Expression and purification of the mGITR-Fc fusion protein and its effect on CD4+ T cells and dendritic cells *in vitro*

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Abstract. Glucocorticoid-induced tumor necrosis factor receptor related protein (GITR) is a member of the tumor necrosis factor receptor superfamily. The present study attempted to obtain the mouse GITR-Fc fusion protein and investigate its function on the proliferation of CD4+ T cells and on the expression of mGITR ligand (mGITRL) on dendritic cells. The sequences of the mouse (m)GITR gene and mouse immunoglobulin G Fc (mIgGFc) were amplified from mouse spleen cells and introduced into a pET-32a(+) vector. Following the induction, purification and validation of the mGITR-Fc fusion protein, the mGITR-Fc fusion protein was used to analyze its function on the proliferation of CD4+ T cells and on the expression of mGITR on dendritic cells. A recombinant plasmid containing the mGITR gene fragment and mIgGFc was constructed, and the recombinant mGITR-Fc fusion protein was successfully expressed. The exogenous mGITR-Fc fusion protein inhibited the proliferation of CD4+ T cells, dependent on the presence of mGITRL. The exogenous mGITR-Fc fusion protein also inhibited the expression of mGITRL on the dendritic cells. In conclusion, the mGITR-Fc fusion protein was confirmed to exhibit biological functions of a co-stimulatory signal and reverse signal. These experiments provide the basis for further investigation of the function of the mGITR-Fc fusion protein on certain autoimmune diseases.

Introduction

Glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) is a member of tumor necrosis factor receptor superfamily (TNFSF) (1,2). It is expressed on inactivated T cells at a basal level and is upregulated when T cells are activated (1). GITR is also expressed on B cells and natural killer cells at low levels (2,3). Mouse (m)GITR is a type I transmembrane protein and encodes a 228 amino acid cysteine-rich protein (4). The ligand of GITR (GITRL) is a member of the TNFSF (5,6). It is predominantly expressed on antigen-presenting cells, including dendritic cells (DCs), freshly isolated splenic B cells and macrophages (1,2,5,6). mGITRL is a type II transmembrane protein and encodes a 173 amino acid protein containing an extracellular C terminus and a short cytoplasmic segment (7).

The GITR/GITRL interaction is known to augment the immune response in the early stage and affect inherent and adaptive immunity (8). It is involved in the onset and progression of several types of tumor and certain autoimmune diseases, including autoimmune thyroid disease and Sjögren's syndrome (9-12). In these diseases, investigations have focused predominantly on the co-stimulatory signaling of the GITR/GITRL system. However, reverse signaling has also been reported in certain TNF family members, including GITRL and cluster of differentiation (CD)70 (13-16). The co-existence of the co-stimulatory signal and reverse signal in the TNF family members is of note and the present study aimed to focus on effects of the two signals in the interference or promotion of GITR or GITRL in certain autoimmune diseases. To achieve this aim, expression of a recombinant mGITR or mGITRL protein with the potential function is required.

In our previous study, the sequence of mGITR (Genebank no. ADB93077) was analyzed using bioinformatics methods (17), and the extracellular region of mGITR was identified as the important domain to express and functionally analyze for mGITR protein. These results were in accordance with those reported by Nocentini *et al* (4). The present study aimed to construct a prokaryotic plasmid to express mGITR-Fc fusion protein containing the extracellular region of mGITR, and the function of the recombinant mGITR-Fc fusion protein on the proliferation of CD4+ T cells (co-stimulatory signal) and on the expression of mGITRL on DCs (reverse signal) was investigated. The results may provide a basis for the further investigation of the function and potential mechanisms underlying the effect of the GITR-Fc fusion protein on certain autoimmune diseases.

Materials and methods

Experimental animals and reagents. A total of 8 healthy male mice (C57BL/6; 6-8 weeks-old, weighing 18-22 g) were

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purchased from the Experimental Animal Center, Veterinary College of Yangzhou University (Yangzhou, China). Mice were administered pathogen-free water and food and housed together under a 12 h light/dark cycle. The room temperature was controlled at 23±2˚C. All the animal procedures in the present study were performed in accordance with the institutional ethical guidelines for laboratory animal care and use of Jiangsu University (Zhenjiang, China). The study was approved by the ethics committee of Jiangsu University. *E. coli* DH5α, *E. coli* Rosetta, the pET-32a(+)prokaryotic expression vector and D2SC/1 cell line, which was kindly provided by Professor Liwei Lu from the University of Hong Kong, were all preserved in the Immunology laboratory at the Institute of Laboratory Medicine, Jiangsu University.

