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Research Article

Producing Soluble Human Programmed Cell Death Protein-1: A Natural Supporter for CD4+T Cell Cytotoxicity and Tumor Cells Apoptosis

Samane Mohammadzadeh[1], Hossein Khanahmad[2], Nafiseh Esmaeil[1], Nahid Eskandari[1], Ilnaz Rahimmanesh[2], Abbas Rezaei[1], Alireza Andalib[1*]

[1] Immunology Department, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
[2] Genetics and Molecular Biology Department, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

[*] *Corresponding author:* Alireza Andalib, Immunology Department, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. P.O.BOX 8174673461, Tel: +98 03137929097, E-mail: andalib@med.mui.ac.ir

Background: Programmed cell death protein-1 (PD-1)/PD-L1 pathway is one of the immune checkpoint pathways involved in the regulation of the immune responses and the suppression of anti-tumor defense. PD-1/PD-L1 blocking antibodies improve immune responses such as cytotoxic activity of $CD8^+/CD4^+$T cells and increase mortality of tumor cells as well; however, their use is accompanied by adverse side effects.
Objectives: We aimed to produce a native blocker of human PD-1/PD-L1, for developing T cells cytotoxicity and tumor cells apoptosis.
Materials and Methods: We designed and cloned soluble human PD-1-GFP-pcDNA3.1/hygro construct in *Escherichia coli* strain TOP10 cells and then transfected this construct into the HEK cells. The concentration of the secreted shPD-1 in the supernatant was measured and the supernatant was used for blocking PD-L1 on the MDA-MB-231 cells. The cytotoxicity of $CD8^+/CD4^+$T cells and the apoptosis of MDA-MB-231 cells, under the influence of shPD-1 in the co-culture of T cells with the MDA-MB-231 cells, were evaluated using flow cytometry technique.
Results: The GFP expression in the transfected cells illustrated the successful designing, transfection, and production of shPD-1. Soluble human PD-1 concentration in the supernatant of the transfected HEK cells was significantly higher than the untransfected cells. In addition, shPD-1 significantly blocked PD-L1 on the MDA- MB-231 cells, improved the cytotoxicity of $CD4^+$T cells, and increased the apoptosis of MDA-MB-231 cells.
Conclusion: Overall, increased $CD4^+$T cell cytotoxicity and tumor cells apoptosis under the influence of shPD-1, confirmed the effectiveness of shPD-1 as a natural blocker of PD-L1and as an augmenter of the anti-tumor immune responses.
Keywords: Apoptosis, PD-L1, Soluble Human PD-1, T Cell Cytotoxicity

1. Background

The programmed cell death protein-1 (PD-1, also known as CD279) and its ligands, PD-L1 (B7-H1 or CD274) and B7-DC (PD-L2 or CD273), constitute one of the immune checkpoint pathways that are involved in regulating the immune cell responses via inhibition, exhaustion, or augmentation of the immune cells (1, 2). PD-1 is expressed on activated lymphocytes and its ligand PD-L1 is expressed on a variety of hematopoietic and non-hematopoietic cells, as well as in various types of cancer cells (3). Once the PD-1/PD-Ls interaction takes place, PD-1 is phosphorylated on its intracellular tyrosine residues, which leads to declining signal transduction through phosphoinositide 3-kinase and T cell receptor complex (4). The interaction of PD-1 with its ligands are accompanied with reduction in perforin/ granzyme/ CD107a, interferon γ (IFNγ), tumor necrosis factor α (TNFα), and interleukin 2 (IL-2) production. PD-1/PD-Ls interaction also augments IL-6, IL-10 and transforming growth factor-β (TGFβ) secretion. Therefore, PD-1/PD-Ls axis is one of the most important modulation pathways in the immune system (5-7).

Studies have shown induction of IFNγ and TNFα secretion could increase PD-1 ligands expression on tumor cells as well as tumor-infiltrated immune cells which inhibit anti-tumor immune responses (8-10). In fact, in tumor cells, PD-L1 acts as an anti-apoptotic molecule and protects cells from apoptosis and the chemotherapy-mediated cytotoxicity (11, 12). Consequently, due to the inhibitory effect of PD-1/ PD-1 interactions on the immune responses, blocking of this axis to target the tumor cells, is an important immuno-therapeutic model (13). Besides, applying anti-PD-1 (e.g., Nivolumab), or anti PD-L1 (e.g., Atezolizumab) antibodies have shown capability in inducing an anti-tumor immune response in several tumors. In addition, experimental studies in *in vivo* melanoma models or *ex vivo* multiple myeloma indicated that anti-PD-1 antibody could restore cytotoxicity of immune cells, and cytokine secretion, as well as a reduced tumor size (14-17). Therefore, it is reasonable to suppose that blocking PD-1/ PD-L1 interaction using antibodies can increase the IFNγ production and the cytotoxicity of T cells in the tumor microenvironment (18). However, immune-toxic side effects are the consequence of using anti-PD-1/PD-Ls antibodies (19). Accordingly, the inhibitory agents, such as the genetically engineered PD-1, could be used for blocking this pathway without having the antibodies side effects. Experiment studies have shown that soluble PD-1, such as the IgV extracellular domain of PD-1, could be used to block the PD-1/PD-Ls pathway in animal models and *in vitro* conditions (20, 21). Hence, some attempts were made to produce a protein similar to a PD-1Δex3 variant product, which contains only extracellular domain without the trans-membrane domain (exon3) of PD-1 (22). This variant product can inhibit signaling of the membranous PD-1 on activated T cells and preserve T cells on activated functional state (23). Various murine PD-1 expressing plasmids, like pPD-1A and pAAV/sPD-1, have an extracellular domain of murine PD-1 which can attach to PD-L1 and block the PD-1/ PD-L1 interaction (24-26). However, the animal soluble PD-1 products can induce immunogenic reactions in human (27). Therefore, production of fully-human suppressors of PD-1/PD-Ls has been recommended to prevent later reactions.

