogram of TIM3 expression on T cells, $CD8^+$ T cells, and $CD8^-$ T cells after 3 days and 6 days of con.A stimulation. E. $PD1^+TIM3^+$ and $PD1^+TIM3^-$ T cells were significantly increased 6 days after con.A stimulation. Representative dot plots show the expression level of PD1 and Tim3 on T cells after 3 and 6 days of stimulation with con.A. F. $PD1^+TIM3^+$ and $PD1^+TIM3^-CD8^+$ T cells were significantly increased 6 days after con.A stimulation ($P<0.05$). Representative dot plots show the expression level of PD1 and Tim3 on $CD8^+$ T cells after 3 and 6 days of stimulation with con.A. G. $PD1^+TIM3^+$ and $PD1^+TIM3^-CD8^-$ T cells after 6 days of con.A stimulation was significantly higher than after 3 days of stimulation ($P<0.05$). Representative dot plots show the expression level of PD1 and Tim3 on $CD8^-$ T cells after 3 and 6 days of stimulation with con.A. Results are presented from three independent experiments. Results are reported as the mean ± SEM from three independent experiments.

($P<0.05$) in shPD-1 treated group, transfected cell line group, and lysate/shPD-1 group and lysate/transfected group compared to control group (**Figure 3**).

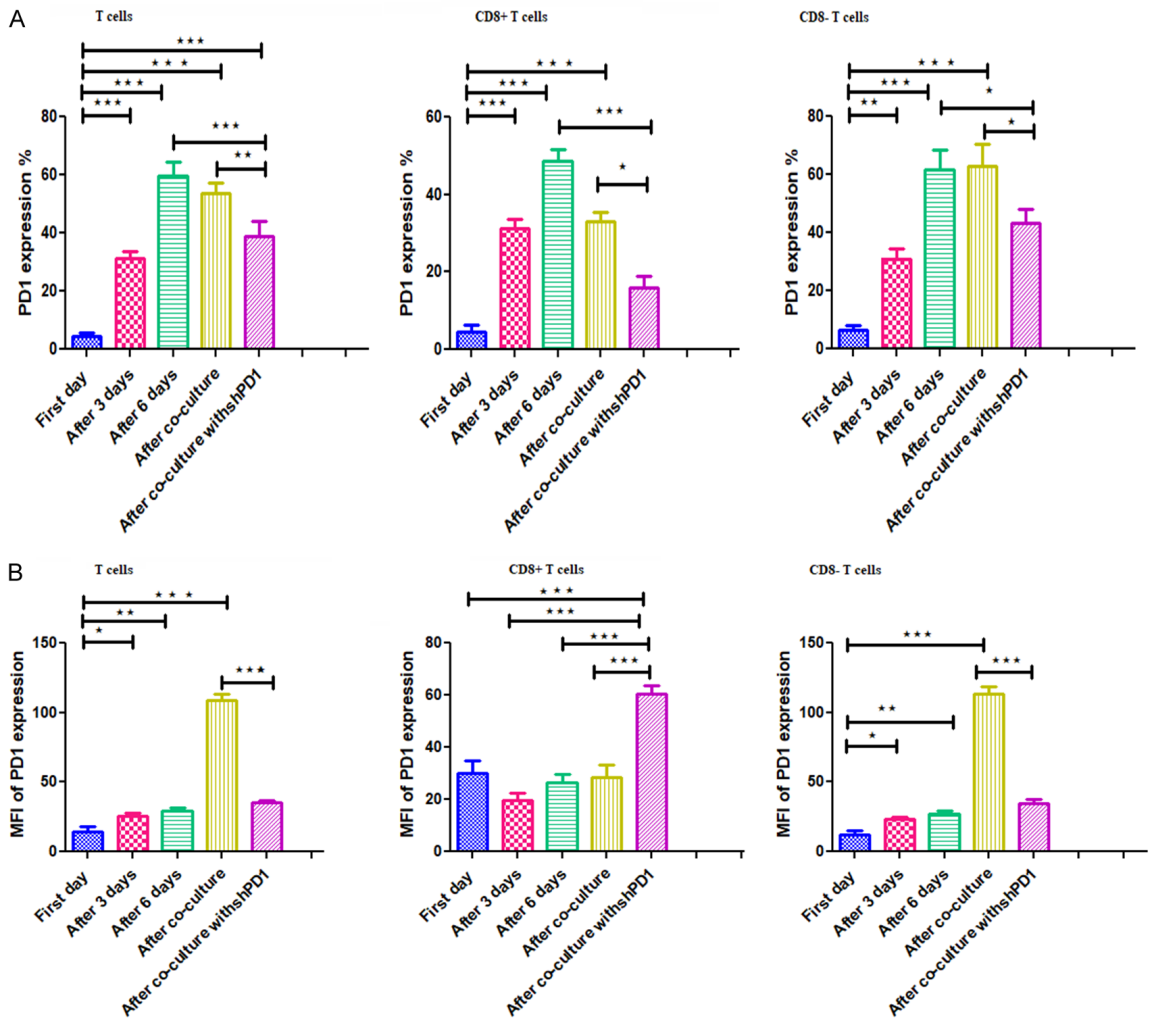

Figure 2. shPD1 reduced PD1 expression on exhausted T cells. A. shPD1 treatment significantly decreased PD1 expression on T cells, $CD8^+$ T cells, and $CD8^-$ T cells after co-culture of exhausted T cells and MDA-MB-231. B. Median fluorescence intensity (MFI) of PD1 expression after 3 and 6 days of con.A