



Stimulation of Camel Polyclonal Antibody against Human T cell Immunoglobulin and Mucin 3

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Background: T cell Immunoglobulin, Mucin (TIM)-3, is a type I transmembrane glycoprotein belonging to TIM family. This receptor expresses on T helper type 1 (Th1) cells that binds to galectin-9 (Gal9); inducing an inhibitory signal. As a result, apoptosis of Th1 cells occurs and cytotoxicity of CD8 T cells becomes evident in vitro. Therefore, this immunomodulatory molecule may be used as a novel target for clinical purposes. The production of camel polyclonal antibodies against TIM-3-expressing cell line was the purpose of this study.

Objectives: In this study, we aimed to use HEK 293 cells expressing human TIM-3 to obtain camel polyclonal antibody against TIM-3 by immunization.

Materials and Methods: A pre-synthesized human TIM-3cDNA was inserted into pcDNA3.1 plasmid and the new construct was transfected in HEK cell. TIM-3 expression was confirmed by qRT-PCR and flow cytometry. A camel (6 months old) was immunized with the lysate prepared from rTIM-3 expressing HEK cells 4 times. The anti-TIM-3 antibody level was evaluated using ELISA method.

Results: TIM-3 was successfully cloned in HEK cells with 88% success rate. High level of anti-TIM-3 antibody was detected in the serum of the camel immunized with the recombinant cell lysate, after final injection.

Conclusions: Our rhTIM-3 cell display system can be useful for future diagnostic or therapeutic approaches.

Keywords: Camel antibody, Gal9, HcAb, HEK 293, TIM-3

1. Background

T-cell Immunoglobulin and Mucin 3 (TIM-3) is a glycoprotein that includes domains of amino-terminal immunoglobulin variable (IgV), mucin, transmembrane and a cytoplasmic tail (1, 2).

The *TIM* gene family consists of eight members (TIM-1-8) on mouse chromosome 11B1.1, and three members (*TIM-1*, *TIM-3*, and *TIM-4*) on human chromosome 5q33.2. This location has been associated with asthma, allergy, and autoimmunity region (1, 3). In human, TIM-3 expresses on differentiated Th1 cells, activated CD8⁺ T cells and at lower levels on Th17 cells as well as on cells of the innate immune system. It can mediate phagocytosis and cross-presentation of antigens (4, 5).

TIM-3 expressing on Th1 cells binds to ligands including galectin-9 (Gal9) and phosphatidyl serine (PtS). Interaction of PtS -TIM-3 leads to clearance of apoptotic cells. Gal-9 binds to a carbohydrate structure on the IgV domain of TIM-3. When Gal-9 binds to TIM-3, induces an inhibitory signal. This may regulate interferon- γ (IFN γ) secretion and blocks the induction of T-cell tolerance (6-8). TIM-3 is also an important player in the CD8⁺ T cell exhaustion. Exhaustion takes place in chronic immune conditions such as chronic viral infection and cancer in both human and experimental models. Furthermore, it has a role in myeloid cells and cancer stem cells by promoting myeloid-derived suppressor cells (MDSC) and regulating the cytokine response. TIM-3 expression

on tumor-infiltrating lymphocytes (TILs) also defines highly suppressive T regulatory (Tregs) in both human and mouse tumors. In addition, TIM-3 blockade and Treg depletion have a synergistic effect on tumor growth inhibition (6, 7, 9-13).

Camels have a unique antibody in their circulation, which consists of about 50% of their total antibody. This antibody has only a 15 kDa heavy chain homodimer (HcAb), with an antigen-binding domain called nanobody or V_{HH} . The small size of the HcAb allows it to penetrate into tissues and pass blood brain barrier (BBB). One may conclude, it would be considered as an effective tool for future therapeutic approaches (14, 15).

Human Embryonic Kidney (HEK) 293 cell line has been extensively used as an expression tool for recombinant proteins in industrial biotechnology to produce therapeutic proteins and viruses for gene therapy (16, 17).