The *Eco*RI, *Sal*I and *Xho*I restriction enzymes were purchased from Takara Bio., Inc. (Otsu, Japan). The PrimeScript RT Reagent kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phycoerythrin (PE)-labeled rat anti-mouse GITRL antibody (monoclonal antibody, eBioYGL-386 clone, cat. no. 12-5854-80) and rat anti-mouse GITR antibody (monoclonal antibody, DTA-1 clone, cat. no. 12-5874-80) were purchased from eBioscience, Inc. (San Diego, CA, USA). Horseradish peroxidase (HRP)-labeled goat anti-rat immunoglobulin (Ig)G antibody was purchased from Beyotime Institute of Biotechnology (Haimen, China). The ToxinEraser Endotoxin removal kit and ToxinSensor Chromogenic LAL Endotoxin assay kit were purchased from GenScript (Nanjing, China). The Nickel nitrilotriacetic acid (Ni-NTA) column was provided by Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Construction of the expression plasmid. Mice were sacrificed via cervical dislocation and splenic cells were isolated from normal mice under aseptic conditions using Red blood cell cracking liquid (Solarbio, Beijing, China). Subsequently, the splenic cells ($1x10^8$) were harvested and total RNA was extracted from the splenic cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA samples were then reverse transcribed into complementary (c)DNA using a PrimeScript RT Reagent kit. The cDNA was used as the template for polymerase chain reaction (PCR) analysis. The extracellular coding sequence of mGITR (20-15 amino acids, not containing signal peptide) was amplified from the cDNA template using a pair of primers: P1, forward 5'-CCGGAATTCCAGCCGAGTGTAGTTGAG-3' and P2, reverse 5'-CGCGTCGACATGGCCGTATTGCTCAGT-3'. The restriction recognition sites of *Eco*RI (GAATTC) and *Sal*I (GTCGAC) were introduced into the 5' ends of P1 and P2, respectively. The coding sequence of mouse IgG Fc (mIgGFc) was amplified from the same cDNA template using another pair of primers: P3, forward 5'-CGCGTCGACGATATCGAAGGAAGAATGGATGGTTGTAAGCCTTGCATA-3' and P4, reverse 5'-GGCCTCGAGTTTACCAGGAGAGTGGGA-3'. The restriction recognition sites of *Sal*I and *Xho*I were introduced into the 5' ends of P3 and P4, respectively. A linker, containing 21 nucleic acid bases (GATATCGAAGGAAGAATGGAT), was also added into the 5' end of the P3 primer. All the primers mentioned above were synthesized by Sangon Biotech, Co., Ltd. (Shanghai, China). PCR was performed in Thermal Cyclers (Applied Biosystems, Foster City, CA, USA) as follows: 94˚C for 5 min, followed by 25 cycles at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 1 min, then held at 72˚C for 15 min, and finally a 4˚C standby. The final concentration of each primer was 0.4 μM and the quantity of cDNA was 200 ng of a 25 μl total volume. Subsequently, the PCR products of mGITR and mIgGFc were double-digested with restriction enzymes, cloned into the pET-32a(+) vector and transformed into *E. coli* DH5α competent cells. Following verification by PCR analysis, restriction enzyme digestion and DNA sequencing (Invitrogen Life Technologies), the positive plasmid was termed pET-32a-mGITR-mIgGFc.

Optimization of mGITR-Fc fusion protein induction. The pET-32a-mGITR-mIgGFc recombinant plasmid was transformed into *E. coli* Rosetta competent cells using a method of heat-shocking at 42˚C for 90 sec and then transferred into an ice-bath for 2-3 min and cultured in lysogeny broth (LB) medium containing ampicillin (100 μg/ml) at 37˚C for 14 h. The bacterial suspension was then inoculated into culture tubes containing 6 ml LB medium (100 μg/ml ampicillin) at a volume ratio of 1:100. Following culture at 37˚C, 250 rpm until the optical density (OD)600 (BioPhotometer; Eppendorf, Hamburg, Germany) of the bacterial liquid reached between 0.4 and 0.6, isopropyl-1-thio-β-D-galactopyranoside (IPTG; Sangon Biotech, Co., Ltd.) was added, at the indicated concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 mM). The bacterial suspension was then cultured at 37˚C for the indicated durations (1, 2, 3, 4, 6 and 8 h). Following culture, the bacterial cells were harvested by centrifugation at 18,500 x g for 5 min at 4˚C, resuspended in 100 μl phosphate-buffered saline (PBS) and separated on 10% SDS-PAGE gels (Sangon Biotech, Co., Ltd.). The pET-32a(+) vector was transformed into *E. coli* Rosetta competent cells using the method described above and induced as a negative control. In addition, to detect that whether protein was expressed in a soluble format, supersonic lysates were harvested by centrifugation at 18,500 x g for 30 min at 4˚C from bacteria cultured at the different temperatures (37, 30 and 25˚C) and separated on 10% SDS-PAGE gels.