stimulation and co-culture of exhausted T cells with MDA-MB-231 cell line increased on the T cells, $CD8^+$ T cells and $CD8^-$ T cells. PD1 expression on T cells and $CD8^-$ T cells decreased with shPD1 treatment. Results are reported as the mean ± SEM from three independent experiments. $^*P<0.05$, $^{**}P<0.001$, and $^{***}P<0.0001$ as determined by one-way ANOVA.

Increase of CD107a expression on T cells in the presence of shPD-1

CD107a expression was evaluated between six groups as a cytotoxic marker of T cells. Significant differences were seen for CD107a expression on T cells between co-cultured groups (**Figure 4A**). CD107a expression was increased on $CD8^+$ T cells in the presence of transfected MDA-MB-231 cells compared with the control group, but it was not significant (**Figure 4B**). The expression of CD107a was significantly elevated ($P<0.05$) in the presence of shPD-1 and transfected MDA-MB-231 cells, on $CD8^-$ T cells compared with the control group (**Figure 5B**). Moreover, treatment of T cells with shPD-1 and MDA-MB-231 cell lysate significantly enhanced CD107a expression on $CD8^-$ T cells ($P<0.05$). T cells treatment with transfected MDA-MB-231 cells and MDA-MB-231 cell lysate significantly increased CD107a expression on $CD8^-$ T cells ($P<0.05$) (**Figure 4B**).

shPD1 decreased $FOXP3^+$ T cells population

The frequency of $FOXP3^+CD25^+CD4^+$ regulatory T cells as suppressor T cells of immune responses was evaluated between 6 co-cultured groups (**Figure 5A**). The percentage of

