

Short Communication

Human Recombinant B7-H3 Expressed in *E. coli* Enhances T Lymphocyte Proliferation and IL-10 Secretion *in Vitro*

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Abstract To explore the biofunctions of human B7-H3 on activated T lymphocyte, the gene of human B7-H3 encoding the extracellular region (IgV-like and IgC-like domains) was obtained by RT-PCR from human lung cells and subcloned into the prokaryotic expression vector pGEX-5X-3 to express glutathione S-transferase (GST) fusion protein. A 49 kD fusion protein (named as GST/hB7-H3 hereafter) was induced by IPTG and purified by standard methods reported in prokaryotic system. In the presence of the first signal imitated by anti-CD3 monoclonal antibody, T lymphocyte proliferation was observed by incubating purified T cells with soluble GST/hB7-H3 fusion protein by MTT assay. The concentrations of IFN- γ and IL-10 in the supernatants of T cells were determined by ELISA. The results showed that the GST/hB7-H3 protein produced in bacteria had modest biological activities to proliferate the T lymphocyte and enhance IFN- γ as well as IL-10 secretion.

Key words B7-H3; GST-fusion protein; T lymphocyte proliferation

Since it was proposed in 1970, the two-signal hypothesis for T lymphocyte activation became widely accepted, which elucidated T cells required two distinct signals for optimal T helper precursor cell expansion. This model hypothesized that peptides presented to antigen-specific T cells in the context of MHC molecule delivers signal 1, whereas costimulatory molecules (CM) on T cell surface triggered signal 2 [1]. The most intensively characterized CMs were those of the B7 family. The classical members B7-1 (CD80) and B7-2 (CD86) of this family interacted with and activated CD28 on naïve T cells, and stimulated IL-2 production [2]. Recently, several new members (B7-H1, B7-H2, B7-H3, B7-DC) of the B7 family were identified, and their structures and functions were elucidated [3–5]. It was proposed that they regulated the function and differentiation of effector lymphocytes in the periphery. B7-H2 (B7h, B7-related protein 1, GL50,

LICOS) bound to inducible costimulator (ICOS) on T cells, and appeared to play major role in regulating Th2 responses [5–7]. B7-H1 (PD-L1) and B7-DC (PD-L2) bound to the receptor PD-1 on T cells, and inhibited T cell proliferation and cytokine production [8–10]. A newest member of the B7 family, designated as B7-H3, was cloned from a human dendritic cell-derived cDNA library [11].

Similar to other B7 homologs, mRNA of hB7-H3 was detected on a broad spectrum of tissues even in some malignant tumor cell lines, such as chronic myelogenous leukemia K562 cells, lung carcinoma A546 cells. The receptor for hB7-H3 was unknown until now, but the counter-receptor that is distinct from CD28, CTLA-4, ICOS, and PD-1 was observed to be rapidly induced following T lymphocytes activation. The soluble hB7-H3 protein could also costimulate the proliferation of T cells and selectively enhanced IFN- γ secretion with modest effects on TNF- α and IL-8 production [11].

Mounting data indicated that hB7-H3 maybe present a novel regulatory way in immune responses. To clarify these current controversial biological activities of hB7-H3, we cloned the full-length cDNA of hB7-H3 and constructed the prokaryotic expression system of hB7-H3. The target

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protein GST/hB7-H3 expressed in *E. coli* showed its biological function to proliferate the T lymphocytes, and for the first time to our knowledge to induce T cell produce IL-10, which might be an important evidence for the hB7-H3 down-regulating characteristic.

Materials and Methods

Materials

The *E. coli* strain TOP10 and expression host BL21 (DE3) was purchased from Invitrogen (USA), Expression vector pGEX-5X-3 (GST fusion protein vector) was product of Amersham Pharmacia Biotech Inc. (USA). RT-PCR Kit, all restriction endonucleases, T4 DNA ligase, *Taq* DNA polymerase, pMD18-T Vector, DNA and protein molecular weight marker were obtained from TaKaRa Biotech (Dalian, China). Gel extraction mini kit and Plasmid mini kit were purchased from Shanghai Watson Biotech (Shanghai, China). Anti-CD3 monoclonal antibody was from Immunotech Company (French). BM Chemiluminescence Western blotting kit (Mouse/Rabbit) was obtained from Boehringer Mannheim (Germany). Protein concentrations were estimated by the Bradford method with Bio-Rad protein assay. All other reagents and apparatuses were of high quality available from commercial sources.