2. Objectives

HEK 293 cells expressing human TIM-3 were used to produce anti-TIM-3 camel antibody. A camel was immunized with these cells to obtain polyclonal antibody against TIM-3.

3. Materials and Methods

3.1. Cloning of pcDNA-TIM-3 Construct

The cDNA clone of *TIM3* (EX-W2682-M67) was synthetically produced by Genecopoeia (MD, USA). The construct was sub-cloned into the pcDNA3.1 hygro⁺ expression vector. The cassette of TIM-3 was amplified using *NheI* forward primer 5'-AAAGCTAGCTGCCACCTGACGTCTAAGA-3' and *MluI* reverse primer 5'-TACGCGTTAAGATACATTGATGAGTTTGGAC-3' (Gene Fanavaran Co. Iran). The PCR-amplified DNA fragment contains CMV promoter, coding sequence of *TIM-3* and SV40 polyA signal. The pcDNA 3.1 hygro⁽⁺⁾ and the PCR product were separately digested by *NheI* and *MluI*. Ligation reaction was performed between pcDNA3.1 hygro⁽⁺⁾ linear plasmid and digested with T4 DNA ligase. The ligation product transformed into competent *E. coli* Top10F['] using CaCl₂ method. The transformed bacterial cells were cultured on LB-agar plate with ampicillin (50 µg.mL⁻¹) and incubated at 37 °C for 16 h. The recombinant plasmid, pcDNA-TIM3, digested with *EcoRV* and the linear plasmid was used for cell transfection.

3.2. Cell Growth and Transfection

About 5 × 10⁶ HEK 293T cells (Pasture Institute of

Iran) were seeded in 24 well plates and incubated for 24 h. Cells were transfected with linearized plasmid (pcDNA-TIM3) according to (18). Briefly, 21 µg of linear plasmid was diluted in 304 µL ddH₂O, 11 µL 1 × TE and 35 µL CaCl₂ (2.5 M) were added to DNA solution. HBS 2× (350 µL) was added drop wise under agitation while it was being vortexed. DNA complex was poured drop wise on cell culture and the plate was swirled to expose all cells to DNA complex. Transfected cells were incubated 48 h and treated with 150 µg.mL⁻¹ hygromycin for 3 weeks. The remaining cells were expanded for one month under hygromycin treatment. The expressions of TIM-3 at both gene and protein levels were evaluated on the cells.

3.3. Measuring recombinant TIM-3 (*rTIM-3*) Transcript Level in Transfected HEK Cells

Total RNA was extracted from transfected HEK cells using the RNX plus solution (Cinagen Inc., Iran). RNA concentration was measured by nanodrop (Biochrom WPA Biowave II, Cambridge. UK). The cDNA was synthesized from total RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc. MA. USA) as instructed by the manufacturer.

The resulting transcripts were quantified on a StepOne plus real-time DNA amplification system (Applied Biosystem, USA) using SYBER Premix Ex Taq II kit (Takara Bio Inc., Japan) according to the manufacturer's protocol. Specific primers for TIM-3 and β-actin cDNA amplification were designed with Allele ID 7.0 software (Table 1). For each sample, transcript quantity was normalized to the amount of β-actin expression. Amplification carried out in a total volume of 20 µL for 40 cycles of 15 seconds at 95 °C, 60 seconds at 60 °C and 15 seconds at 75 °C preceded by a 10 min at 95 °C as a starting step.

3.4. Evaluation of *rTIM-3* Protein Expression on Transfected HEK Cells

The percentage of transfected cells expressing *rTIM-3* was evaluated by flow cytometry using anti-TIM-3-PE antibody (BD Biosciences, USA). Selected cells (10⁶) under hygromycin treatment were mixed with 5 µL of the antibody and incubated for 20 min in dark. After washing with PBS, samples were evaluated by flow cytometer and analyzed using Cell Quest Pro software (FACS Caliboure, USA).

3.5. Camel Immunization with HEK Cells Displaying *rTIM-3*

A 6-months old healthy male camel (*Camelus deromedaricus*) was immunized and the animal was

Table 1: Sequences of the specific primers applied in the current study.