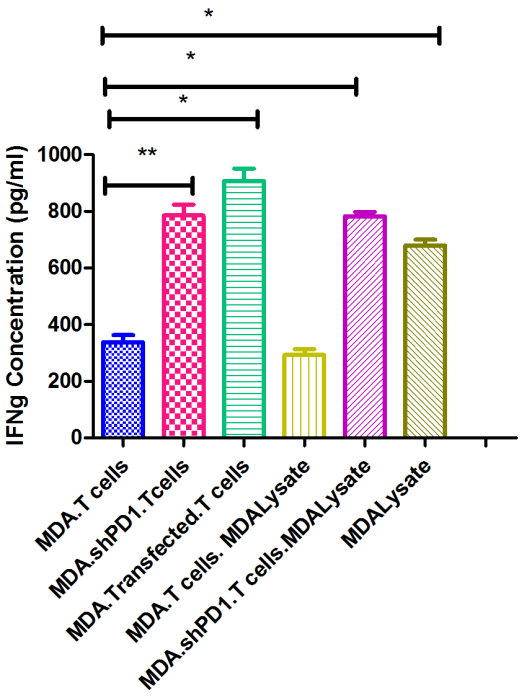

Figure 3. shPD1 restored IFNγ secretion by exhausted T cells. After co-culture of exhausted T cells with MDA-MB-23, IFNγ levels were significantly decreased. IFNγ secretion by exhausted T cells is restored by shPD1 treatment. Results are reported as the mean ± SEM from three independent experiments. *P<0.05 and **P<0.001, as determined by one-way ANOVA.

regulatory T cells was significantly decreased in co-culture groups in the presence of shPD-1, transfected MDA-MB-231 cell lines or MDA-MB-231 cell lysate (P<0.05) compared to the control group (**Figure 5B**). Also, regulatory T cells were reduced significantly in the presence of shPD-1 and MDA-MB-231 cell lysate compared to the control group. However, co-culture of MDA-MB-231 cell lysate with transfected MDA-MB-231 cells could not substantially reduce regulatory T cells (**Figure 5B**).

Comparison of proliferation of MDA-MB-231 cell lines between co-cultured groups

We assessed MDA-MB-231 cell lines' proliferation by flow cytometry (**Figure 6A**) and found that their proliferation was significantly higher in co-culture groups of MDA-MB-231 cell lines with T cells compared with other groups (**Figure 6B**).

In addition, the MTT assay was used for evaluation of proliferation between co-cultured groups. Proliferation of MDA-MB-231 cells was significantly decreased in the presence of shPD-1 and transfected MDA-MB-231 cells and also in the presence of shPD-1/MDA-MB-231 cell lysate and transfected MDA-MB-231 cells/MDA-MB-231 cell lysate compared with the control group (**Figure 6C**).

Discussion

To test shPD1's ability to inhibit PD1/PDL1 interaction, we used con.A, an antigen-independent mitogen, to create a model of exhausted T cells (23). In our study, the exhausted model was similar to studies of viral and bacterial infections, as well as extinguished T cells in tumor-infiltrated lymphocytes (TIL) or chronic infections such as LCMV [18, 19]. In this study, our model exhibited two dominant subsets of exhausted T cells, $PD1^+TIM3^+$ and $PD1^+TIM3^-$ T cells, which were responsible for the dysfunctional activity of T cells. Other studies have indicated that $PD1^+TIM3^-$ T cells have reduced characteristics of exhausted T cells [20]. In solid tumor models such as melanoma, $PD1^+TIM3^-$ T cells are the dominant T cell population, whereas in the AML model, $PD1^+TIM3^+$ T cells are the dominant population [13]. As previously shown in human studies, these subsets show a severe state of exhaustion with high expression of eomesodermin and inhibitory molecules, with a disability to induce effector cytokine secretion, such as IFNγ [21]. In our model, $PD1^-TIM3^+$ was a recessive subset that had minimal effector functionalities. This subset is found at low frequency in the AML model. In previous studies, it has been demonstrated that the $PD1^-TIM3^+$ subset has a high concentration of T-bet and a low ability to secrete cytokines.

These cells are the progenitors of exhausted T cells with high levels of PD1 and eomesodermine [22, 23]. A reduction in $PD1^-TIM3^-$ T cells as non-exhausted T cells was observed in the present study. A decrease in the population of effector T cells was caused by a decrease in the number of non-exhausted T cells ($PD1^-TIM3^-$T cells) and an increase in the progenitor of exhausted T cells ($PD1^-TIM3^+$ T cells). Enhancement of the exhausted population ($PD1^+TIM3^+$ and $PD1^+TIM3^-$ T cells) by con.A stimulation confirmed T cell exhaustion and the capability of these cells for tumor model studies.