Cloning of hB7-H3 and construction of expression vector

Using the total RNA of human lung tissue cells as template, the first strand cDNA of hB7-H3 was obtained with Oligo dT as a reverse primer following the manufacturer's protocol by RT-PCR. Then the PCR amplification was carried out with the following primers: B7-H3-A: 5'-ATG CTG CGT CGG CGG GGC AGC CCT GGC A-3'; B7-H3-B: 5'-TCA GGC TAT TTC TTG TCC ATC ATC-3'. The PCR product was subcloned into pMD18-T vector. Confirmed by sequencing, the recombinant vector pMD18-T/hB7-H3 containing the sequence encoding entire human B7-H3 protein was conserved as a template for future work. Other two primers were constructed to facilitate cloning of the extracellular domain of hB7-H3 from pMD18-T/hB7-H3 as follows: BH73-A: 5'-CGTGGATCCTGGAGGTCCAGGTCCCTGAAG-3' (*Bam*HI site underlined); BH73-B: 5'-CCTCTCGAGT-CAGGGGAATGTCATAGGCTG-3' (*Xho*I site underlined). Digested with *Bam*HI and *Xho*I, the PCR product was inserted into corresponding region of pGEX-5X-3 vector. Identified by the restriction enzyme digestion and

sequencing, the correct recombinant prokaryotic expression vector was named as pGEX-5X-3/hB7-H3 [12].

Expression of GST/hB7-H3 protein

The *E. coli* strain BL21(DE3) was transformed with the recombinant vector pGEX-5X-3/hB7-H3 and then grown overnight in 10 ml LB medium containing 100 µg/ml ampicillin. Next day, 2 ml overnight bacteria culture was added to 200 ml fresh medium, and incubated at 37 °C, 225 rpm. When the A_{600} value reached to 0.6–0.8, the IPTG was added to the different final concentrations. The bacteria cells were collected at different induced time by centrifugation at 8000 g for 10 min. Finally, all bacteria cells were collected and resuspended in 30 ml dual-boiling water, centrifuged again, and frozen at –70 °C for use.

Purification of the recombinant protein

Preparation of extracts The bacteria expressed fusion protein was analyzed by SDS-PAGE and then was resuspended in 6 ml distilled water. After sonication at a high setting (duty time = 30 s, rest time = 30 s) at 480 W on ice bath until the viscosity disappeared, the samples were centrifuged at 5000 g for 10 min. The supernatant was saved and the pellet was resuspended in 12 ml washing buffer [20% glycerol (V/V), 2% Triton X-100 (V/V), 2 M urea, 50 mM NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH 8.0)]. After being kept for 10 min at room temperature, the samples were centrifuged at 5000 g for 10 min at 4 °C. Following three cycles of washing, the deposit was denatured in 7 M urea with 5 mM DTT for 4 hours at room temperature. The supernatant and deposit collected respectively by centrifugation for 15 min at 12,000 g were analyzed by SDS-PAGE (15%).

Purification by GST-affinity chromatography The supernatant was dropped into 100 ml renaturation solution (2 M urea, 100 µM PMSF, 50 mM EDTA, 100 mM Tris-HCl, pH 8.2) with constant agitation at 4 °C for 24 h. Then the renaturation procedure was performed at room temperature. Another 24 h later, the renaturation solution containing the fusion protein was dialyzed for 24 h against 1 L PBS (pH 7.3) at room temperature, replacing the PBS every 4 h. Then the renatured protein was loaded onto the pre-equilibrated GST affinity chromatography column (Amersham Pharmacia Inc.) according to the manufacturer's protocol. Following another equilibration, the bound GST-fusion protein was then eluted with 5 ml washing solution (10 mM reduced form glutathione, 50 mM Tris-HCl, pH 8.0). The peak fraction of elution was pooled and subjected to SDS-PAGE to determine the purity of the GST/hB7-H3 protein [13–15].