Gene name	Direction of primer	Primer sequence
TIM-3	Forward	5'-CCA TCA GAA TAG GCA TCT ACA TC-3'
	Reverse	5'-CTT TGC TAT GAG AAT ACC ATT TGA-3'
B-Actin	Forward	5'-TTC GAG CAA GAG ATG GCC A 3'
	Reverse	5'-CAC AGG ACT CCA TGC CCA G-3'

kept under vet surveillance for the period. A serum sample was collected and stored at -80°C before the first injection of the antigen. The camel immunized with the lysates prepared from 5×10^7 rTIM-3 expressing cells for 4 times every 2 weeks. Injections were carried out intramuscularly in triceps and hip muscles. The sera samples were collected before each injection and 10 days after the final injection.

3.6. Evaluation of the Camel Anti-human TIM-3 Antibody

Enzyme-linked immunosorbent assay (ELISA) was applied for measurement of polyclonal Antibody against human TIM-3 (hTIM-3) in the camel serum samples. A 96-well ELISA plate was coated with $8 \mu\text{g.mL}^{-1}$ of a purified recombinant TIM-3 (Sino Biological, Inc., China) using carbonate/bicarbonate buffer (pH 9.6). Skimmed milk (2%) used as a blocker. After washing with PBS containing 0.05% (v/v) Tween-20, 1:100, 1:1000 and 1:10000 dilutions of camel serum was added to positive and negative (without antigen) wells and incubated for 1 h at 22°C . The next step, $100 \mu\text{L}$ of 1: 8000 diluted rabbit anti-camel monoclonal antibody (Sigma-Aldrich, Germany) was added to each well and incubated for 1 h at 22°C . After washing, 1:3000 diluted HRP (horseradish peroxidase) conjugated goat anti-rabbit polyclonal antibody (Sigma-Aldrich, Germany) was applied for 1 h at 22°C as a secondary antibody. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich, Germany) added and colorimetric reaction was stopped. The optical density (OD) was read at 450 nm wavelengths with a microplate reader (ELx800).

3.7. Statistical Analyses

Mann-Whitney U test used for comparison between the two groups of the cells and One Way ANOVA applied for comparison among sera samples of different injections. All experiments carried out in triplicate and data are presented as mean \pm standard error of mean (SEM) in graphs. All the statistical tests were performed using SPSS 16.0 software (Chicago, USA).

4. Results

4.1. Recombinant Construct of pcDNA-TIM-3

TIM-3 cDNA inserted into the pcDNA3.1 hygro+ and resulted construct was successfully transformed in *E. coli* Top10F'. Restriction Enzyme (*Nhe*I and *Mlu*I) sites and sequencing map of TIM-3 is shown in (Fig. 1). The pcDNA-TIM-3 was extracted from the bacteria and linearized with *Eco*RV.

The linear recombinant vector pcDNA-TIM-3 was transfected in HEK cells. After 14 days of transfection and treatment with hygromicine, the cells were alive and morphologically normal.

4.2. Expression of rTIM-3 in Transfected HEK Cells

As indicated in Figure 2, the expression level of rTIM-3 in transfected HEK cells was highly increased ($p < 0.05$).

4.3. HEK Cells Expressing rTIM-3 Protein was Elevated

The result of flow cytometry analysis on stable transfected HEK cells revealed that more than 88% of cells express TIM-3 protein on their surfaces (Fig. 3) ($p < 0.0001$). The

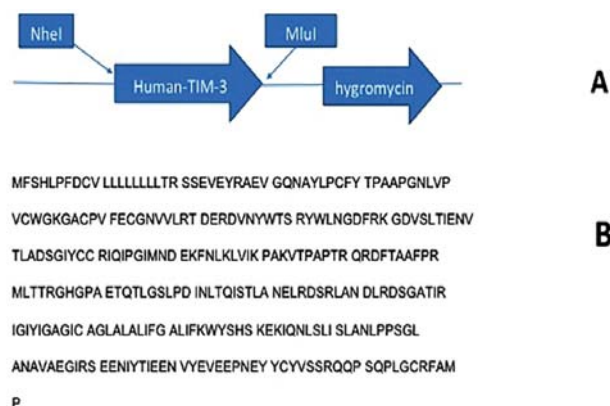


Figure 1. A) Schematic representation of the expression cassette of pcDNA-TIM-3; **B)** Amino acid sequence of human TIM-3.