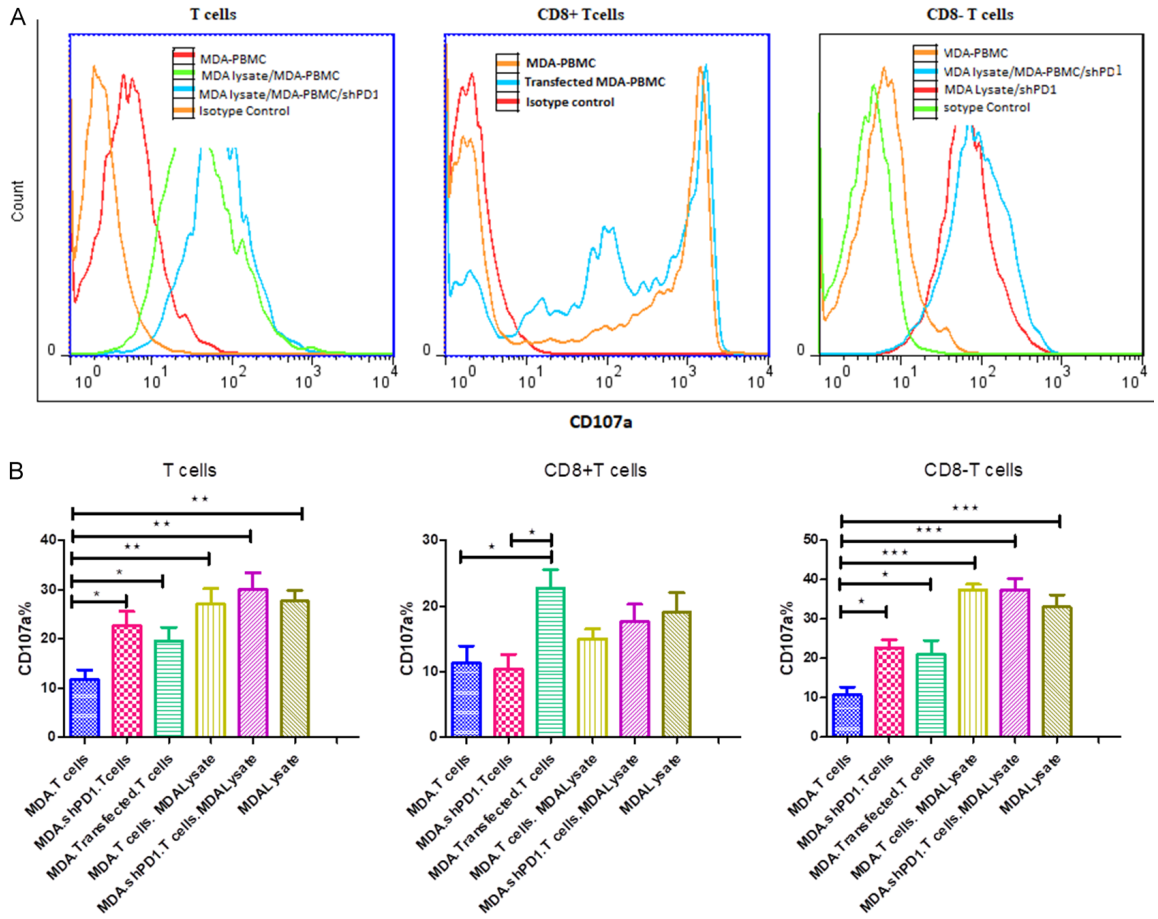

Figure 4. shPD1 increased CD107 expression by T cells. A. Representative histogram plots show CD107a expression on T cells, $CD8^+$ and $CD8^-$ T cells of co-cultured groups. B. Comparison of CD107a expression on T cells, including $CD8^+$ and $CD8^-$ T cells between co-culture groups. The data are presented as the mean ± SEM of three independent experiments. $^*P<0.05$, $^{**}P<0.001$, and $^{***}P<0.0001$ as determined by one-way ANOVA.