2. Objective

The aim of this study was to design a soluble human PD-1 expressing construct for producing natural soluble human PD-1 in place of the membranous PD-1 gene. This efficacy of this product to block PD-L1 was evaluated. Its effects on T cells cytotoxicity and tumor cells apoptosis after blocking PD-L1 were determined. There may be advantages to our method of production beyond creating antibodies for blocking the PD-1/PD-L pathway.

3. Materials and Methods

3.1. Materials

The following substances were used in the present work: GeneJET™ Plasmid Miniprep Kit (Thermo Scientific, the USA); DMEM high glucose, RPMI1640, and fetal bovine serum (FBS, Gibco Ltd, USA); Pen-strep (Inoclon, Iran); Luria Bertani broth, Lennox (BIOMARK, India); Ficoll-Hypaque (Biosera, the UK); ConcanavalinA (conA, Sigma-Aldrich, USA); Polyfect (Qiagen, Germany); Dialysis tube, TUB2012 (12~14 kD) (Scientific Laboratory Supplies, UK); Anti-human PD-1 ELISA kit (R&D Co, the USA); and Monensin, FITC- Annexin V, mouse anti-human IFNγ antibody, FITC mouse anti-human CD274 (MIH1), FITC- mouse anti-human CD4 antibody, PerCP/ CY7.7- mouse anti-human CD8 antibody, PE- mouse anti-human CD107a antibody, and FITC- mouse anti-human isotype control (BioLegend, the USA).

3.2. Cell Culture

Human embryonic kidney (HEK 293, ATCC® CRL-1573™) and human invasive ductal carcinoma (MDA-MB-231 cells, ATCC® HTB-26™) were purchased from Pasteur Institute of Iran and cultured in Dulbecco's minimal essential medium (DMEM) with high glucose and RPMI 1640, respectively. These media were supplemented by 10% FBS and 1% Pen strep. Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll-hypaque density gradient from human donor venous blood. PBMCs were stimulated with 4 $\mu g.mL^{-1}$ conA at 37 °C and 5% CO for 3 and 6 days in a total volume of $500\mu l.well^{-1}$ RPMI 1640 plus FBS 10%, and Pen-strep 1% in 24 well plates. HEK 293 cells were used for transfection of pshPD-1 construct and production of shPD-1 protein. In the co-culture, MDA-MB-231 cells were applied as target cells which express PD-L1. PBMCs were used in the co-culture as the effector cells and inducer of PD-L1on MDA-MB-231 cells.

3.3. Co-Cultured Groups

The co-cultured groups of this work included a 6 days-conA stimulated PBMCs, co-cultured with MDA-MB-231 cell lines at the 5:1 ratio of effector/target cells for 24 h (group 1). In addition, 4500 $pg.mL^{-1}$ shPD1 was added to MDA-MB-231 cells 1 h before co-culturing with stimulated PBMCs (group 2). Triplicate wells were used for each experimental condition. The experiments were repeated three times.

3.4. Construction of PshPD-1-GFP

In order to achieve a soluble human PD-1 construct (shPD-1), the sequence of the extra cellular domain

of the human PD-1 cDNA (NCBI gene ID 5133, from 131-580n) was used, similar to the PD-1Δex3 variant. To convert membranous PD-1 to secretory and soluble PD-1, the human PD-1 signal peptide was compared with three secretory signal peptides (IL-2, IgK chain V-1 region HK102 and IgK chain V-1 region HK101) using signalP3.0, signalP4.1 (http://www.cbs.dtu.dk/services/SignalP), and target P1.1 (http://www.cbs.dtu.dk/services/TargetP/) softwares. The designed signal peptide sequence was placed in the shPD-1 construct instead of the native PD-1 signal peptide.

Additionally, green fluorescent protein (GFP) sequence located at the C-terminal of the construct was used as a reporter gene for detection of PD-1 expression in the transfected cells. A linker sequence (G4S1)3 was inserted between the PD-1 and GFP sequence and two restriction enzymes sites were chosen for 5' and 3' of the shPD-1 construct. This construct was cloned in pCDNA3.1 hygro and was expressed by the promoter of cytomegalovirus. The recombinant clones were selected by colony PCR using forward primer for the backbone of plasmid (5'-CACTGCTTACTGGCTTATCG-3) and reverse primer for the backbone of designed construct (5'-CTTGTGGCCGTTTACGTC3'). To confirm the recombinant pshPD-1-GFP, the positive clones were selected by colony PCR and then were subjected to plasmid extraction by GeneJET™ Plasmid Miniprep Kit according to the instruction of the manufactures. The extracted plasmids were digested with the restriction enzymes. Afterwards, the PCR and digestion products were detected on 1% agarose gel. The confirmed clones were cultured in the Luria-Bertani (LB) broth media with 100µg.mL^{-1} ampicillin and then the plasmid was extracted for utilization in cell transfection.