Identification of the GST/hB7-H3 fusion protein by Western blot

The rabbit-anti human B7-H3 polyclonal antibody was prepared in our laboratory (submitted to publish). Purified proteins were analyzed by 12% SDS-PAGE according to the standard procedure. After electrophoresis, the gel was immersed in the transfer buffer (0.24% Tris-HCl, 1.153% glycine, 5% methanol, pH 8.8), and the proteins were transferred to nitrocellulose membrane by electrophoresis at 100 mA for 1 h. The membrane was incubated 1 h in the blocking buffer (5% blocking reagent, BM) at room temperature. After being washed three times (10 min each) with TBS-Tween buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20), the membrane was incubated with the anti-hB7-H3 polyclonal antibody for 1 h at room temperature. After being washed, the membrane was incubated for another 1 h with Ap-conjugated goat anti-rabbit IgG at room temperature. After briefly being rinsed with Dig-buffer III (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂), the specific protein bands were visualized by BCIP/NBT (Watson Biotech, Shanghai).

T cell proliferation and cytokine assay

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human peripheral blood by Ficoll gradient centrifugation. Monocytes were removed by plastic adherence for 2 h in RPMI 1640/10 % FCS at 37 °C, CD3⁺ T cells were collected by passing over a nylon wool column (>90%) [16]. For T cell proliferation assay, a 96-well flat-bottom plate was pre-coated with anti-CD3 monoclonal antibody (mAb, 0.4 mg/L) in PBS at 4 °C overnight. Wells were washed extensively and the purified T cells were then diluted into 1×10⁵ cells/ml to seed into every well at 200 μl/well together with different concentration of two protein: GST/hB7-H3 protein or soluble GST protein. After incubated for 96 h in a 37 °C, 5% CO₂ incubator, the supernatant of T cells was harvested immediately before adding MTT. The concentrations of IFN-γ and IL-10 in the culture supernatant were determined with ELISA kits according to the manufacturer's instructions. MTT solution was added to each well to the final concentration of 10 mg/L and the cells were incubated for 5 h. After the supernatant was removed by centrifugation for 5 min at 3000 g and the wells were washed with PBS 3 times. 100 μl iso-propylalcohol was added to dissolve the crystal. The absorbance of solution in each well was measured at 595 nm by using a microplate reader (Bio-Rad). All the measurements were performed

in triplicate [17].

The inhibition assay for T cell proliferation

The purified T cells were diluted into 5×10⁵ cells/ml and seeded into 96-well flat-plate that were pre-coated with anti-CD3 mAb (1 mg/L). Then, the fusion protein GST/hB7-H3 (1.25 mg/L) with or without the rabbit anti-human B7-H3 polyclonal antibody was also added into the wells with the GST protein as control. After a 96 h culture at 37 °C, 5% CO₂, proliferation of T cells was analyzed by MTT assay. The other inhibition assay was performed under the same condition except that T cells were pre-stimulated by anti-CD3 mAb (1 mg/L) for 48 h.

Results

Cloning and construction of expression vector of hB7-H3

With RT-PCR, a 951 bp fragment was amplified from human lung tissue cells [Fig. 1(A)]. Confirmed by DNA-sequencing (Bioasia Inc. Shanghai, China), the cloned sequence was entirely identical to the published sequence of human B7-H3 in the GenBank database (gi: 13376851). The extracellular region of hB7-H3 was cloned from the recombinant vector pMD18-T/hB7-H3 [Fig. 1(B)].