Purification and identification of the mGITR-Fc fusion protein. The mGITR-Fc fusion protein was purified using Ni-NTA column, according to the manufacture's instructions. Briefly, the bacteria pellets were harvest, as mentioned above, and resuspended (10 ml/g bacteria) in binding buffer, containing 50 mM $NaH_2PO_4{\cdot}2H_2O$, 300 mM NaCl and 5 mM imidazole (pH 8.0). Following mixing on a rocker, the suspended bacteria were lysed by supersound and the supernatant, containing the soluble target protein, was harvested by centrifugation at 4˚C at 18,500 x g for 30 min. The supernatant was loaded onto a Ni-NTA column, which had been equilibrated with binding buffer, according to the Ni-NTA instructions. Following being washed with washing buffer, containing 50 mM $NaH_2PO_4{\cdot}2H_2O$, 300 mM NaCl and 10 mM imidazole (pH 8.0), the recombinant mGITR-Fc fusion protein was eluted from the column using elution buffer, containing 50 mM $NaH_2PO_4{\cdot}2H_2O$, 300 mM NaCl and 250 mM imidazole (pH 8.0) and collected into a clean tube.

The purified mGITR-Fc fusion protein was separated on 10% SDS-PAGE gel and electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA, USA). The PVDF membrane was then incubated with the primary antibody against mGITR protein (1:1,000, diluted in 5% nonfat dry milk) at 4˚C overnight), and HRP-conjugated goat anti-rat IgG (1:1,000, diluted in 5% nonfat dry milk), was used as the secondary antibody at room temperature for 2 h. The membrane was then visualized using enhanced chemiluminescence (ECL; Millipore). The endotoxin in the protein solution was then removed using endotoxin removal kit and detected using an endotoxin detection kit. Subsequently the mGITR-Fc fusion protein was de-germed using a filter (0.22 μm, Millipore). The concentration of the mGITR-Fc fusion protein was quantified using a bicinchoninic acid assay and stored at -70˚C for the subsequent experiments.

Functional characterization of recombinant mGITR-Fc fusion protein on the proliferation of CD4$^+$ T cells. The CD4$^+$ T cells were isolated from the mouse spleen tissues and sorted using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells ($10x10^4$/well) were then plated into 96-well plates, which were coated with anti-mouse CD3 antibody at 4˚C overnight. Subsequently mGITRL protein (0.25 μg/ml) and the prokaryotic mGITR-Fc fusion protein were added to the cells (0.1, 0.25, 0.5, 1.0 or 2.0 μg/ml). Following culture for 54 h at 37˚C, 0.5 μCi tritiated thymidine (^{3}H-TdR) was added to each well, and the cells were incubated for another 18 h at 37˚C. Following incubation, the cells were harvested into the specific filter of the β liquid scintillation counter (Beckman, Brea, CA, USA) and dried overnight. Then the filter was immersed into a clean eppendorf tube with 500 μl of scintillation solution and the incorporation of ^{3}H-TdR was determined using liquid scintillography. The data (counts per minute, cpm) were collected and the stimulating index was calculated as the cpm value of experimental group / cpm value of control group.

Functional characterization of recombinant mGITR-Fc fusion protein on the expression of mGITRL on DCs. D2SC/1 cells ($2.0x10^5$/well) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and treated with mGITR-Fc fusion protein at concentrations of 0, 0.25, 1.0, 5.0 and 10 μg/ml for 24 h at 37˚C, respectively. The cells were then harvested and labeled with PE-mGITRL antibody (0.5 μl) and incubated at 4˚C for 30 min. The cells were then washed and resuspended in 200 μl PBS. After 100 μl of 4% paraformaldehyde was added, the cells were prepared to perform flow cytometry analysis using a FACSCalibur flow cytometer (BD Biosciences, Sparks, MD, USA). The data were collected and analyzed using WinMDI software (The Scripps Research Institute, San Diego, CA, USA).