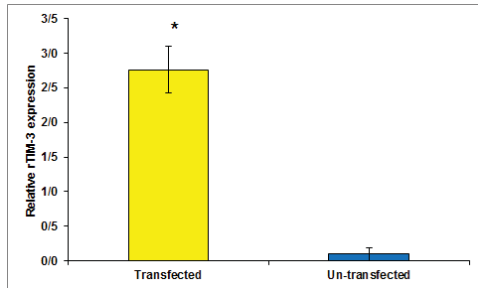


Figure 2. Measurement of TIM-3 mRNA level in HEK cells using qRT-PCR method. TIM-3 is highly expressed in transfected HEK cells while it is almost undetectable in un-transfected cells. Asterisk (*) represents statistical significance. Results are shown as mean \pm SEM obtained from three identical repeats of each experiment.

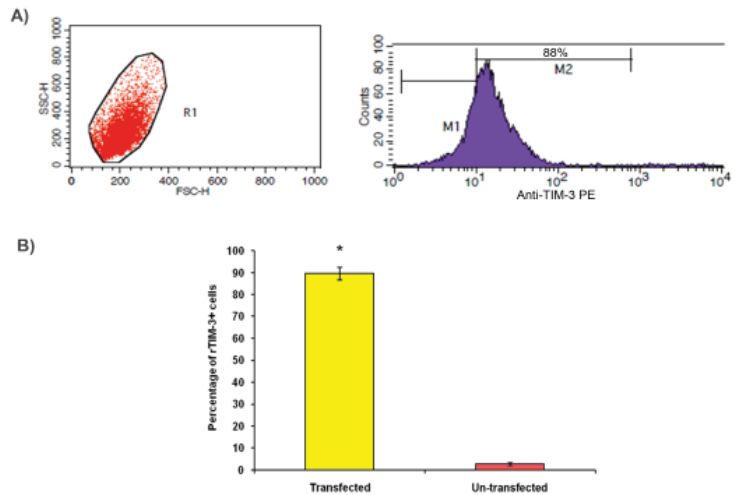


Figure 3. Evaluation of TIM-3 expression on HEK cells surface using flow cytometry method. **A)** Left panel shows gating area of the work. Right panel is the histogram plot of a positive transfected HEK cell sample. **B)** Bar chart compares the percentage of TIM-3+ cells between transfected and un-transfected samples. It shows high percentage of TIM-3+ cells in transfected group. The difference between the two groups is highly significant (*: $p < 0.0001$). Results are shown as mean \pm SEM obtained from three identical repeats of each experiment.

majority of the TIM-3 positive cells showed high median fluorescent intensity (MFI) (30.4) representing for over-expression of TIM-3 on transfected HEK cells.

4.4. Reactivity of Camel Antibody with Human rTIM-3 Protein

The dilution of 1:100 of the camel anti-serum was highly reactive with rTIM-3 ($p < 0.001$). The reactivity of other dilutions (1:1000 and 1:10000) was not significant; however, it was higher than the negative control reactivity level (Fig. 4). Anti-human TIM-3 antibody level was