The recombinant prokaryotic expression vector pGEX-

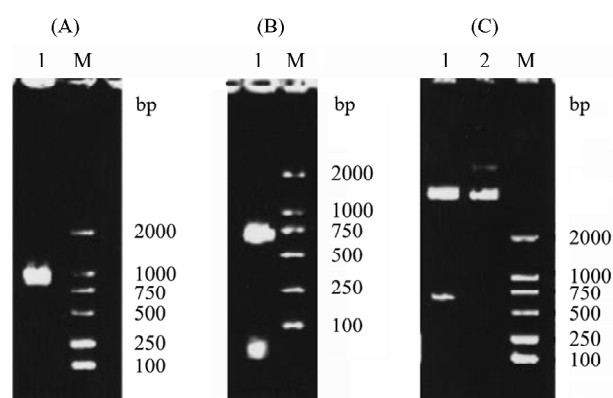


Fig. 1 Amplification of hB7-H3 gene by RT-PCR and construction of pGEX-5X-3/hB7-H3 recombinant vector

(A) Full-length hB7-H3 cDNA amplified from human lung cells by RT-PCR. 1, RT-PCR product of hB7-H3; M, DL2000 DNA marker. (B) PCR amplification of extracellular region of human B7-H3. 1, PCR product of hB7-H3 extracellular region; M, DL2000 DNA marker. (C) Identification of pGEX-5X-3/hB7-H3 recombinant vector by the restriction endonuclease digestion. 1, pGEX-5X-3/hB7-H3 digested by *Bam*HI and *Xho*I; 2, pGEX-5X-3/hB7-H3; M, DL2000 DNA marker.

5X-3/hB7-H3 digested by *Bam*HI and *Xho*I could release the target fragment [Fig. 1(C)], showing that the recombinant vector was constructed successfully.

Expression and purification of recombinant protein GST/hB7-H3

Transformed with pGEX-5X-3/hB7-H3, *E. coli* BL21 (DE3) cells were induced to express fusion protein GST/hB7-H3. The electrophoretic mobility of the expressed protein approximated to molecular weight of 23 kD plus the 26 kD GST protein, which was in agreement with the molecular weight 49 kD calculated based on its deduced amino acid sequence. And the expressed protein constituted about 49 % of the total bacterial protein after being induced with 1 mM IPTG for 7 h at 37 °C. The concentration of IPTG had no obvious effect on the expression

efficiency of GST/hB7-H3 fusion protein. However, the efficiency of the recombinant protein expression was time-dependent, reaching the highest level at the 7th hour after adding IPTG [Fig. 2(A,B)]. The insoluble protein following denaturation and renaturation was purified through GST affinity chromatography column. The elution profile was monitored and the analysis of SDS-PAGE showed that the purity reached to 90 % [Fig. 3(A,B)].

The identification of fusion protein

Western blot showed the GST/hB7-H3 could be recognized by the polyclonal antibody against hB7-H3. But in the groups of lysates from bacteria without induction or only expressed GST protein, no obvious band was detected. This result confirmed that the fusion protein had same immuno-domain as hB7-H3 (Fig. 4).