3.5. Cell Transfection

The HEK cells were cultured in 24-well tissue plates (4 x 10^4 cells/well) in DMEM high glucose medium at 37 ℃, 5% CO2. Then the pshPD-1-GFP was transfected into the HEK cells by polyfect according to the manufacturer's instructions. The pGFP was transfected alone into the HEK cells as a control. Next, the PD-1-GFP expression was evaluated using fluorescence microscopy and flow cytometry 24 h after the transfection.

3.6. Measurement of Secreted Shpd-1 and IFNγ

The shPD-1 secretion was determined in the supernatants of the transfected HEK cells, two days after the transfection by a quantitative sandwich ELISA kit using mouse anti-human PD-1 antibody. The proteins were salted out by ammonium sulfate 50% and then centrifuged (15200 g, at 4 ℃) for 20 min. The protein pellet was dissolved in PBS and dialyzed overnight in dialysis tube (TUB2012). Also, the concentration of shPD-1 in the dialyzed medium was determined by ELISA kit to the manufacturer's protocol. Two days after stimulation of PBMCs by conA, supernatants of the PBMCs were collected and then the concentrations of IFNγ was measured by ELISA kit. Using mouse anti-human IFNγ antibody. The limit of sensitivity of kits was 0.0 and 10,000 pg.mL^{-1} for the shPD-1 and 0.0 to 500 pg.mL^{-1} for IFNγ assays, respectively.

3.7. Functional Assay of Human Secreted PD-1

MDA-MB-231 cell line, which natively expresses PD-L1, was co-cultured with conA-stimulated PBMCs in a media containing dialyzed shPD-1 (48 h), to increase expression of PD-L1on the MDA-MB-231 cells. The MDA-MB-231 cells were harvested using trypsin/EDTA 1% and shPD-1/ PD-L1interaction was evaluated by using FITC- conjugated mouse anti-human PD-L1antibody. FITC- conjugated mouse anti-human isotype antibody was used as the negative control in the cell processing for flow cytometry.

3.8. Evaluation of CD107a Expression

To evaluate the cytotoxic activity of T cells under the influence of shPD-1, stimulated PBMCs were co-cultured with MDA-MB-231 cells and PE conjugated mouse anti-human CD107a antibody and monensin (0.35 mg.mL^{-1}) were added in the wells. After 6 h incubation in 37 ℃, suspending lymphocytes were collected and stained by PerCP/CY5.5 conjugated mouse anti-human CD8 and FITC conjugated mouse anti-human CD4 antibodies. Then, the CD107a expression on the T cells was assayed by a flow cytometer (FACSCalibur, Becton Dickinson, USA).

3.9. Evaluation of MDA-MB-231 Cell Lines Apoptosis

To evaluate the apoptotic effect of shPD-1 on co-cultured MDA-MB-231 cell lines, 24 h after co-culturing, the suspended cells were removed and adherent MDA-MB-231 cell lines were detached by 1X trypsin/EDTA. MDA-MB-231 cell lines were suspended in AnnexinV buffer set and stained by FITC AnnexinV. Apoptosis of MDA-MB-231 cell lines was investigated by a flow cytometer and data were analyzed using FlowJo software.

3.10. Statistical Analysis

The data were analyzed using the SPSS software (version 16.0, USA). Independent Student's t-test and ANOVA

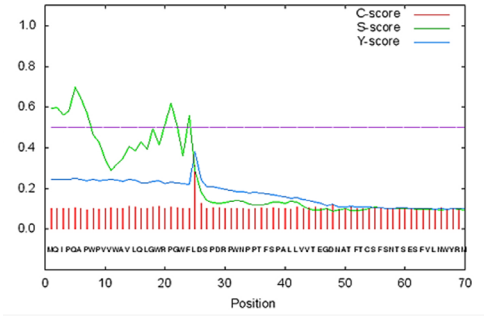
test were applied to compare two groups and more than two groups, respectively. Flow cytometry results were analyzed using the FlowJo software version 7. 6.1 for CD107a and AnnexinV assessments. $p<0.05$ was considered to indicate statistically significant differences.

4. Results

4.1. Selections of Secretory Signal Peptide for ShPD-1 Gene Construct

A new recombinant PD-1 construct, shPD-1-GFP, was designed in this study. The gene construct had IgK chain V-1 region HK101 signal peptide on 5' instead of the native signal peptide of human PD-1. The mentioned computer programs were used for comparing the secretory probability of membranous PD-1 signal peptide with IL-2, IgK chain V-1 region HK102, and IgK chain V-1 region HK101 signal peptides. The results showed that IgK chain V-1 region HK101 signal peptide is the most powerful secretory signal peptide with the highest D score, signal peptide probability, maximum cleavage site probability, and secretory pathway/class reliability ratio, but it had the lowest signal peptide anchor probability **(Fig. 1)**.

