Cellular & Molecular Immunology (2010) 7, 316–322

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RESEARCH ARTICLE

# An isoleucine-zipper motif enhances costimulation of human soluble trimeric GITR ligand

Dawei Cui[1,2], Shengjun Wang[1], Yu Chen[2], Jia Tong[1], Jie Ma[1], Li Tang[1], Xianzhi Yang[1], Ye Shi[1], Jie Tian[1], Liwei Lu[3] and Huaxi Xu[1]

**Glucocorticoid-induced tumor-necrosis factor receptor (GITR) and its ligand, GITRL, play significant roles in regulating immune responses. It is clear that human soluble GITRL (hsGITRL) transduces signal activity through multiple oligomerization states. To develop human soluble trimeric GITRL protein as a potential therapeutic target, we explored the link of the isoleucine-zipper (ILZ) motif to the N-terminus of the human soluble GITRL with two leucine sequences. hsGITRL, with the ILZ motif (ILZ-hsGITRL), was firstly expressed in *Escherichia coli*, which exhibited a predominant trimer when identified by Sephadex G-100 filtration and non-reducing SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The significantly higher biological activity of the ILZ-hsGITRL compared with hsGITRL was confirmed by $CD4^+$ T proliferation, interferon-γ (IFN-γ) secretion and binding activity assay. To reveal and compare the underlying mechanisms, the level of extracellular signal-regulated kinase-1/2 (ERK1/2) phosphorylation was examined, indicating that ILZ-hsGITRL induced more persistent and stronger ERK1/2 activation than hsGITRL. In conclusion, the incorporation of an ILZ motif could markedly improve the costimulation of hsGITRL.**
*Cellular & Molecular Immunology* (2010) **7,** 316–322; doi:10.1038/cmi.2010.7; published online 15 March 2010

**Keywords:** GITR ligand; isoleucine zipper; T cells

## INTRODUCTION

The glucocorticoid-induced tumor-necrosis factor receptor (GITR, also known as TNFRSF18) and its ligand, GITRL, belong to the tumor-necrosis factor (TNF)/TNF receptor superfamily.[1,2] GITR is expressed constitutively on $CD4^+CD25^+$ regulatory T cells at high levels, but it is also expressed on resting $CD4^+CD25^-$ effector T cells at low levels and upregulated after activation.[3,4] In addition, expression of GITR is detected on natural killer cells and macrophages.[5,6] The natural GITRL is a type II transmembrane protein predominantly expressed on antigen-presenting cells, including dendritic cells, B cells, macrophages, endothelial cells and some tumor cells.[1,2,5]

Engagement of GITR and GITRL is correlated with the functional interplay among T cells, antigen-presenting cells and some tumor cells. GITR triggered by soluble GITRL, cell surface GITRL or anti-GITR monoclonal antibody (mAb) (DTA-1) abrogates regulatory T-cell suppression[3,4,7,8] and results in exacerbation of organ-specific autoimmune diseases,[3,9,10], enhancement of antitumor immunity and the immunity to viral pathogens.[11–13] Thus, the functional interaction between GITR and GITRL is important for regulatory T cells to induce immune tolerance and regulate homeostasis.[14,15] Structure analysis has revealed that human soluble GITRL (hsGITRL) increases its receptor-binding affinity and enhances costimulatory activity through self-assembly into a trimer. However, hsGITRL exhibits a relatively weak tendency to trimerize and a monomer–dimer–trimer–supercluster exhibits a dynamic equilibrium, while the dimer is relatively stable in solution.[16,17] Interestingly, mouse GITRL displays a monomer–dimer dynamic equilibrium and shows biological activity with self-assembly into dimer states in solution.[18,19]

Leucine zippers are short coiled-coil domains that serve to dimerize or oligomerize protein subunits.[20,21] Crystal structural analysis indicates that an ILZ forms trimers through self-assembly.[22] Soluble TNF/TNF receptor modified by an ILZ motif can form a stable trimeric or multiple oligomerization states.[23] For example, soluble CD40L with an ILZ motif folds into a stable trimer, and its biological activity is significantly enhanced.[24,25] Soluble type I natural killer cell receptor with the ILZ-fusion proteins can form a trimer in solution and exhibit a higher binding avidity with related ligand than classical immunoglobulin-fusion proteins.[26] Accumulating evidence indicates that ILZ-fusion proteins can serve as a valuable tool to facilitate the study of receptor–ligand interactions in many cellular systems.