Statistical analysis. A total of three independent experiments were performed and the data are presented as the mean ± standard deviation. Significant statistical differences were determined using one-way analysis of variance (least significant difference) on SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA).P<0.05 was considered to indicate a statistically significant difference.

Results

Construction of the mGITR prokaryotic expression plasmid. A sketch map of the recombinant plasmid pET-32a-mGITR-mIgGFc is shown in Fig. 1A. The mGITR (414 bp) and mIgGFc (696 bp) fragments were amplified from the splenic cells of mice (Fig. 1B). These cells were then cloned together into the pET-32a(+) vector, and the recombinant plasmid was verified using PCR, enzyme digestion and DNA sequencing. As shown in Fig. 1C, a band at ~1107 bp was identified following digestion of the recombinant plasmid pET-32a-mGITR-mIgGFc with *EcoR*I and *Xho*I enzymes. The length of the restriction enzyme digestion product was identical to the length of the PCR product using the P1 and P4 primers. The recombinant plasmid was then sequenced to confirm that it had been successfully constructed.

Optimization of the induction conditions, and purification and verification of the mGITR-Fc fusion protein. To express the mGITR-Fc fusion protein, the pET-32a-mGITR-mIgGFc recombinant plasmid was transformed into *E. coli* Rosetta, and the proteins were extracted and separated using 10% SDS-PAGE. As shown in Fig. 2, the recombinant mGITR-Fc fusion protein was expressed at the site of ~66 kDa, as expected, while no band was observed at the corresponding site in the control group. The results, as shown in Fig. 2A-C, demonstrated that the soluble mGITR-Fc fusion protein was expressed successfully, and predominantly when the recombinant plasmid was induced by IPTG (0.5 mM) at 25˚C for 6 h. The soluble mGITR-Fc fusion protein was then purified using a Ni-NTA column and identified using SDS-PAGE and western blotting. The results, as shown in Fig. 2D and E, indicated that the soluble mGITR-Fc fusion protein had been successfully purified.

Function of the recombinant mGITR-Fc fusion protein on the proliferation of CD4$^+$ T cells. As is already known, GITR is expressed on resting CD4$^+$ T cells at a low level, however it can be upregulated when T cells are activated (1). As shown in Fig. 3A, the proliferation of the CD4$^+$ T cells increased when the cells were treated with mGITRL (0.25 μg/ml) only, compared with the control group, which containing cells treated without the mGITR-Fc fusion protein or mGITRL (P<0.05). However, the mGITRL-induced increased proliferation of CD4$^+$ T cells was markedly inhibited when the cells were co-treated with mGITRL (0.25 μg/ml) and mGITR-Fc fusion protein (1.0, and 2.0 μg/ml; (P<0.05). No significant difference (P>0.05) was observed between the mGITRL (0.25 μg/ml) and mGITR-Fc fusion protein (1.0 μg/ml) group and the mGITRL (0.25 μg/ml) and mGITR-Fc fusion protein (2.0 μg/ml) group. In addition, as shown in Fig. 3B, no significant difference (P>0.05) were observed between the values for the control group and the mGITR-Fc fusion protein (2 μg/ml) group. These results indicated that exogenous mGITR-Fc fusion protein inhibited the proliferation of T cells, dependent on the engagement of mGITRL. The mGITR-Fc fusion protein did not affect the proliferation of the CD4$^+$ T cells when the cells were treated with the mGITR-Fc fusion protein alone.

Functional characterization of recombinant mGITR-Fc fusion protein on the expression of mGITRL on DCs. A previous study

