Development of a Stable Cell Line, Overexpressing Human T-cell Immunoglobulin Mucin 1

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Background: Recent researches have demonstrated that human T-cell immunoglobulin mucin 1 (TIM-1) glycoprotein plays important roles in regulation of autoimmune and allergic diseases, as well as in tumor immunity and response to viral infections. Therefore, targeting TIM-1 could be a potential therapeutic approach against such diseases.
Objectives: In this study, we aimed to express TIM-1 protein on Human Embryonic kidney (HEK) 293T cell line in order to have an available source of the TIM-1 antigen.
Materials and Methods: The cDNA was synthesized after RNA extraction from peripheral blood mononuclear cells (PBMC) and TIM-1 cDNA was amplified by PCR with specific primers. The PCR product was cloned in pcDNA™3.1/Hygro (+) and transformed in *Escherichia coli* TOP 10 F'. After cloning, authenticity of DNA sequence was checked and expressed in HEK 293T cells. Finally, expression of TIM-1 was analyzed by flow cytometry and real-time PCR.
Results: The result of DNA sequencing demonstrated correctness of TIM-1 DNA sequence. The flow cytometry results indicated that TIM-1 was expressed in about 90% of transfected HEK 293T cells. The real-time PCR analysis showed TIM-1 mRNA expression increased 195-fold in transfected cells compared with un-transfected cells.
Conclusions: Findings of present study demonstrated the successful cloning and expression of TIM-1 on HEK 293T cells. These cells could be used as an immunogenic source for production of specific monoclonal antibodies, nanobodies and aptamers against human TIM-1.

Keywords: Cloning; Gene expression; HEK 293T; Immunogenic source; TIM-1

1. Background

TIM-1 is a type I membrane glycoprotein expressed on wide variety of cells consisted of T helper (Th) cells, B cells, mast cells, macrophages and kidney proximal tubular epithelial cells (1, 2). TIM-1, also known as hepatitis A virus cellular receptor-1 (HAVCR-1) and kidney injury molecule-1 (KIM-1), is considerably upregulated in proximal tubular epithelial cells of kidney following ischemic and toxic injury (3).

Human TIM-1 gene is located on 5q32.2 locus, which is a region linked with susceptibility to asthma and allergy, and also is highly polymorphic among TIM gene family members (4, 5). Structurally, TIM-1 include an NH_2-terminal IgV-like domain with an *N*-linked glycosylation site, a mucin domain containing many *O*-linked glycosylation sites, a transmembrane domain, and a COOH-terminal short cytoplasmic domain (6).

TIM-1 protein as a co-stimulatory molecule regulates the expansion and effector functions of T helper

cells, particularly Th-2 immune responses (7, 8). Thus, associations between TIM-1 and allergic diseases are evident that makes it a major worldwide health issue (5). Moreover, TIM-1 plays critical role in Th-1 and Th-17 cell homing into the central nervous system (CNS). Thus, TIM-1 might be an important tool to achieve selective tissue homing of T cells in neurodegenerative disorders like multiple sclerosis (MS) (9). TIM-1 also regulates responses to viral infections and homeostasis of tumor microenvironment (2, 10). TIM-1 as a phosphatidylserine receptor on the surface of antigen-presenting cells helps in the detection and removal of apoptotic cells (2, 11). Filoviruses, ebolavirus, alphaviruses and baculoviruses use TIM-1 as a receptor for their entry to host cells (12-14). Phosphatidylserine on the viral envelope through interaction with TIM-1 enhances infection by these viruses (10, 15).

Therefore, interference with TIM-1 signaling, stimulating or blocking TIM-1 activity offers a novel approach for potential treatments for T cell-mediated diseases (9, 16). For example, blocking TIM-1 would inhibit Th-2 proliferation and cytokine production in allergic diseases (7). Also, blocking TIM-1 might be useful for preventing entry of Th1 and Th17 cells to CNS in order to help for treatment of multiple sclerosis (9).

2. Objectives

Anti-human monoclonal antibody, nanobody and aptamer could be considered as novel therapeutic agents for manipulation of TIM-1 associated immune-disregulations. Producing such agents requires stable source over-expression of TIM-1 antigen on a suitable cell line; not only for having an available source of the antigen, but also to achieve proper folding in TIM-1 protein structure. Hence, we aimed to produce a human TIM-1 expressing cell line using HEK 293T cells.