To block PD1/PDL1 attachment, we used our shPD1 in co-culture of the MDA-MB-231 cell line with exhausted T cells. Our results revealed high PDL1 expression on the co-cultured MDA-MB-231 cell lines (data not shown) and also high PD1 expression on the co-cultured T cells. It seems that PD1 expression was up-regulated following TCR/MHC I and TCR/MHC II engagement between T cells and the MDA-MB-231 cell line, without blockade of PD1/PDL1 interactions. It has been suggested that this could be a protective mechanism against excessive chronic stimulation (28). Nevertheless, by adding shPD1, PD1 expression on T cells was reduced. Consistent with our finding, it has been reported that PD1 expression has diminished on T cells after treatment with the anti-PD1 antibody [24, 25]. This shows that anti-PD1/PDL1 antibodies and shPD1, compete with PD1 for attachment to PDL1. In addition, masked PDL1 could reduce PD1 excitation, so TCR signaling suppression is diminished and stimulatory/co-stimulatory signals of T cells are transduced. Accordingly, the PD1 expression on T cells, especially $CD8^-$ T cells is reduced and the $PD1^-$ T cell subsets are expanded. We discovered that the presence of shPD1 had no effect on $PD1^+CD8^+$ T cells. It might be associated with the dosage of shPD1 which had no effect on this subset of T cells. Interestingly, Verma et al. recently indicated that PD-1 blockade by anti-PD1 therapy induces the generation of dysfunctional $PD\text{-}1^+$ $CD38^{hi}CD8^+$ cells. The presence of this population is accompanied by resistance to anti-PD1 antibodies and ineffective treatment [26]. They have suggested that resistance to anti-PD1 can be reserved by optimal priming conditions and

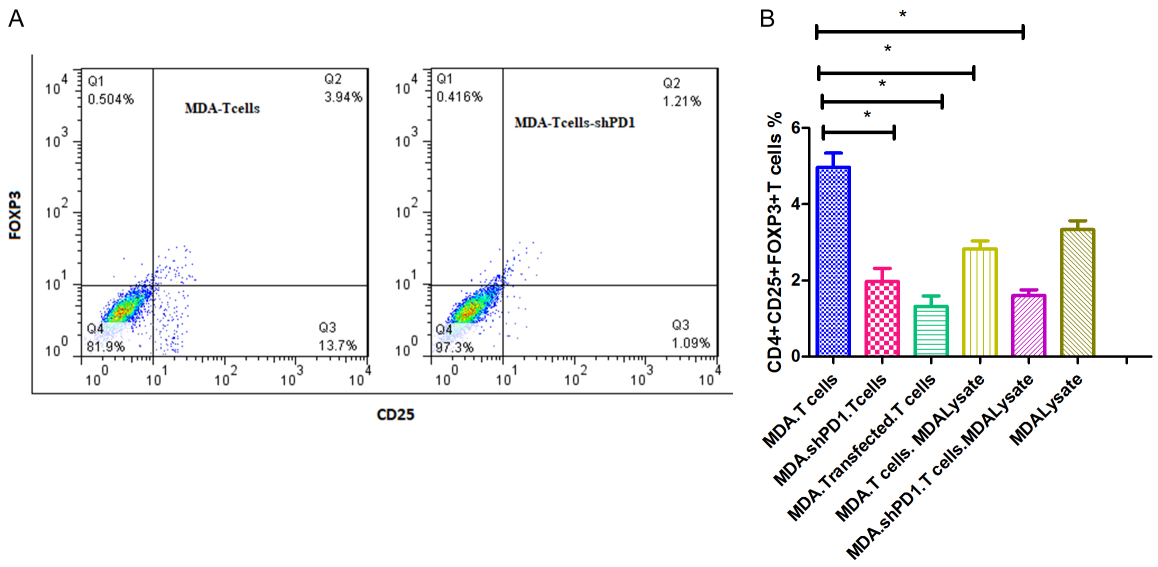

Figure 5. shPD1 treatment decreased regulatory FOXP3$^+$ T cells. A. Representative flow cytometry dot plots show CD4$^+$, CD25$^+$, FOXP3$^+$ T cells. First the lymphocytes population was gated and then CD4$^+$ cells were selected (figures are not shown). CD25$^+$, FOXP3$^+$ T cells were determined in CD4$^+$ T cells. B. Treatment with shPD1 reduced the number of FOXP3$^+$CD25$^+$CD4$^+$ T cells in the co-culture of exhausted T cells with MDA-MB-231 and exhausted T cells. The data is presented as the mean ± SEM of three independent experiments. *P<0.05 as determined by one-way ANOVA.