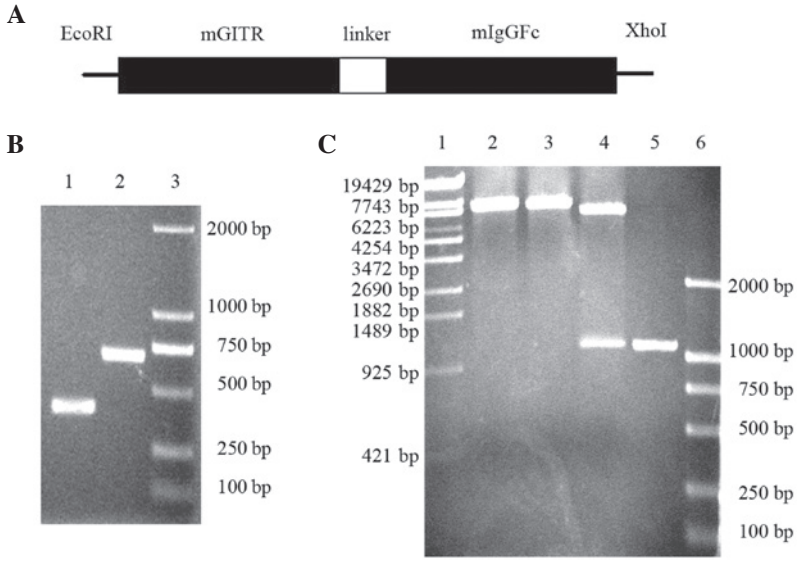

Figure 1. Construction of the pET-32a-mGITR-mIgGFc prokaryotic expression plasmid. (A) Sketch map of the pET-32a-mGITR-mIgGFc recombinant plasmid. mGITR and mIgGFc were cloned into the pET-32a(+) vector MCS in the sites of *EcoR*I and *Xho*I. (B) mGITR and mIgGFc fragments were amplified using RT-PCR respectively. Lane 1 represents mGITR (414 bp) and lane 2 represents mIgGFc (699 bp). Lane 3 represents the DL 2000 DNA marker. (C) A band at the 1107 bp site was identified on the agarose gels when the pET-32a-mGITR-mIgGFc recombinant plasmid was confirmed using PCR (lane 5) and double-digested with *EcoR*I and *Xho*I enzymes (lane 4). Lanes 2 and 3 contain the pET-32a-mGITR-mIgGFc recombinant plasmid, which was mono-digested with *EcoR*I and *Xho*I, respectively. Lane 1 represents the λ-EcoT14I digest DNA marker and lane 6 represents the DL 2000 DNA marker. GITR, glucocorticoid-induced tumor necrosis factor receptor related protein; mIgGFc, mouse immunoglobulin G Fc; RT-PCR, reverse transcription-quantitative polymerase chain reaction.