In this study, we expressed soluble forms of both the extracellular domain of hsGITRL and the hsGITRL with an ILZ motif (ILZ-hsGITRL) in *Escherichia coli* strain Rossetta (DE3). We demonstrated that the ILZ-hsGITRL mainly existed as trimers, whereas the hsGITRL mainly existed as monomers, dimmers and trimers in solution. Moreover, ILZ-hsGITRL proteins had a significantly higher potency in driving $CD4^+$ T-cell proliferation, interferon-γ (IFN-γ) secretion and extracellular signal-regulated kinase-1/2 (ERK1/2) phosphorylation than hsGITRL. These findings demonstrated that the ILZ motif could facilitate the formation of trimeric hsGITRL and enhance its

[1]Department of Immunology, School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang, China; [2]The First Affiliated Hospital of Medical School of Zhejiang University, Hangzhou, China and [3]Department of Pathology, University of Hong Kong, Hong Kong, China
Correspondence: Dr SJ Wang, Department of Immunology, School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang 212013, China.
E-mail: sjwjs@ujs.edu.cn
Received 27 October 2009; Revised 6 January 2010; accepted 17 January 2010

biological activity, which would provide a basis for further studying the relationship between GITR and GITRL.

## MATERIALS AND METHODS

### Construction of ILZ-hsGITRL expression plasmids

Using standard PCR and cloning techniques, an ILZ sequence was linked to the N-terminus of hsGITRL (amino acids E52–S177) by two leucine sequences. The recombinant sequences (ILZ-hsGITRL) were cloned into the PET32a$^+$ vector (Novagen, Madison, WI, USA) between the *Bam*HI and *Hin*dIII sites. At the same time, the hsGITRL (amino acids E52–S177) was also cloned into the PET32a$^+$ vector. The PET32a$^+$-hsGITRL and PET32a$^+$-ILZ-hsGITRL plasmids were identified by nucleic acid sequence analysis (Sangon, Shanghai, China); the DNA sequence showed 100% homology with the sequence for GITRLaa52-177 in GenBank (BC112032).

### Production of ILZ-hsGITRL proteins

The PET32a+ vector contained Trx-Tag, His-Tag and S-Tag sequences for detection and purification. In addition, it contained cleavable enterokinase sequences for obtaining pure protein. *E. coli* strain Rossetta (DE3) (Novagen) was used as the expression host. The cells were grown in a medium (10 g of trypton, 5 g of yeast extract and 5 g of NaCl in 1 liter of water) with 100 μg/ml ampicillin at 37 °C until optical density (OD)=0.6–0.8, and the expression was induced with 0.6 mM isopropyl-β-D-thiogalactoside for 8 h at 25 °C. The soluble proteins were produced, obtained by ultrasound sonication on the ice, and then identified by anti-hGITRL mAb (eBioscience, San Diego, CA, USA) and anti-His mAb (Cell Signal Technology, Danvers, MA, USA).

### Purification of ILZ-hsGITRL proteins

The hsGITRL and ILZ-hsGITRL proteins were purified by Ni$^+$-IMAC (Bio-Rad, Irvine, CA, USA) and was dialyzed for 24 h at 4 °C in phosphate-buffer solution (PBS), then was condensed with PEG 4000 (Merk, Darmstadt, Germany) and digested by recombinant enterokinase (Novagen) to remove the Trx-Tag, His-Tag and S-Tag sequences. The digested soluble protein mixtures were dialyzed overnight at 4 °C in PBS, and then further purified by Ni$^+$-IMAC to a purity of 94% as revealed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). In the stage of the second purification, we collected filtering peak protein, because Trx-Tag and other tags were bound to the Ni$^+$-IMAC column by His-Tag. The filtering peak proteins, hsGITRL or ILZ-hsGITRL, were examined by SDS-PAGE and western blotting, respectively.