proper antigenic stimulation [26]. Therefore, the presence of PD1$^+$CD8$^+$ T cells in treatment with shPD1 may be related to the resistance of these T cells to shPD1. However, more detailed studies are required to assess the impact of shPD1 treatment on the function of PD1$^+$CD8$^+$ T cells.

Also, in the present study, IFNγ secretion was evaluated in co-culture of exhausted T cells with the MDA-MB-231 cell line in the presence of shPD1. IFNγ secretion as a functional marker of T cells was reduced by the exhausted T cell [27]. It is related to reduced ratios of T cells with high expression of T-bet to T cells with high expression of eomesodermin and PD1. It implies that ratios of PD1$^-$TIM$^-$ T cells/PD1$^+$TIM3$^-$ T cells, PD1$^-$TIM$^-$ T cells/PD1$^+$TIM3$^+$ T cells, PD1$^-$TIM$^+$ T cells/PD1$^+$TIM3$^-$ T cells and PD1$^-$TIM3$^+$ T cells/PD1$^+$TIM3$^+$ T cells have lessened in exhausted T cells than non-exhausted T cells [27]. Moreover, in co-culture without shPD1, IFNγ secretion was diminished, but in the presence of shPD1, it was enhanced to the secretion of functional T cells. Low IFNγ concentration is related to an increased percentage of PD1$^+$CD8$^-$ T cell subsets, which probably have high eomesodermin. Additionally, increased IFNγ secretion is related to the decreased percentage of eomesoderin [28]. It seems that, similar to the usage of anti-PDL1 or anti-PD1 antibodies in microenvironments of tumors, administration of shPD1 inhibits PD1 engagement with PDL1 and could stopover ITIM/ITSM phosphorylation and phosphatase SHP1/SHP2 recruitment [29]. Therefore, signaling of TCR and co-stimulatory receptors resulted in the functionality of T cells as well as IFNγ secretion [30]. The third consequence of shPD1 on the T cell population was a decrease of FOXP3$^+$CD25$^+$CD4$^+$ T cells in co-culture of exhausted T cells with MDA-MB-231 cell lines. FOXP3$^+$ T cells are key immune-inhibitory cells that can cause T cell exhaustion by secreting inhibitory cytokines and consuming local IL-2 [31]. In this study, we demonstrated that blockade of PD1/PDL1 by using shPD1 reduced FOXP3 expressing cells, including CD25$^+$ FOXP3$^+$CD4$^+$ T cells and PD1$^+$FOXP3$^+$CD8$^-$ T cells, but induced FOXP3$^-$CD8$^-$ T cells. It has been demonstrated that PD1 signaling inhibits the Akt/mTOR pathway, induces FOXP3 expression, and accelerates the differentiation of CD4$^+$ T cells into CD4$^+$CD25$^+$FOXP3$^+$ Treg cells [32]. On the other hand, it has been reported that PD1$^+$FOXP3$^+$ Treg cells are activated Treg cells that have a great suppressive function against CD8$^+$ T cell. These cells are