3. Materials and Methods

3.1. Cells and Bacteria

HEK 293T cells were purchased from Pasteur Institute of Iran and cultured in DMEM (Sigma-Aldrich, St., MO, USA) medium supplemented with 1% (w/v) penicillin/streptomycin (Gibco BRL, Karlsruhe, Germany) and 10% FBS (Sigma-Aldrich, St., MO, USA) in 5% CO_2 at 37ºC. *E. coli* TOP 10 F' was purchased from Pasteur Institute of Iran and cultured in Luria Bertani (LB) culture medium (Sigma-Aldrich, St., MO, USA) containing 100 µg.mL^{-1} tetracycline (Sigma-Aldrich, St., MO, USA).

3.2. PBMC Isolation

PBMC were isolated from heparinized whole blood samples using gradient centrifugation in Ficoll-Paque solution according to its manufacturer's instruction (Sigma-Aldrich, St., MO, USA).

3.3. RNA Extraction and cDNA Synthesis

Total RNA was extracted from PBMC using RNX kit (CinnaGen, Inc, Iran) based on the manufacturer's protocol. Using spectrophotometry and electrophoresis, quantity and quality of the extracted RNA were measured. The extracted RNAs were treated with *DNase I* (Thermo Fisher Scientific, Inc., MA, USA) and cleaned up with DNA extraction kit (Bioneer Inc., Seoul, South Korea) according to the manufacturer's instruction. cDNA was synthesized from 1 µg of the total RNA using first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc., MA, USA) as instructed by the manufacturer.

3.4. TIM-1 Amplification

PCR was carried out on the cDNA for specific amplification of TIM-1 using designed specific primer: TIM-1-forward containing *Nhe*I restriction site

Table 1. Oligonucleotide primers using in PCR

Name	Sequences (5'-3')	Length (bp)	Tm (°C)
TIM-1	Forward: GCTAGCATGCATCCTCAAGTGGTC	24	63.8
	Reverse: CTCGAGTTAGTCCGTGGCATAAAG	24	61.2
pcDNA	Forward: ACTAGAGAACCCACTGCTTACTG	23	60.6
	Reverse: ATGGCTGGCAACTAGAAGG	19	56.7
RTIM	Forward: ACATAGTCTACTGACGGCCAATACC	25	61.9
	Reverse: CTCGAGTTAGTCCGTGGCATAAAGAC	26	63.2
β-actin	Forward: TTCGAGCAAGAGATGGCCA	19	59
	Reverse: CACAGGACTCCATGCCCAG	19	60

at 5′ end, and TIM-1-reverse containing *Xho*I restriction site at 5′ end (Table 1). The PCR program started with 1 cycle at 95ºC for 4 min, continued by 30 cycles at 95ºC for 30 sec, 58ºC for 30 s and 72ºC for 2 min and 40 s, and ended with 1 cycle at 72°C for 10 min in a thermo cycler (Bio-Rad Laboratory, CA, USA). PCR products were electrophoresed on 1% (w/v) agarose and purified using Gel Purification kit (Bioneer Inc., Seoul, South Korea).

3.5. Cloning of TIM-1 cDNA

The pcDNA™3.1/Hygro (+) (100 ng.mL^{-1}) (Pasteur Institute, Iran) and TIM-1 amplicons (120 ng.µL^{-1}) were separately digested with *Xho*I and *Nhe*I enzymes (Thermo Fisher Scientific, Inc., MA, USA) according to the manufacturer's instruction. The digestion products (backbone of pcDNA™3.1/Hygro (+) and TIM-1 PCR product) were electrophoresed on 1% agarose and gel purified. Linearized pcDNA™3.1/Hygro (+) and TIM-1 were ligated using T4 DNA ligase enzyme (Thermo Fisher Scientific, Inc., MA, USA) according to the manufacturer's instruction. Ligation product was transformed into chemically competent *E. coli* TOP 10 F′ using heat shock method (17). The pcDNA™3.1/Hygro (+) contains ampicillin resistance gene, therefore, *E. coli* TOP 10 F′ bacteria could growth in LB culture medium with ampicillin as a selection marker.

To confirm the transfected clones, DNA sequencing was performed using a specific primer: pcDNA forward and reverse (Table 1).

3.6. Linearization of pcDNA/TIM-1

The pcDNA/TIM-1 plasmid was extracted with plasmid extraction kit (SolGent Co., Ltd., Deajaon, Korea). The extracted pcDNA/TIM-1 was linearized using *Bgl* II enzyme (Thermo Fisher Scientific, Inc., MA, USA) according to the manufacturer's instruction. After electrophoresis of the product on 1% agarose, the linear plasmid was gel purified.