A)



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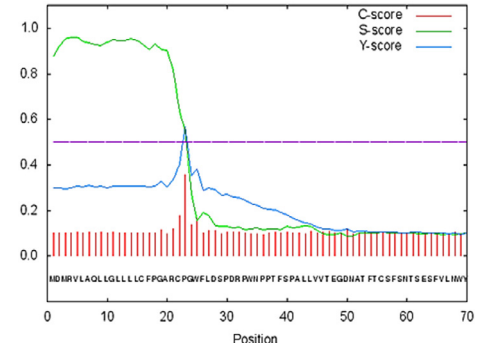
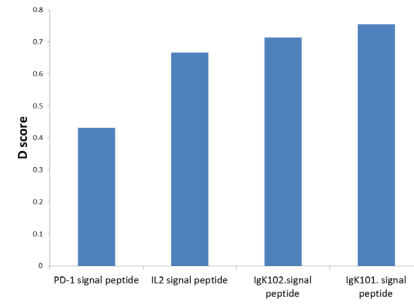


Figure 1. A) the "SignalP 4.1" software analysis for native PD-1 signal peptide, **B)** IgK chain V-1 region HK101 signal peptide

4.2. Plasmid Construction

The shPD-1-GFP construct was sub-cloned in pcDNA3.1 hygro. The PCR amplification of the recombinant pcDNA3.1/hygro containing the shPD-1-GFP resulted in a 700-bp PCR product **(Fig. 3A)**. Furthermore, digestion of pshPD-1-GFP with restriction enzymes resulted in two restricted DNA fragments, 5500-bp, and 1300-bp **(Fig.3B)**, which confirmed a successful cloning of shPD-1-GFP expression cassette in the pcDNA3.1/hygro vector.

A)



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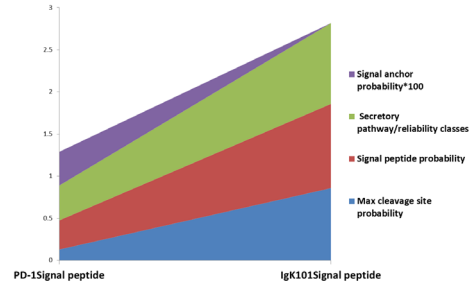


Figure 2. A) The D scores of three secretory signal peptides and native PD-1 signal peptide, compression of secretory probabilities (max cleavage site probability, signal peptide probability, **B)** signal anchor probability, and secretory pathway/Reliability class ratio) with native PD-1 and IgK chain V-1 region HK101 signal peptides.

4.3. Plasmid Expression and Characterization

The pshPD-1-GFP-cDNA3.1 hygro was transfected into the HEK cell line to confirm shPD-1-GFP expression. Fluorescent microscopy revealed an obvious green fluorescence in the transfected HEK cell line **(Fig. 3C)**. Also, flow cytometry analysis data indicated that 51.96%±4.3 of transfected HEK cell line had a high level of GFP expression in comparison with un-transfected HEK cell line **(Figs. 3D and 3E)**.

4.4. Expression of shPD-1 and IFNγ proteins

The obtained results showed high expression levels of shPD-1 proteins in the supernatant of transfected the HEK cell line **(Fig. 4A)**. In the supernatant of pshPD-1-GFP transfected HEK cells after two days, the levels of shPD-1 protein were significantly higher (4550±685.5 $pg.mL^{-1}$) than those of the un-transfected HEK cells (19.2±4.3$pg.mL^{-1}$) and the pGFP transfected cells (12.7±6.1 $pg.mL^{-1}$) ($p<0.001$). Also, the shPD-1 protein of the dialyzed medium was significantly higher (4625.5±585.2$pg.mL^{-1}$) than the transfected HEK cell by pGFP and un-transfected cells ($p<0.001$).