### Identification of ILZ-hsGITRL proteins

ILZ-hsGITRL or hsGITRL proteins were identified with Sephadex G-100 size exclusion column (Pharmacia, GE Healthcare, Piscataway, NJ, USA), western blotting and non-reducing SDS-PAGE. According to the manufacturer's protocol, the purified ILZ-hsGITRL/hsGITRL proteins were eluted from Sephadex G-100 column. The protein peaks were collected and identified with SDS-PAGE and western blotting. The anti-His mAb or anti-hGITRL mAb and horseradish peroxidase-conjugated goat antimouse mAb (Fermentas, Vilnius, Lithuania) were utilized in the analysis. Western blotting was developed with ECL reagent (GE Healthcare). ILZ-hsGITRL or hsGITRL proteins were separated from non-reducing SDS-PAGE sample buffer with 10% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). After electrophoresis, the gels were stained with Coomassie blue.

### Isolation of CD4$^+$ T cells

Human peripheral blood CD4$^+$ T cells were isolated from healthy donors by human CD4$^+$ T-cell isolation kit (Miltenyi, Bergisch Gladbach, Germany). CD4$^+$ T cells were further stained with PE conjugated anti-CD4$^+$ mAb (BD PharMingen, San Diego, CA, USA). The purity of CD4$^+$ T-cell population was >95% by flow cytometry.

### hsGITRL/ILZ-hsGITRL binding assay

The purified anti-hGITRL mAbs were coated onto a 96-well plate (Corning, New York, USA) overnight at 4 °C. After washing with PBS containing 0.05% Tween-20, the wells were blocked with Tris-phosphate-buffered saline containing 5% bovine serum albumin for 2 h at 37 °C. The plates were washed with PBS containing 0.05% Tween-20 and incubated with PBS (blank control), purified hsGITRL and ILZ-hsGITRL fusion protein (serial dilutions) for 1 h at 37 °C. After washing with PBS containing 0.05% Tween-20, biotin-conjugated anti-hGITRL mAb and biotin-conjugated mouse immunoglobulin G (IgG) (negative control) were added into the wells for 1 h at 37 °C. Avidin-conjugated horseradish peroxidase was added into the plates for 1 h at 37 °C. The wells were incubated with 3,3′,5,5′-tetramethylbenzidine (Sigma, St Louis, MO, USA) for 15 min at 37 °C, and then the reaction was stopped with 1 M $H_2SO_4$. The optical density was measured at 450 nm.

To further analyze binding activity, we analyzed binding ability of ILZ-hsGITRL protein to natural GITR of actived CD4$^+$ T cells by flow cytometry. CD4$^+$ T cells ($2\times10^5$/well) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$, and stimulated with antihuman CD3 mAb (100 ng/ml) for 24 h. After washing twice with PBS, hsGITRL, ILZ-hsGITRL and control protein were coincubated for 30 min on the ice, respectively. After washing twice with PBS, the FITC-conjugated anti-hGITRL mAbs were added into the CD4$^+$ T cells for 30 min. The CD4$^+$ T cells were analyzed by flow cytometry using the FACSCalibur system (BD Biosciences, San Jose, CA, USA).

### Proliferation assay

CD4$^+$ T cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$. CD4$^+$ T cells ($2\times10^5$/well) were cultured with hsGITRL or ILZ-hsGITRL in the presence of antihuman CD3 mAb (50 ng/ml) for 72 h. In addition, different protein concentrations were performed. The cultures were added with Cell Counting Kit-8 solution (Dojindo Laboratories, Kumamoto, Japan) and incubated for the final 4 h, and then the OD values were measured at 450 nm with a microplate reader.