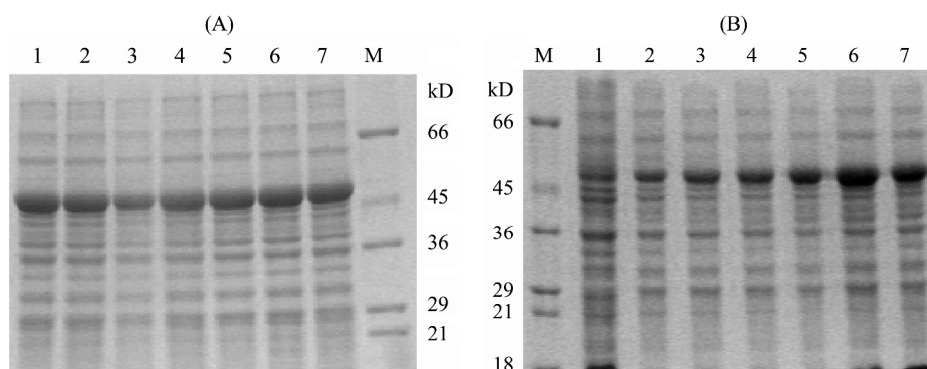


Fig. 2 Expression of the recombinant protein GST/hB7-H3

(A) GST/hB7-H3 expression at different IPTG concentration. M, protein molecular weight marker; 1, 0.02 mM IPTG; 2, 0.05 mM IPTG; 3, 0.1 mM IPTG; 4, 0.2 mM IPTG; 5, 0.5 mM IPTG; 6, 1 mM IPTG; 7, 2 mM IPTG. (B) GST/hB7-H3 expression at different inducing time (1 mM IPTG). M, protein molecular weight marker; 1, BL21(DE3)/pGEX-5X-3/hB7-H3 without induction; 2-7, BL21(DE3)/pGEX-5X-3/hB7-H3 induced by IPTG for 1, 2, 3, 5, 7, and 9 h, respectively.

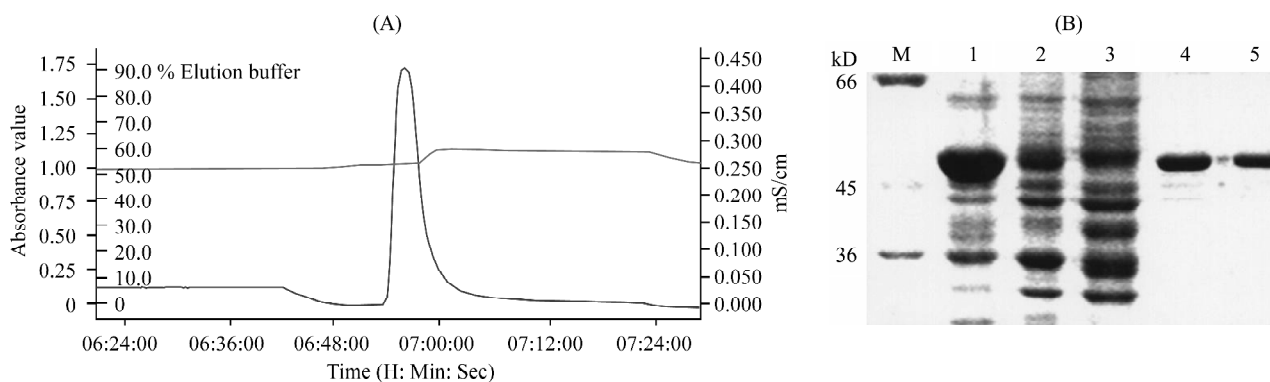


Fig. 3 Purification of the recombinant protein GST/hB7-H3

(A) Elution profile of the recombinant protein GST/hB7-H3. mS/cm: conductance. (B) SDS-PAGE analysis. M, protein molecular weight marker; 1, BL21(DE3)/pGEX-5X-3/hB7-H3 induced by IPTG; 2 and 3, uninduced BL21(DE3)/pGEX-5X-3/hB7-H3; 4 and 5, purified fusion protein GST/hB7-H3.

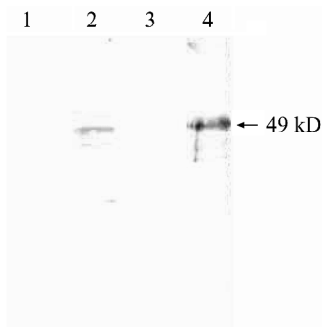


Fig. 4 GST/hB7-H3 fusion protein was recognized by the polyclonal antibody against human B7-H3

1, GST protein; 2, purified GST/hB7-H3 protein; 3, uninduced BL21(DE3)/pGEX-5X-3/hB7-H3; 4, BL21(DE3)/pGEX-5X-3/hB7-H3 induced by IPTG.