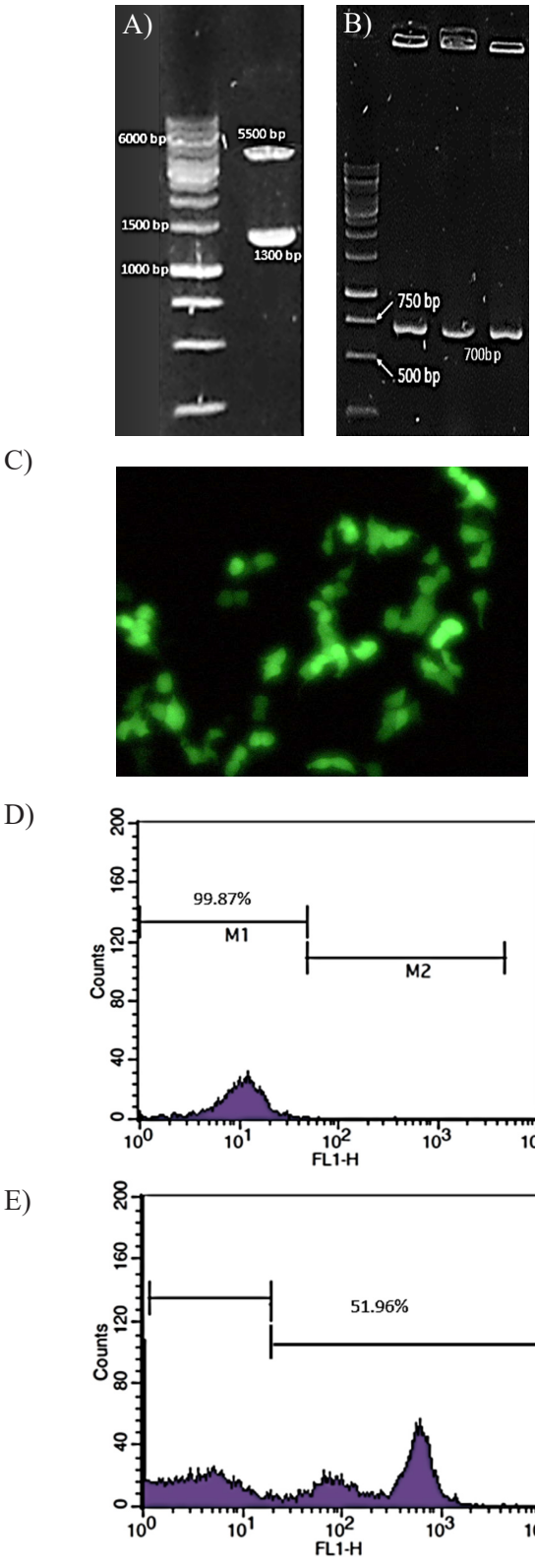


Figure 3. A) The shPD-1-GFP construct cloning in pcDNA3.1/hygro on 1% agarose gel, **B)** ligated pshPD-1-GFP-pcDNA3.1/hygro by digestion, **C)** GFP expression in transfected HEK cells, **D)** flow cytometry analysis of GFP expression in untransfected/, transfected HEK cells, **E)** flow cytometry analysis of GFP expression in untransfected/transfected HEK cells.

To evaluate the secretion of IFNγ, we determined the IFNγ levels in supernatants of stimulated PBMCs **(Fig. 4B)**. Our data indicated that the levels of IFNγ were significantly higher in the culture of the conA-stimulated PBMCs (933±76pg.mL^{-1}) than the culture of un-stimulated PBMCs (26±22pg.mL^{-1}), P<0.001.

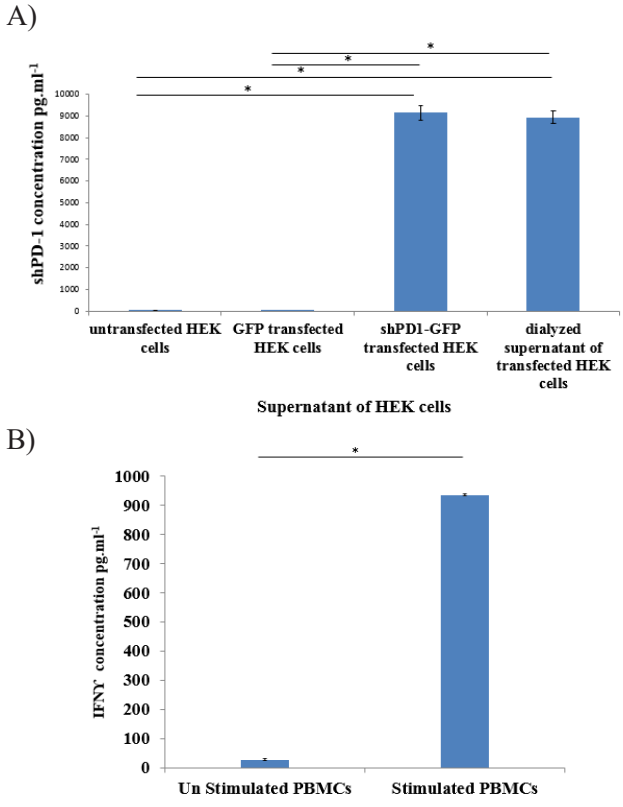
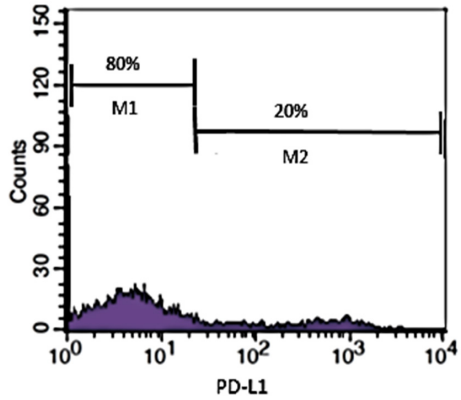


Figure 4. A) The concentration of secreted shPD-1 in the supernatant of transfected/untransfected HEK cells, **B)** the concentration of IFNγ secretion in the supernatant of the conA-stimulated and unstimulated PBMCs.

4.5. Functionality of ShPD-1

To induce PD-L1overexpression on MDA-MB-231 cell line and evaluate whether the secreted shPD-1 by the transfected HEK cells could bind to PD-L1 and block its interaction, we co-cultured MDA-MB-231 cells with ConA-stimulated PBMCs for 48 h. Our flow cytometry analysis data showed that PD-L1 positive MDA-MB-231 cells were significantly higher (p<0.001) in co-cultured MDA-MB-231 cells (97.3%±2) compared to the untreated MDA-MB-231 cells (20%±2) **(Figs. 5A and B)**. Furthermore, PD-L1 over-expressing cells were used as target cells in the interaction of shPD-1 with PD-L1. We found that 94.17%±1 of MDA-MB-231 cells were covered by shPD-1 and therefore attachment of anti- PD-L1 to PD-L1 was greatly hindered (p <0.001) **(Fig. 5C)**.