### Detection of cytokine production

Supernatants from similar cultures were collected and IFN-γ production was measured by ELISA Kit (eBioscience).

### Western blotting assay

CD4$^+$ T cells were stimulated with hsGITRL or ILZ-hsGITRL (5.0 μg/ml) in the presence of antihuman CD3 mAb (50 ng/ml), harvested at the indicated time points (10, 20 and 40 min), washed twice with ice-cold PBS and lysed with RIPA lysis buffer; the level of ERK1/2 was analyzed by western blotting. Polyclonal antibodies for phosphorylation and total ERK1/2 were purchased from Cell Signal Technology, and the secondary horseradish peroxidase-conjugated antibodies (GE Healthcare) were used at a 12000 dilution and visualized with ECL

regents. Bands were detected with the Typhoon FLA 9000 scan system (GE Healthcare). Experiments were repeated at least three times.

### Statistical analysis

Data were presented as mean±SD. A value of $P<0.05$ was considered statistically significant. Statistical differences between two groups in the same conditions were determined by using Student's *t*-test for paired samples. For multiple groups means were compared by repeated-measures analysis of variance.

## RESULTS

### Production and purification of ILZ-hsGITRL proteins

In the current study, the ILZ motif was linked to the N-terminus of the hsGITRL. The structural comparison between the two human GITRL molecules was diagrammed in Figure 1a. An ILZ motif was firstly conjugated to hsGITRL's N-terminus, and the two fragments were performed by two leucines. Soluble proteins including hsGITRL (E52-S177) without the ILZ motif or ILZ-hsGITRL expressed in *E. coli* strain Rossetta (DE3) were found by ultrasound sonication in solution. Two soluble proteins have Trx-Tag, His-Tag and S-Tag sequences and cleavable enterokinase sequences. We randomly selected His-Tag for purification and detection. Each protein was analyzed by Coomassie gels (Figure 1b, lanes 2–9) and western blotting (Figure 1b, lanes 10–11). The calculated peptide molecular masses for hsGITRL and ILZ-hsGITRL were almost 28 and 32 kDa, respectively. A few soluble proteins were in supernatant, while many proteins were in inclusion body. After purification, identification and dialysis concentration, to obtain pure hsGITRL or ILZ-hsGITRL by removing Trx-Tag, HisTag and S-Tag sequences and enterokinase sequences, each soluble GITRL preparation was digested by recombinant enterokinase according to the instruction manual. The digested mixtures were secondly purified with $Ni^+$-IMAC, The perforation peaks were collected and identified with Coomassie gels (Figure 1c, lanes 2–3), western blotting (Figure 1c, lanes 4–5). In the phase of western blotting, we detected the purity of hsGITRL or ILZ-hsGITRL protein with anti-His mAb (data not shown) and anti-hGITRL mAb. The results indicated that the perforation peaks were pure hsGITRL or ILZ-hsGITRL proteins without cleavable residual protein consisting of Trx-Tag, His-Tag and S-Tag, the calculated peptide molecular masses for pure hsGITRL and ILZ-hsGITRL were almost 14 and 18 kDa, respectively.

### Identification of ILZ-hsGITRL proteins

In this study, to compare the biophysical characterization of ILZ-hsGITRL and hsGITRL, the comparison between the purified proteins of hsGITRL and ILZ-hsGITRL was done in Sephadex G-100 filtration and non-reducing SDS-PAGE experiments. The hsGITRL was confirmed as a mixture of dimmers and trimers in solution by Sephadex G-100 filtration (Figure 2a), the protein isolated from the dimmer and trimer peaks were hsGITRL identified by SDS-PAGE (Figure 2c; lane 3: trimer; lane 4: dimer) and western blotting (Figure 2c; lane 5: trimer; lane 6: dimer). ILZ-hsGITRL existed mainly as trimers in solution (Figure 2b), and the protein isolated from the trimer peak was also ILZ-hsGITRL identified by SDS-PAGE (Figure 2c, lane 2) and western blotting (Figure 2c, lane 7). The non-reducing SDS-PAGE experiments with the purified protein indicated that both hsGITRL and ILZ-hsGITRL existed as a mixture of monomers, dimmers and trimer species, especially significant amounts of dimmers of hsGITRL in solution were observed (Figure 2d, lane 1). However, significant amounts of trimer species of ILZ-hsGITRL existed in solution were observed (Figure 2d, lane 2).