Positive responses of T cells costimulated by GST/hB7-H3 *in vitro*

The purified T cells were stimulated by the anti-CD3 monoclonal antibody in the presence of different concentration of GST/hB7-H3 protein with GST protein as control. The proliferation of T cells was determined by MTT assay after 96 h incubation. The results showed that GST/hB7-H3 protein could obviously increase T cell proliferation with anti-CD3 mAb (0.4 mg/L) [Fig. 5(A)]. The concentrations of IFN- γ and IL-10 from the cultured T cells were measured by ELISA, indicating that the concentrations of IFN- γ and IL-10 from the samples stimulated by different concentration of GST/hB7-H3 protein were obviously higher than those from the corresponding control groups [Fig. 5(B,C)]. In addition, the curve of the concentration of IFN- γ and IL-10 also showed a dose-

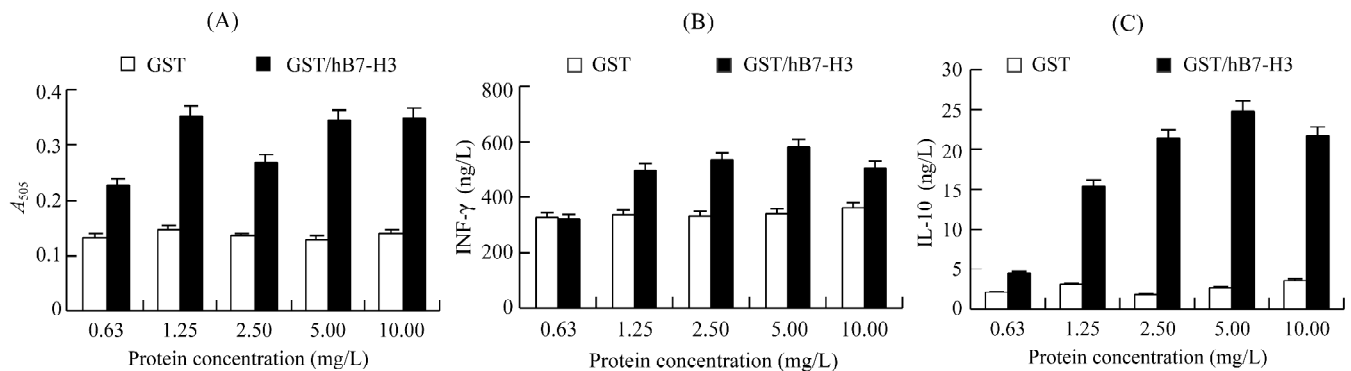


Fig. 5 The recombinant protein GST/hB7-H3 costimulated T cell proliferation and enhanced IFN- γ and IL-10 secretion

(A) Proliferation of T cells stimulated with GST/hB7-H3 protein at different concentrations by MTT assays, repeated three times. (B, C) Detection of IFN- γ and IL-10 from the supernatant of T cells costimulated by GST/hB7-H3 fusion protein at different concentrations by ELISA, repeated three times.

dependent model.

Inhibitory effect of the polyclonal antibody on T cell proliferation mediated by GST/hB7-H3 fusion protein

As Fig. 5 showed, the fusion protein could enhance T cell proliferation modestly comparing with GST control groups. Moreover, the polyclonal antibody against human B7-H3 could inhibit the proliferative effect mediated by the fusion protein (Fig. 6). However, when the fusion protein was added after T cells were firstly stimulated by anti-CD3 mAb for 48 hours, the proliferation effect had no remarkably difference from GST control group (Fig. 6). All these confirmed the biological specificity of fusion protein for T cell proliferation *in vitro*.

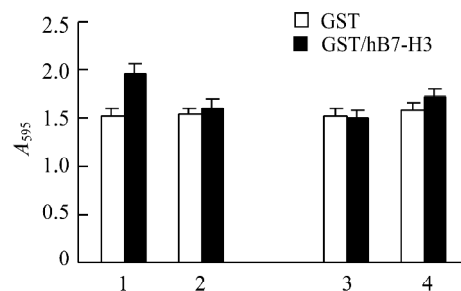


Fig. 6 Inhibitory effect of the polyclonal antibody on T cell proliferation mediated by GST/hB7-H3 fusion protein was analyzed by MTT assay

1, GST or GST/hB7-H3 fusion protein; 2, GST or GST/hB7-H3 fusion protein plus the polyclonal antibody against human B7-H3; 3, GST or GST/hB7-H3 fusion protein added after 48 hours stimulation of anti-CD3 mAb; 4, GST or GST/hB7-H3 fusion protein with the polyclonal antibody against human B7-H3 after 48 hours stimulation of anti-CD3 mAb.