A)



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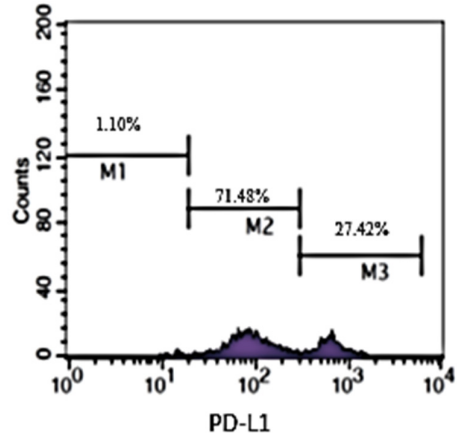


C)

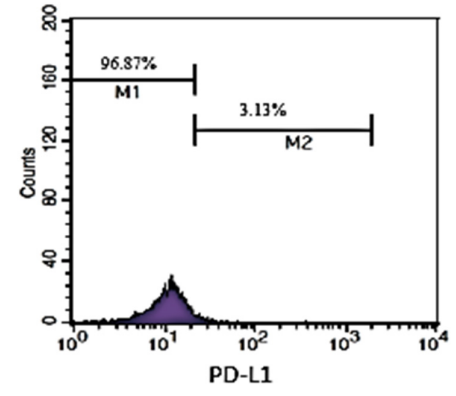
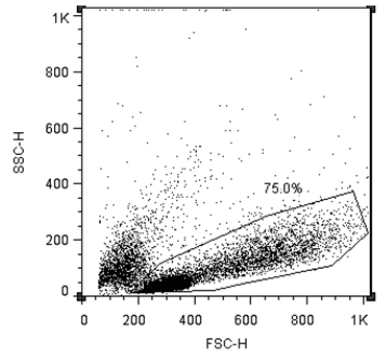


Figure 5. A) Flow cytometry analysis of PD-L1 expression in A) MDA-MB-231 cells, **B)** co-cultured MDA-MB-231 cells with stimulated PBMCs, **C)** co-cultured MDA-MB-231 cells with stimulated PBMCs and shPD-1.

4.6. Evaluation of the Purity of CD3$^+$T Cells

To analyze the effect of shPD1 on the T cells, PBMCs were stimulated by conA and CD3$^+$T lymphocytes were evaluated by PE anti-human CD3 antibody. CD3$^+$ T cells population was 92.3% in PBMCs after 6 days conA stimulation **(Fig. 6)**.

A)



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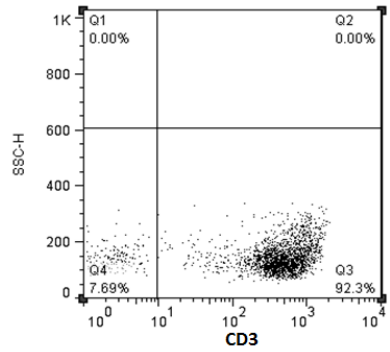

Figure6. A) The PBMCs isolation by ficoll and **B)** CD3$^+$T lymphocyte population with 92% purity after 6 days of stimulation

4.7. CD107a Expression Between Co-Cultured Groups

CD107a expression was evaluated as a cytotoxic marker of the T cells. CD107a expression on CD4$^+$ T cells was significantly higher (p<0.05) in the presence of shPD-1; however, no significant difference was detected for CD107a expression on CD8$^+$T cells between the co-cultured groups **(Figs. 7A and 7B)**.

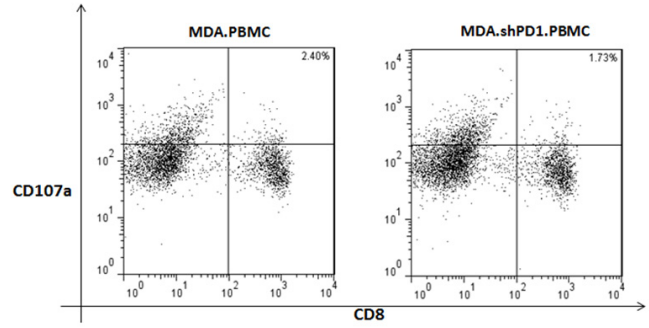

Figure 7. The flow cytometry chart of CD107a expression for **A)** CD8$^+$T cells and **B)** CD4$^+$T cells in co-cultured groups

4.8. Apoptosis of MDA-MB-231 Cells Between the Co-Cultured Groups

AnnexinV has high affinity to phosphatidylserine on the outer membrane of apoptotic cells. FITC-annexinV

was used to evaluate apoptosis of MDA-MB-231 cells. We found that apoptosis of MDA-MB-231 cells was significantly increased (p<0.05) in the treated cells in presence of shPD1 **(Figs. 8)**.