3.7. Transfection

Transfection was performed using TurboFect reagent (Thermo Fisher Scientific, Inc., MA, USA) based on the provided protocol. Prior transfection (24 h earlier), $1×10^6$ HEK 293T cells were sub-cultured in two culture flasks so that confluency of the cells on the time of transfection was 60-70%. The linear pcDNA/TIM-1 plasmid (7.5 µg) were added to 750 µL serum free-DMEM medium, suspended by pipetting, and incubated for 2 min. About 15 µL of TurboFect

reagent was added to the plasmid suspension and incubated for 15-20 min at 22ºC. The content of the tube was added to the cell culture flask drop-wise, mixed by swirling and incubated at 37ºC overnight. In parallel, HEK 293T cells were transfected by linear pcDNA devoid of TIM-1 cDNA as control (mock transfection). On the next day, culture medium was refreshed. Following transfection, the cells were treated with 175 µg.mL^{-1} hygromycin at day 3 (Roche Diagnostics, Indianapolis, IN, USA) to select the transfected cells.

3.8. Polymerase Chain Reaction (PCR) on HEK 293T Genomic DNA

To confirm the integration of linear pcDNA/TIM-1 into genome of HEK 293T cells, one month after transfection, genomic DNA of the transfected and un-transfected cells were extracted by DNA extraction kit (Genetbio, Deajaon, Korea) according to the manufacturer's instruction. PCR with pcDNA forward and reverse primers (Table 1) was carried out. The PCR program was started with 1 cycle at 94ºC for 3 min, continued by 30 cycles at 94ºC for 30 sec, 58ºC for 30 sec and 72ºC for 1 min and 30 sec, and ended with 1 cycle at 72°C for 10 min in a thermo cycler (Bio-Rad Laboratory). Genomic DNA of un-transfected HEK 293T cells was used as the negative control. PCR products of the control and the positive samples were analyzed by electrophoresis on 1% agarose gel. Expression of human TIM-1 in HEK 293T cell line was examined by flow cytometry and real-time quantitative PCR methods.

3.9. Flow Cytometry

Transfected and control cells were placed in 2 distinct flow cytometry tubes (suspension of $1×10^6$ cells/200 µL medium in each tube), 30 days following transfection. The cells were stained with 1 µL PE conjugated monoclonal anti-TIM-1 antibody (Biolegend, Inc., CA, USA), and with appropriate isotype control antibody (Biolegend, UK), separately. Cells were evaluated using FACS Callibour flow cytometry (BD bioscience, NJ, USA). The flow cytometric data were analysed by Cell quest pro software (IBM, CA, USA).

3.10. Real-Time Quantitative PCR

Total RNA of selected cells was isolated and cDNA was synthesized as described above. Primers was designed, RTIM forwardand reverse, and β-actin-forward and reverse (Table 1). Real-time PCR was performed in a total volume of 20 µL in the StepOnePlus™ real-time PCR system (AB Applied

Biosystems, CA, USA) using SYBR$^{®}$ Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan) with the following program: 1 cycle 95°C for 10 min, continued by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Data analysis was performed using $2^{-\Delta\Delta Ct}$ method (18). The results were normalized against β-actin expression level as a reference gene (19). All the reactions were performed in triplicate.

3.11. Statistical Analyses

The results were analyzed by SPSS 20 (Chicago, IL, USA) software. Mann-Withney U nonparametric test used to compare the results of groups. All the qPCR results were reported as Mean±SD (standard deviation) and mean was obtained from three identical repeats of each test.

4. Results

4.1. Amplifying TIM-1 cDNA

For the purpose of producing a human TIM-1 expressing cell line, we needed the human TIM-1 encoding cDNA. TIM-1 molecule expresses on T helper cells, B cells, mast cells and macrophages. Therefore, PBMC could be an appropriate source for this purpose. After amplification of TIM-1 cDNA with specific primers bearing *Nhe*I and *Xho*I specific restriction sites, agarose gel electrophoresis of PCR product demonstrated a 1107 bp band (1095 bp for TIM-1 cDNA and 12 bp for two restriction sites) (Figure 1).
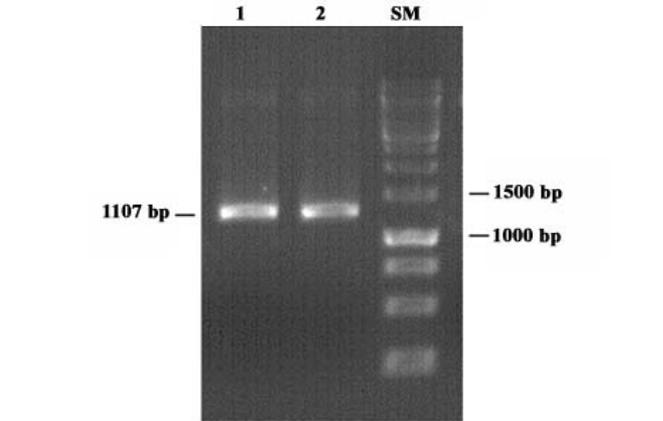
Figure 1. Amplification of specific band for human *TIM-1* cDNA using Pfu DNA polymerase. Lane 1 and 2, *TIM-1* fragments with size of 1107 bp. Lane SM, DNA size marker (1kb)