Discussion

B7-H3, the sixth member of the B7 super-family, was identified recently. The hB7-H3 was first observed to promote T cell proliferation and selectively enhance IFN- γ expression, with modest effect on TNF- α and IL-8 production [11]. Ling *et al.* [18] found B7-H3 played an important role in regulating cell-mediated immune responses against cancer by CD8⁺ T and NK cells. However, Suh *et al.* reported [19] hB7-H3 protein down-regulated immune responses mediated by Th1 and inhibited the secretion of cytokine IFN- γ . Analyzing the B7-H3-deficient mice, Ling *et al.* [18] and Sun *et al.* [20] reported B7-H3 inhibited Th1-type responses *in vivo*; whereas, they found B7-H3 could enhance antitumor immunity in mice challenged with EL-4 cells transfected to express B7-H3. More recently, Steinberger *et al.* [21] found no data supported a costimulatory role of hB7-H3 in anti-CD3-mediated activation of the TCR-complex resulting in T cell proliferation and IFN- γ production. These inconsistent results suggested hB7-H3 probably had more than one receptor on activated T cells. The presence of hB7-H3b [22] may be another explanation to demonstrate the different functions of B7-H3 between human and mouse [16]. And we speculated that the active degree of T cells might be another factor related to the function of hB7-H3. We found IL-10 was induced to a high level by hB7-H3 protein, which might be an important evidence for the hB7-H3 down-regulating characteristic. Contradictory results regarding the costimulatory role of B7 homologues were reported previously in the case of PD-1 ligands PDL1 and PDL2 [23–27]. These reports suggested there's still a long way to go to illuminate the mechanism of hB7-H3 in immune responses.

The work presented in this paper demonstrated that hB7-H3 could be expressed in bacteria and readily purified in reasonable quantities for the research on its bio-functions. In the theory, the GST/hB7-H3 protein could be specially digested with thrombin to delete the GST part. And we had a try, but the result was not as expected. So we explored the bio-functions of hB7-H3 with the GST/hB7-H3 fusion protein, and it showed the recombinant protein GST/hB7-H3 maintained the bioactivity to stimulate T cell proliferation and enhance IFN- γ and IL-10 secretion. This indicated we got a convenient tool to study hB7-H3 in the future work about the functions of hB7-H3 in regulating immune responses. Besides, the full-length cDNA of human B7-H3 cloned by RT-PCR from lung cells would facilitate the preparation of the anti-hB7-H3 monoclonal

antibody for further study. In our work, the efficiency of the recombinant protein expression was time dependent, reaching the highest level at the 7th hour after adding IPTG. In the refolding study, we found GSH/GSSH had not obvious effect on the purification. Meanwhile, the entire lysis by sonication was the key step to obtain high purity recombinant protein. We first found hB7-H3 protein could obviously enhance IL-10 secretion, which suggested hB7-H3 might have effect on B cell-mediated immune response [28]. Our result showed the special antibody against hB7-H3 could inhibit the proliferation of T cells mediated by GST/hB7-H3 fusion protein. Interestingly, we also found GST/hB7-H3 fusion protein had no effect on pre-activation T cells, which might be associated with the expression time of its counter-receptor on T cells. The putative counter-receptor of hB7-H3 could be rapidly expressed on T cells after stimulated by PHA for 24 hours [11]. We also found the receptor of hB7-H3 could be detected on activated T cells stimulated by anti-CD3 mAb for 24–48 hours (data not shown).

In conclusion, our study demonstrated that the prokaryotic expression system could be used to generate hB7-H3 protein with natural spatial conformations and biological functions, which provided an efficient and economical way for the preparation of this target protein, and we first found the hB7-H3 protein could enhance IL-10 secretion, which provided a clue to clarify the mechanism of hB7-H3 in immune responses.

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