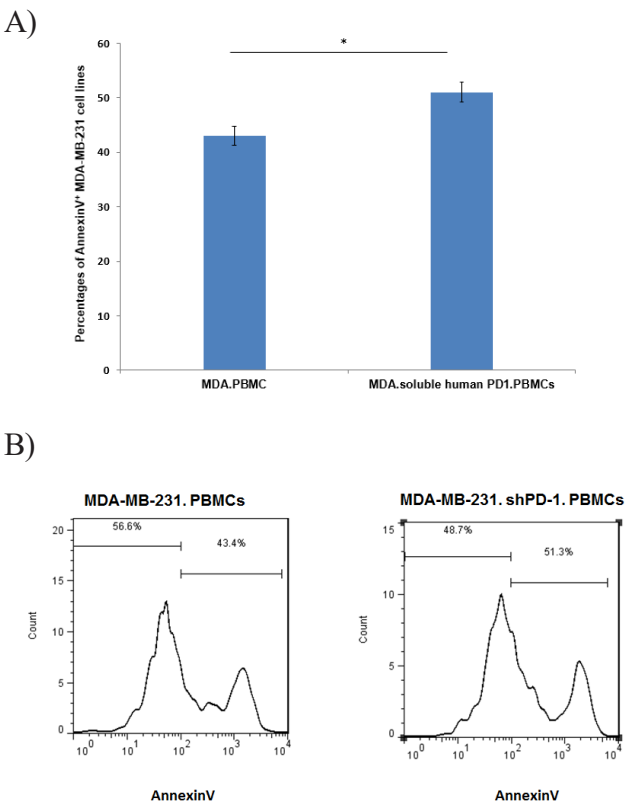

Figure 8. A) AnnexinV$^+$ staining MDA-MB-231 cell lines in the co-cultured groups and **B)** the histogram of AnnexinV$^+$ MDA-MB-231 cell line percentage in the co-cultured groups. *: P<0.05.

5. Discussion

PD-1/ PD-L1 blockers such as anti PD-1 and anti PD-L1 antibodies can block PD-1/ PD-L1 pathway and enhance the cytotoxicity of CTLs (15). Elevating soluble PD-1 levels in autoimmune disease through encoding PD-1Δex3 splice variant blocks PD-1/ PD-L1 signaling pathway. This soluble marker regulates the T cells activity and enhances proliferative response of CD4$^+$ and CD8$^+$T cells (23). Accordingly, in this study, we hypothesized that a native soluble human PD-1 (shPD-1) through binding to PD-L1 on tumor cells blocks PD-1/ PD-L1 interaction and can restore anti-tumor T cell responses. We, therefore, constructed and expressed a secretory shPD-1-GFP cassette gene and examined its product interaction with PD-L1.

To construct a native shPD-1 gene, the secretory signal peptide of human IgK was used in the construct containing the reference sequences of the extracellular domain of human PD-1. Modification of PD-1 signal peptide was performed for enhancement of PD-1 secretion (28). This modification was carried out based on prediction analyses of two computer programs (signalP and TargetP). Our analysis showed that signal peptides of secretory proteins, such as IgK signal peptide, are more powerful (2x) than native PD-1 signal peptide for secretion. The present study confirmed the results of a similar study by Hui Qiu *et al* (29). Also, native PD-1 signal peptide could secret PD1 by using the extracellular domain of the PD-1 without the transmembrane domain (20, 21). We suggest that IgK signal peptide could be a more potent signal peptide for PD-1 extracellular. To avoid some modifications such as mutations or SNPs that affect the affinity and ligand-specificity of PD-1 (30), we synthesized shPD-1 construct based on reference sequence of the PD-1 extracellular domain. Our study indicated that, similar to the human PD-1Δex3 variant, shPD-1 construct gene had no trans-membrane and intracellular domains (22). In contrast to our work, some researcher achieved murine PD-1 extracellular domain by PCR and then compared their sequences with murine reference sequences (20, 21). However, we used reference sequence of human PD-1 to prevent undesirable SNP interrupting in final shPD-1 function and to assure complete activity of its product.

To investigate whether shPD-1 product interacts with its ligand PD-L1, we provided the PD-L1expressing target cells; i.e., MDA-MB-231 cell line. To make our experimental conditions similar to those of tumor microenvironment, the cell line was co-cultured with ConA-stimulated PBMCs, wherein IFNγ secretion induced the transcription and the expression of PD-L1 in the cells (12, 31). Our findings indicated that high concentration of IFNγ and overexpressing PD-L1 in the target cells would cause the tumor cells could escape from immune responses. Afterwards, in *in vitro* experiments, we used shPD-1 for blocking PD-L1 in MDA-MB-231 cells. The results showed that shPD-1 interacted with PD-L1on MDA-MB-231 cells and inhibited its interaction with PD-1 in the T cells. Similarly, other studies revealed that secreted sPD-1 in the supernatant of cells transfected by psPD-1 plasmids would be able to block PD-Ls on DCs as well as H22 cell line, and spleen cells (25, 32). In addition, Lázár-Molnár E et al. have shown that mutant PD-1-Ig could block PD-1/PD-L1 interactions between CD4$^+$T cells and dendritic cells (30). Therefore, shPD-1 product can be used to block membranous PD-1/ PD-L1 interactions. For instance, these are similar to the situation like tumor environments or chronic viral infections, where membranous PD-1/PD-L1 interactions reduce cytotoxicity for anti-tumor T cells or anti-viral specific T cells (1).