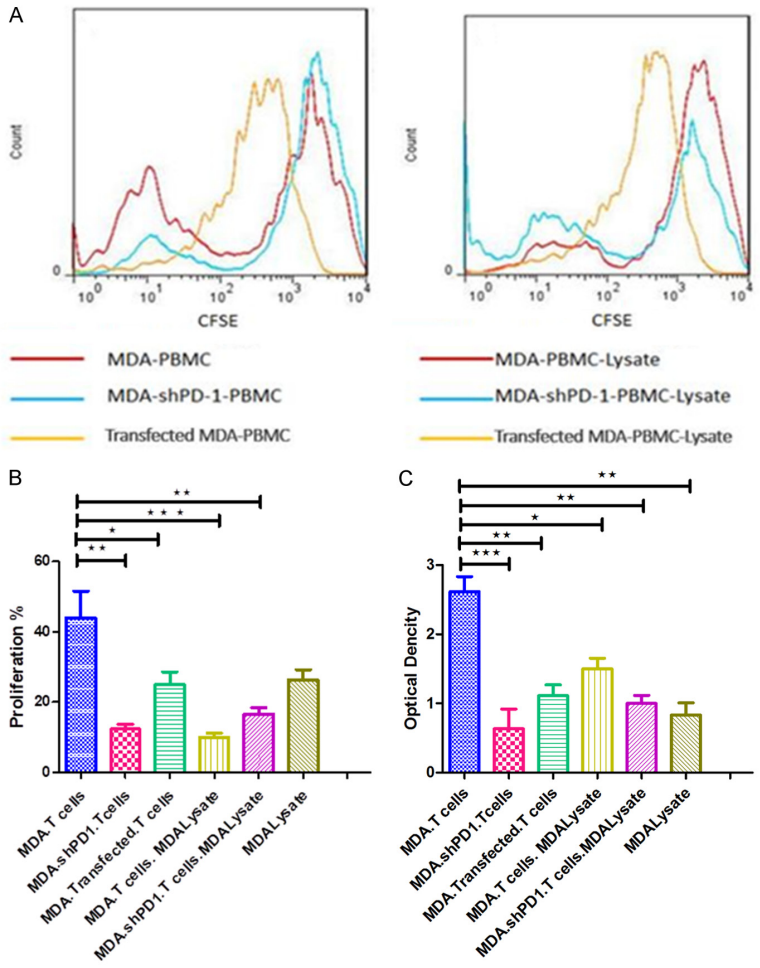

Figure 6. shPD1 treatment decreased the proliferation of the MDA-MB-231 cell line. A. Representative CFSE histogram plots show MDA-MB-231 cell proliferation in different co-culture groups. B. Graphical representation of the proliferating MDA-MB-231 cells in different co-culture groups, which detected by CFSE staining. C. Graphical representation of the proliferating MDA-MB-231 cells in co-culture groups by MTT method. The data are presented as the mean ± SEM of three independent experiments. *P<0.05, **P<0.001, and ***P<0.0001 as determined by one-way ANOVA.

able to block $CD8^+$ T cell function and enhance tumor progression through PD1/PDL1 interactions [33]. Therefore, negative targeting of $PD1^+$ regulatory T cells augments effector T cells activation [8, 34]. Treg suppressive activity is probably impaired due to blocking of PD1/PDL1 by shPD1, which down-regulates PD1 signaling and FOXP3 intracellular expression. Our finding is similar to other published reports that found an increase in IFNγ as a result of PD1/PDL1 blockage, could interfere with Treg induction [10]. In addition, blocking of PD1/PDL1 prevents direct interaction between Treg cells and effector T cells; therefore, the resistance of effector T cells to Treg mediated suppression is promoted [35]. In our study, $PD1^-$ T cells such as $PD1^-FOXP3^+CD8^-$ T cells were the population of regulatory T cells, which were not affected by shPD1. Given that there is no PD1 on the surfaces of these cells to compete with shPD1 for attachment to PDL1, cells remain intact, and FOXP3 expression was not decreased.

Conclusion

This study showed the inhibitory function of shPD-1 protein as a potential blocker for PD-1/PD-L1 interactions between T cells and the MDA-MB-231 cell line. PD-1/PD-L1 blockade by shPD-1 was accompanied by increased IFNγ secretion, cytotoxicity of T cells, decrease of Treg cells and apoptosis of the tumor cell line. As a result, shPD-1 can be used as a therapeutic option in tumor environments by inhibiting the PD-1/PD-L1 pathway.

Overall, we suggest that our shPD1 could be an optimal choice for treatment of cancer and chronic viral infections through augmentation of effector cytokine secretion and suppression of inhibitory cells.

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Disclosure of conflict of interest

None.

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