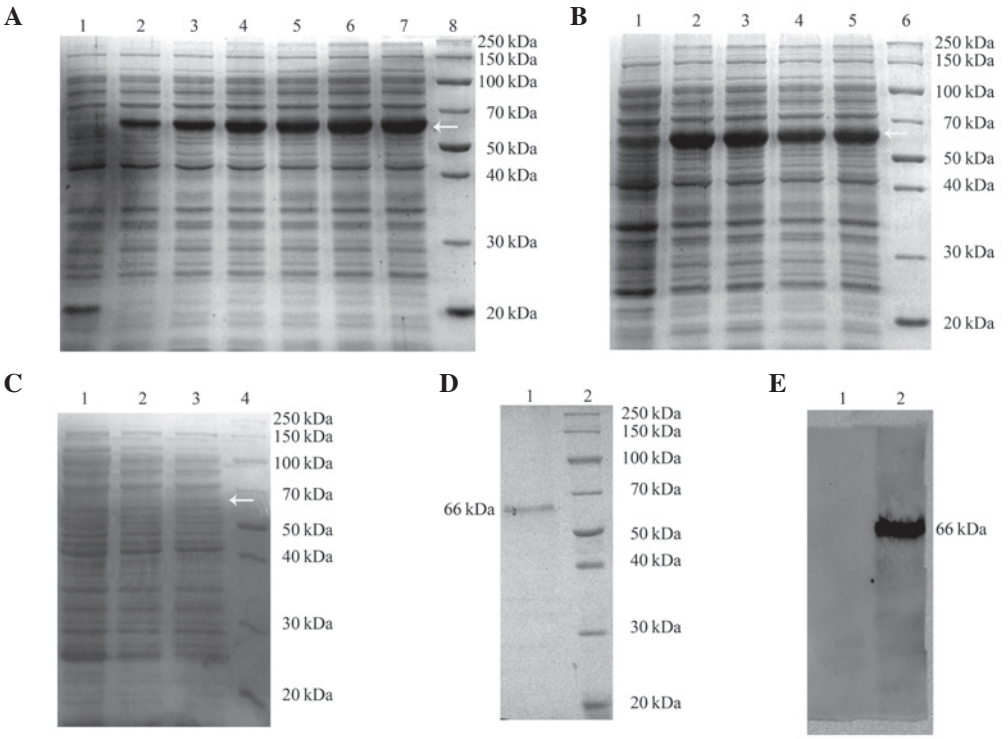

Figure 2. Optimization of mGITR-Fc fusion protein induction, and purification and verification of the protein. (A) mGITR-Fc fusion protein was induced at 37˚C for different durations. Lane 1, pET-32a(+) induced with 0.5 mM IPTG for 8 h. Lanes 2-7, pET-32a-mGITR-mIgGFc induced with 0.5 mM IPTG for 1, 2, 3, 4, 6 and 8 h, respectively. Lane 8, protein marker. The recombinant mGITR-Fc fusion protein was expressed at the site of 66 kDa (lanes 2-7). (B) mGITR-Fc fusion protein was induced at 37˚C for 6 h with IPTG at different concentrations. Lanes 1-5, pET-32a-mGITR-mIgGFc induced with IPTG at 0.1, 0.5, 1.0, 1.5 and 2.0 mM, respectively. Lane 6, protein marker. The recombinant mGITR-Fc fusion protein was expressed at the site of 66 kDa (lane 1-5). (C) mGITR-Fc fusion protein was induced with 0.5 mM IPTG for 6 h at different temperatures, and the supernatants of each were analyzed using electrophoresis. Lanes 1-3, supernatants obtained from bacteria cultured at 37, 30 and 25˚C and lysed ultrasonically, respectively. Lane 4, protein marker. (D) mGITR-Fc fusion protein was induced and purified successfully, as detected using SDS. A specific band at 66 kDa was observed when the protein was purified (lane 1). Lane 2, protein marker. (E) mGITR-Fc fusion protein was identified using western blotting. A band at ~66 kDa was observed on the polyvinylidene difluoride membrane, which was incubated with rat anti-mouse GITR antibody as the primary antibody (lane 2). Lane 1, negative control (protein extracted from bacterial cells containing the pET-32a(+) vector). GITR, glucocorticoid-induced tumor necrosis factor receptor related protein; mIgGFc, mouse immunoglobulin G Fc; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

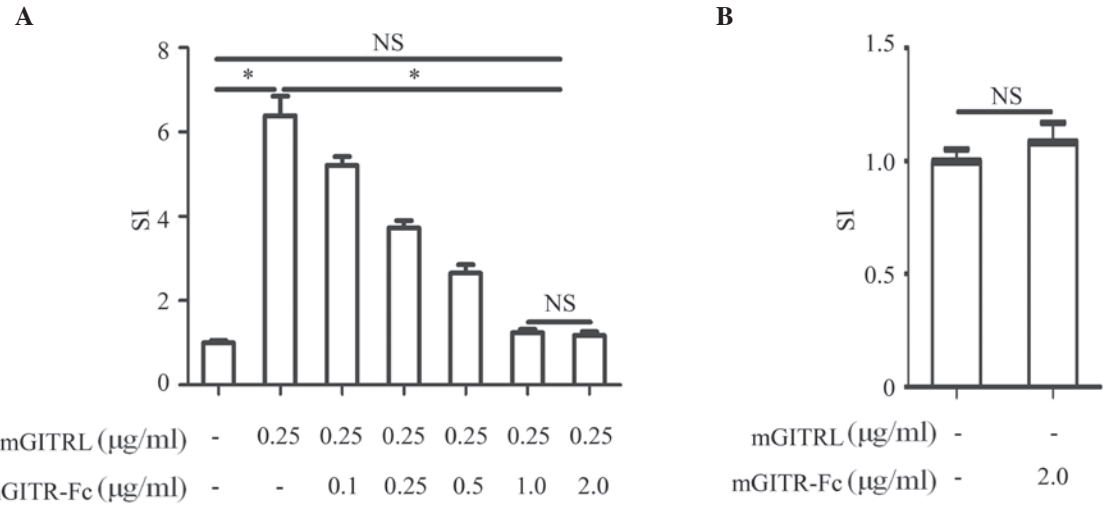

Figure 3. Function of recombinant mGITR-Fc fusion protein on the proliferation of $CD4^+$ T cells. (A) Proliferation of $CD4^+$ T cells was increased when the cells were treated with mGITRL (0.25 μg/ml) only, compared with the control cells, which were treated without mGITR-Fc fusion protein or mGITRL ($^*P<0.05$). The proliferation response of the $CD4^+$ T cells co-treated with mGITRL and mGITR-Fc fusion protein (final concentration of mGITR-Fc fusion protein of 1.0 or 2.0 μg/ml) were significantly lower, compared with that in the $CD4^+$ T cells treated with mGITRL only ($^*P<0.05$). (B) No significant difference ($P>0.05$) was observed in the values between the control group and the mGITR-Fc fusion protein (2.0 μg/ml) group. Data are presented as the mean ± standard deviation. SI = cpm value of experimental group / cpm value of control group. GITR, glucocorticoid-induced tumor necrosis factor receptor related protein; mIgGFc, mouse immunoglobulin G Fc; NS, not significant; SI, stimulating index.