Figure 2. Nucleotide sequence alignment of cloned TIM-1at NCBI. Cloned TIM-1 sequence showed complete identity with human TIM-1 reference sequence. Only 5′ to 3′ ends of sequence have been briefly shown

4.2. Sequencing of pcDNA/TIM-1

After transformation of pcDNA/TIM-1 construction into competent *E. coli* TOP 10 F' bacteria, to confirm cloning of pcDNA/TIM-1 construction, DNA sequencing proved the presence of similar sequence with human TIM-1 reference sequence in the recombinant plasmid (Figure 2).

4.3. Expression of Human TIM-1 Protein

Following transfection of HEK 293T cells with liner pcDNA/TIM-1, to assess integration of linear pcDNA/TIM-1 into the genome of HEK 293T cells, PCR was performed and gel electrophoresis demonstrated the presence of pcDNA/TIM-1 in HEK 293T genomic DNA (band of 1240 bp) (Figure 3). The results of flow cytometry showed 88.15% of transfected cells expressed TIM-1 protein on their own surfaces. On the other hand, surface staining of un-transfected and mock transfection cells have no expression of TIM-1(1.72 and 1.59%, respectively) (Figure 4). Real-time PCR results were in parallel with flow cytometry results revealed that levels of TIM-1 expression increased up to 195-fold (p<0.05) (Figure 5).

5. Discussion

Considering the role of the TIM-1 in development of autoimmune diseases and allergy, regulation of rolling of T cell subsets particularly Th1 and Th17 cell homing into the central nervous system (CNS), homeostasis of tumor microenvironment and receptor for virus entrance into host cells (2, 8-10), TIM-1 could be considered as a target for therapy of T cell-mediated diseases and virus infection using its specific monoclonal antibody, nanobody or aptamer (7, 15).
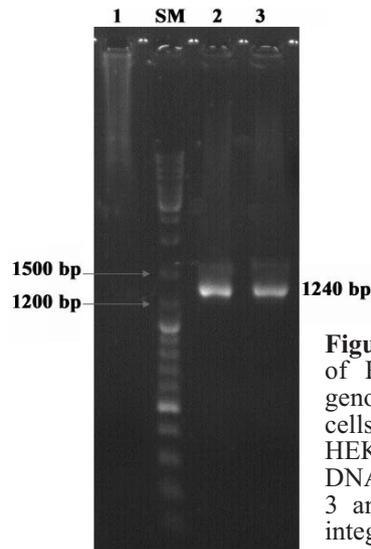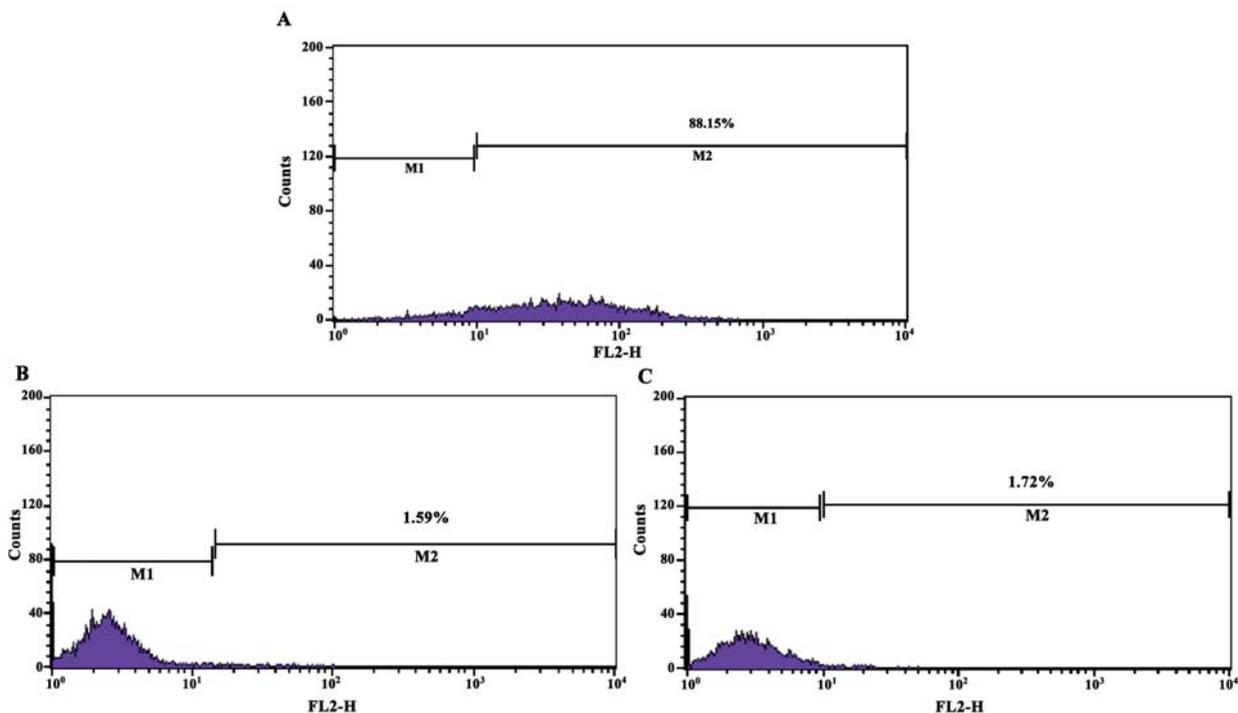