In the present study, the cytotoxicity of T cells was evaluated by measuring the lysosome degranulation. We showed that in the presence of shPD-1 the cytotoxicity of CD4+T cells was significantly increased, however there were no significant differences in the cytotoxicity of $CD8^+$T cells in the presence or absence of shPD1. Granzyme B or Granzyme B-associated molecules such as perforin or CD107a are usually assessed for cytotoxicity of T cells. Granzyme B is expressed by activated memory $CD8^+$ and $CD4^+$T cells, which can kill virally infected and tumor cells (33). Previous studies have shown that $CD8^+$T cells express intracellular Granzyme B more than $CD4^+$T cells; however, secretion of this serine protease by the activated memory $CD8^+$ is similar to the activated memory $CD4^+$T cells (34). In a mouse model of viral infection, elimination of target cells by $CD4^+$T cells was equivalent to $CD8^+$T cells (35). In this study, evaluation of CD107a was a stronger indicator of cytotoxicity and lysosome degranulation of T cells than intracellular Granzyme B. On the other hand, recent studies recommended that persistent antigenic stimulation could not induce cytotoxic $CD4^+$ T cells because co-stimulatory signals and cytokines stimulation were required simultaneously (36). Therefore, in our study, shPD1 as a co-stimulatory signal along with antigen expression through MHCII by MDA-MB-231 cells likely increased cytotoxic $CD4^+$ T cells population. This co-stimulatory signal inhibits PD-1/ PD-L1interactions and improves cytotoxicity of $CD4^+$T cells. Consistent with our findings, other studies have indicated that anti- PD-1 antibodies (e.g. nivolumab) and anti- PD-L1antibodies (e.g. BMS936559) inhibit PD1/ PD-L1interaction and increase the proliferation and the cytotoxicity of $CD8^+$T cells in patients with advanced melanoma (14-16). In an ex vivo study, anti-PDL1 antibodies were able to increase cytotoxicity of HCV specific $CD8^+$T cells (37). Additionally, in mouse models of hepatocarcinoma or melanoma, cytotoxicity of CTLs were increased using soluble PD-1 expressing plasmids (20, 21, 29). Furthermore, mutant PD-1-Ig has improved proliferation and cytokine production of T cells in a mouse model with tumor and also in co-cultures of the $CD4^+$ T cells/dendritic cells (30). Interestingly, in a macaque AIDS model study, elicited $CD107a^+$ $CD4^+$T cells were resistant to depletion following infection (38). Therefore, improvement the cytotoxicity of CD4+T cells population by shPD-1 in the present study would be useful to augment protective immunity of cytotoxic $CD4^+$T cells in tumor environments and also in viral infections.

We observed no enhancement of cytotoxic $CD8^+$T cells in the co-culture of stimulated PBMCs with MDA-MB-231 cells. Consistent with our findings, Nakamoto et al. have indicated that ex vivo blockade of PD-1 by anti-PD-L1 antibodies was not sufficient to improve the proliferation of $CD8^+$T cells and IFN-γ or cytolytic molecules (perforin, CD107a) secretion (39). The existence of other inhibitory molecules such as CTLA-4 (39), and shPD-1 concentration are causing for dysfuntionality of $CD8^+$T cells during the treatment processing.

In addition, we showed the influence of shPD1 on augmentation of apoptosis in MDA-MB-231 cells. Previous studies have reported that the expression of PD-L1 in tumor cells regulates cell biological activity, such as apoptosis, and in the absence PD-L1, apoptosis would induced and cell cycling would grind to halt (11). In fact, PD-L1expression and signaling play an anti-apoptotic role in tumor cells and reduce T cell cytotoxicity (12). In this study, shPD-1 suppressed PD-L1 signaling and induced extracellular pathway of apoptosis through mediators, which were released by cytotoxic $CD107^+CD4^+$T cells in the presence of shPD-1. In fact, an inflammatory environment containing secreted cytokines of stimulated T cells could increase the susceptibility of tumor cells to apoptosis (40, 41). The previous studies indicated that, blockade of PD-L1 signaling and induction of apoptosis by using anti-PD1, anti-PD-L1 antibodies, anti-PD1 siRNA, or soluble mouse PD1 were able to reduce tumor mass in patients with melanoma or in mouse bearing tumor (11, 12, 42). Additionally, soluble murine PD1 expressing plasmids caused CTL mediated lysis of tumor cells and reduced tumor in murine models of melanoma and hepatocarcinoma (20, 21). Therefore, based on our study shPD-1, similar to other blockers of PD-1/PD-L1, could suppress anti-apoptotic signaling of PD-L1 through mitogen-activated protein kinases pathway and inhibit survival or tumor proliferation (12). However, more detail studies are needed to assess the effects of shPD-1 on anti-apoptotic signaling pathways and cell survival.

6. Conclusion

The present study showed that inhibitory function of a shPD-1 protein is a potent blocker of the PD-L1 interaction with the anti- PD-L1 antibody. Therefore, the shPD-1 product would be a potential candidate for blocking the PD-1 ligands interactions on the immune or non-immune cells in the following interactions: PD-L1/membranous PD-1 and PD-L1/CD80. In addition, the shPD-1 increased the cytotoxicity of T cells, as well as mortality of tumor cells. Therefore, pshPD1 or shPD1 potentially could be used as a therapeutic DNA vaccine or protein in tumor microenvironments.

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Conflicts of Interest

The authors disclose no potential conflicts of interest.

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