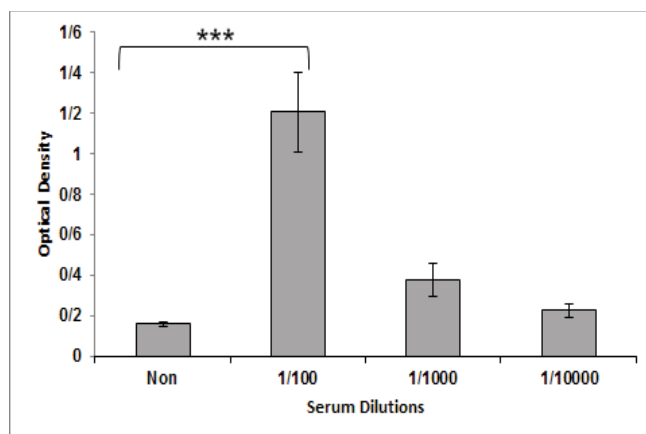


Figure 4. The dilution of 1:100 of the camel anti-serum compared with control serum was highly reactive with rTIM-3. Other dilutions (1:1000, 1:10000) were also reactive but not significant. (***) $p < 0.001$.

increased significantly after the first injection and its level was incremental in the next injections of immunization, while it was at the baseline in the control camel (Fig. 5).

5. Discussion

TIM-3 as a negative regulator of IFN- γ producing CD4 and CD8 cells, has a key role in regulation of T cells and is an interesting target for medical interventions. In chronic viral infection and cancer, blocking TIM-3 *ex vivo* or *in vivo* increases the functionality of exhausted T cells to restore viral control or to inhibit tumor growth (10, 13).

Monoclonal nanobody against TIM-3 can stimulate or inhibit its function and could be used for therapeutic approaches. In addition, this target might be applied for diagnostic purposes or in research.

It was shown that TIM-3 blockade can restore proliferation of both CD4⁺ and CD8⁺ T cells (18) Treatment with anti-TIM3 and anti-PDL1 (programmed death ligand 1) as an immune checkpoint like TIM-3 together resulted in reduction of tumor growth (13). In order to produce and evaluate such therapeutic and/or diagnostic tools, a suitable amount of the native antigen with proper folding and post-translational modifications are necessary. The antigen can be used in immunization, and/or panning and phage display (16). In the current study, human rTIM-3 was over-

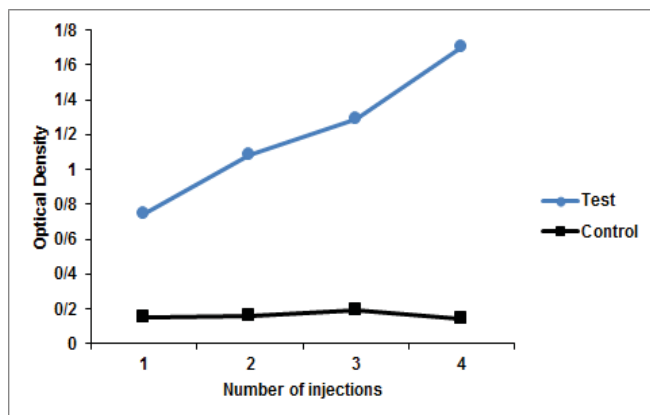


Figure 5. The immune response (Ab) level of camel before and after injections of the antigen (TIM-3). The immunization was successful and there was significant increase in antibody response after each injection compared with control serum.

expressed in HEK cells to produce a suitable, low cost and available source of the antigen. The rTIM-3 expressing HEK cells were used for immunizing a camel to produce specific anti-serum, confirming proper folding towards exciting immunoreactivity. When a TIM-3-EGFP fusion protein construct in pcDNA3.1, no quantification result was reported (19). So, it was not possible to compare their results with ours. Factors that affect the level of expression of protein in transfected cells include: plasmid size, amount of plasmid used per transfection, promoter strength, cell type, efficiency of the transfection, and potential toxicity of transfection reagent (20). In the present study, camel immunization was performed successfully to provoke Humeral immune responses and demonstrated that the expressed rhTIM-3 has good immunogenicity to produce HcAbs (Fig. 4). We did not find any report of camel immunization with cells expressing human TIM-3 antigen, for comparison. Conclusively, our rhTIM-3 cell display system might be useful for future studies of TIM-3 such as producing monoclonal antibody, nanobody and aptamers.

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