Figure 3. Gel electrophoresis of PCR product related to genomic DNA of transected cells. Lane 1, untransfected HEK 293T cells, Lane SM, DNA size marker (1kb), Lane 3 and 4,a 1240 bp band of integrated human TIM-1

Figure 4. Flow cytometric analysis. Transfected cells displayed significantly positive signal, whereas mock transfected and untransfected cells showed no positive signal. A: pcDNA/TIM-1 transfected HEK 293T. B: Mock transfected HEK 293T. C: untransfected HEK 293T. FL2: Anti human TIM-1-PE
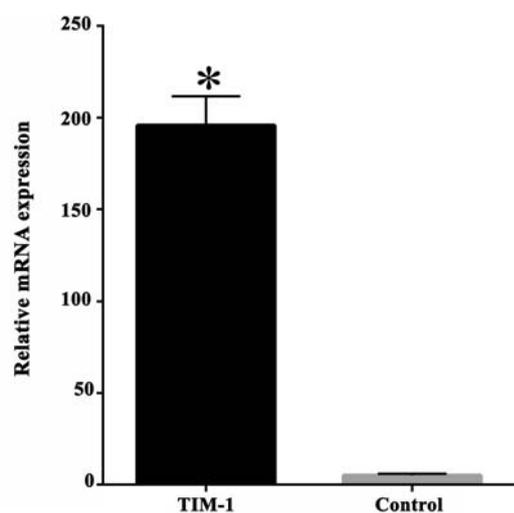
Figure 5. Real-time quantitative PCR analysis. TIM-1 column represents human TIM-1 mRNA expression level in transfected cells with linearized pcDNA/TIM-1 and control column represents human TIM-1 mRNA expression in untransfecetd ones. The results are represented as mean±SD (n=3); *p<0.05 versus control cells

Several anti-TIM-1 monoclonal antibodies have been used in treatment of experimental autoimmune diseases, allergy and viral infections. Xiao *et al.* (20) using anti-TIM-1 antibodies showed that these antibodies could have a dual function: inhibiting and increasing of T cell responses in autoimmune encephalomyelitis. The results of Encinas *et al.* (21) research indicated that anti-TIM-1 antibody can decrease Th2 responses in a mouse model of asthma. Study of Soo Hoo *et al.* (22) showed that TIM-1 antibodies caused induction of cellular proliferation and IFN-γ production against influenza virus infection.

The cells over-expressing a certain molecule are an appropriate model as target for the production of monoclonal antibodies, nanobodies and selection process of aptamers. Eukaryotic cells provide a suitable source for recombinant protein production with accurate folding and proper post translational modifications. Consistent with previous data, the HEK 293T cells are suitable source for this aim (23-26).

Kondratowicz *et al.* (13) have reported transient transfection of a TIM-1 expressing plasmid into HEK 293T cells have been resulted in TIM-1 surface expression in about 20% of the population. Also Moller-Tank *et al.* (14) have reached to 70% TIM-1 surface expression; whereas our results showed 88.15% of cells have stably expressed TIM-1 protein. This very high expression was obtained by

pcDNA™3.1/Hygro (+) vector, which contain cytomegalovirus promoter. Hence, these cells, as an available "cell display" source, could be used for production of specific molecules against TIM-1.

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