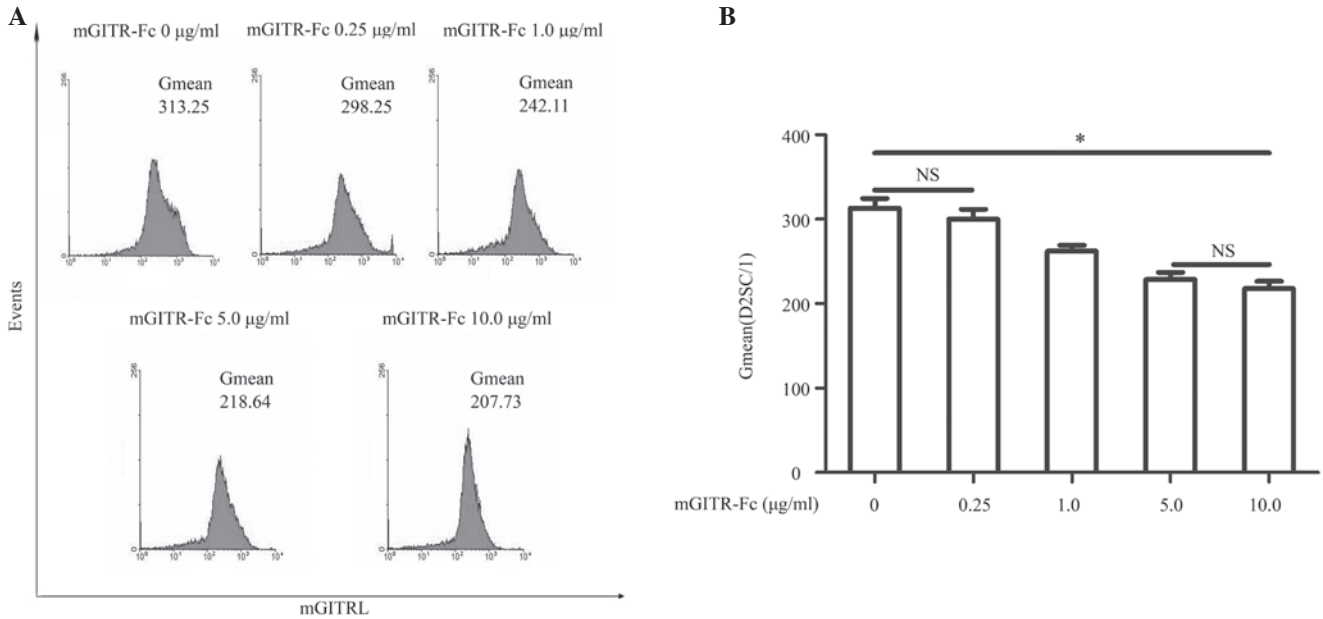

Figure 4. Function of the recombinant mGITR-Fc fusion protein on the expression of mGITRL on DCs. (A) Expression of mGITRL on DCs was detected using FCM. (B) Histogram of Gmean data obtained from FCM. The mGITR-Fc fusion protein at concentrations of 1.0, 5.0 and 10.0 μg/ml inhibited the expression of mGITRL on the DCs, compared with the untreated control group ($^*P<0.05$). No significant difference were observed in the group treated with 0.25 μg/ml mGITR-Fc fusion protein, compared with the control group ($P>0.05$). Data are presented as the mean ± standard deviation. GITR, glucocorticoid-induced tumor necrosis factor receptor related protein; mIgGFc, mouse immunoglobulin G Fc; FCM, flow cytometry; NS, not significant; Gmean, geometric mean.

demonstrated that GITR initiates GITR/GITRL reverse signaling on DCs in the progression of candidiasis (16). Therefore, the present study examined the function of the mGITR-Fc fusion protein on the expression of mGITRL on DCs. As shown in Fig. 4A and B, the expression of mGITRL decreased in the DCs when the cells were treated with the mGITR-Fc fusion protein at concentrations of 1.0, 5.0 and 10.0 μg/ml, compared with the control group ($P<0.05$). No significant difference ($P>0.05$) was observed between the group treated with mGITR-Fc fusion protein (0.25 μg/ml) and the control group. Therefore, the expression of mGITRL on the DCs was be inhibited by the mGITR-Fc fusion protein, and the mGITR-Fc fusion protein may function through reverse signaling.

Discussion

The GITR-GITRL interaction has been suggested to be important in certain diseases and in organ transplantation.

Galuppo *et al* (18) found that the GITR-GITRL system is critical to the progression of acute pancreatitis in mice. Kim *et al* (19) reported that the GITR-GITRL system can regulate the activation of macrophages to increase atherogenesis and destabilize atherosclerotic plaques by the expression and activation of pro-inflammatory cytokine and matrix metalloproteinase-9. Treg-dependent allograft survival is also facilitated via the inhibition of GITR (20). To focus on the function and potential mechanisms of the GITR-GITRL system on certain autoimmune diseases, particularly arthritis, the present study provides essential preparation for functional investigations of the GITR-GITRL system.

In the present study, the extracellular region of mGITR and mIgGFc fragment were first introduced into the pET-32a(+) vector and his-tagged recombinant mGITR-Fc fusion protein was successfully expressed in *E.coli* Rosetta. The expressed form of prokaryotic protein is a factor, which affects the function of a protein, and the fusion protein expressed in a prokaryotic system often maintains the prokaryotic protein characteristics to a certain extent. The fusion protein is also readily purified and is used for the investigations for animal experiments (21). Therefore, in the present study, the soluble mGITR-Fc fusion protein was obtained successfully, rather than the inclusion-body protein (data not shown), by optimizing the conditions for induction. In addition, the mIgGFc segment fused to the recombinant mGITR-Fc fusion protein also enabled the target protein to be expressed more readily and be more soluble. The mIgGFc segment also prolongs the half-life and improves the stability of the mGITR-Fc fusion protein, providing suitable for use in subsequent animal experiments *in vivo* (22,23).

Following successful construction of the mGITR-Fc fusion protein, the present study examined its function *in vitro*. $CD4^+$ T cells can be activated by the GITR/GITRL co-stimulatory signal, and GITRL can promote the proliferation of $CD4^+$ T cells by targeting GITR expressed on $CD4^+$ T cells (2). Therefore, analyzing the proliferation of $CD4^+$ T cells provides an optimal method of analyzing the function of GITR *in vitro*. The results of the present study demonstrated that the proliferation of $CD4^+$ T cells, induced by mGITRL, was inhibited markedly when the cells were co-treated with the exogenous mGITR-Fc fusion protein. Notably, the exogenous mGITR-Fc fusion protein did not inhibit or promote the proliferation of $CD4^+$ T cells with no engagement of mGITRL. These results demonstrated that the mGITR-Fc fusion protein obtained in the present study may function as the inhibitor of GITR/GITRL the co-stimulatory signal.

Although the co-stimulatory signal of GITR/GITRL is the recognized and common mechanism by which GITR/GITRL promotes the immune response in certain diseases (9-12), the reverse signal is another mechanism by which GITR and GITRL can be involved in these diseases (15,16). Grohmann *et al* (15) reported that reverse signaling in the GITR/GITRL system can regulate immune responses in animals with allergic airway inflammation. In the progression of candidiasis, GITR can also modulate the expression of Toll-like receptor 4 on DCs through GITR/GITRL reverse signaling (16). This signaling pathway was also reported to exist in certain tumor necrosis family members. Kuka *et al* (13) reported that the expression of CD70 on DCs can be self-regulated when the cells were treated with CD27, the receptor of CD70. In the present study, the exogenous mGITR-Fc fusion protein was also used to treat DCs, and the results confirmed that expression of mGITRL on the DCs was possibly regulated by GITR, through the reverse signal.

In conclusion, the present study demonstrated that the mGITR-Fc fusion protein inhibited the GITR/GITRL co-stimulatory signal *in vitro*. The results also demonstrated that the mGITR-Fc fusion protein inhibited the expression of mGITRL on DCs, in which the reverse signal may be the explanation. These conclusions indicated the mGITR-Fc fusion protein as optimal for use in subsequent animal experiments to determine which signal is the dominant mechanism, by which GITR/GITRL modulates the immune responses in certain autoimmune diseases.

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