### ILZ-hsGITRL proteins possess stronger binding activities

To demonstrate binding activities of hsGITRL or ILZ-hsGITRL, our study displayed that the purified proteins could especially bind to anti-hGITRL mAb. Although the OD values showed changes after serial concentrations of the purified proteins, the difference of OD values between ILZ-hsGITRL and hsGITRL was not notable (Figure 3a). But using flow cytometric analysis, we demonstrated that the hsGITRL or ILZ-hsGITRL could bind to natural GITR of actived $CD4^+$ T cells, and the binding activity was obviously different (Figure 3b).

### ILZ-hsGITRL proteins have powerful costimulation

The biological activities of hsGITRL and ILZ-hsGITRL were evaluated for their potencies in inducing human $CD4^+$ T-cell proliferation in the presence of 50 ng/ml antihuman CD3 mAb as a stimulator (Figure 3c). The extent of $CD4^+$ T-cell proliferation depended on the protein concentrations, although both hsGITRL and ILZ-hsGITRL induced

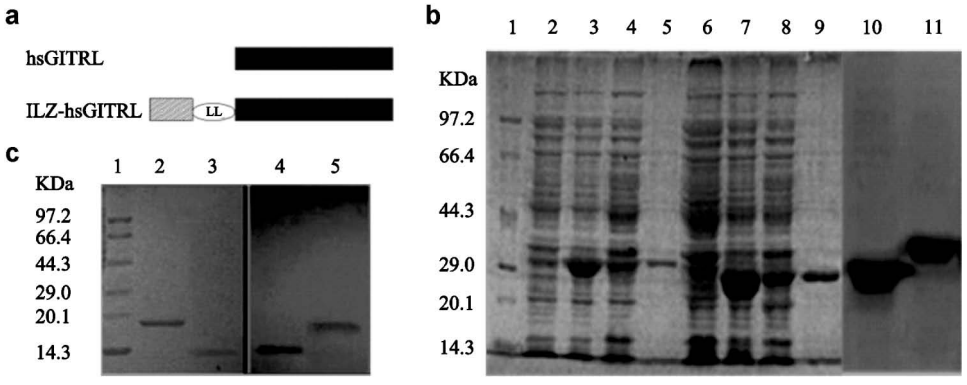


**Figure 1** Expression of recombinant hsGITRL proteins. (**a**) Diagram showing soluble GITRL molecules. hsGITRL consisted of the extracellular domain (black box). ILZ-hsGITRL consisted of hsGITRL, an ILZ motif (gray box) and two leucine sequences. (**b**) Coomassie stain (lanes 1–9) and western blotting (lanes 10–11) of various Trax-hsGITRL proteins. Lanes 1, marker; lanes 2–5, Trax-ILZ-hsGITRL. Lane 2, not induced; lane 3, induced; lane 4, ultrasonic supernatant; lane 5, purified; lanes 6–9, Trax-hsGITRL. Lane 6, not induced; lane 7, induced; lane 8, ultrasonic supernatant; lane 9, purified; lane 10, Trax-hsGITRL; lane 11, Trax-ILZ-hsGITRL. (**c**) Lane 1, marker; lane 2–3, Coomassie stained gel. Lane 2, ILZ-hsGITRL; lane 3, hsGITRL; lanes 4–5, western blotting; lane 4, hsGITRL; lane 5, ILZ-hsGITRL. These experiments were repeated more than three times with essentially the same results. GITRL, glucocorticoid-induced tumor-necrosis factor receptor ligand; hsGITRL, human soluble GITRL; ILZ-hsGITRL, isoleucine-zipper hsGITRL.

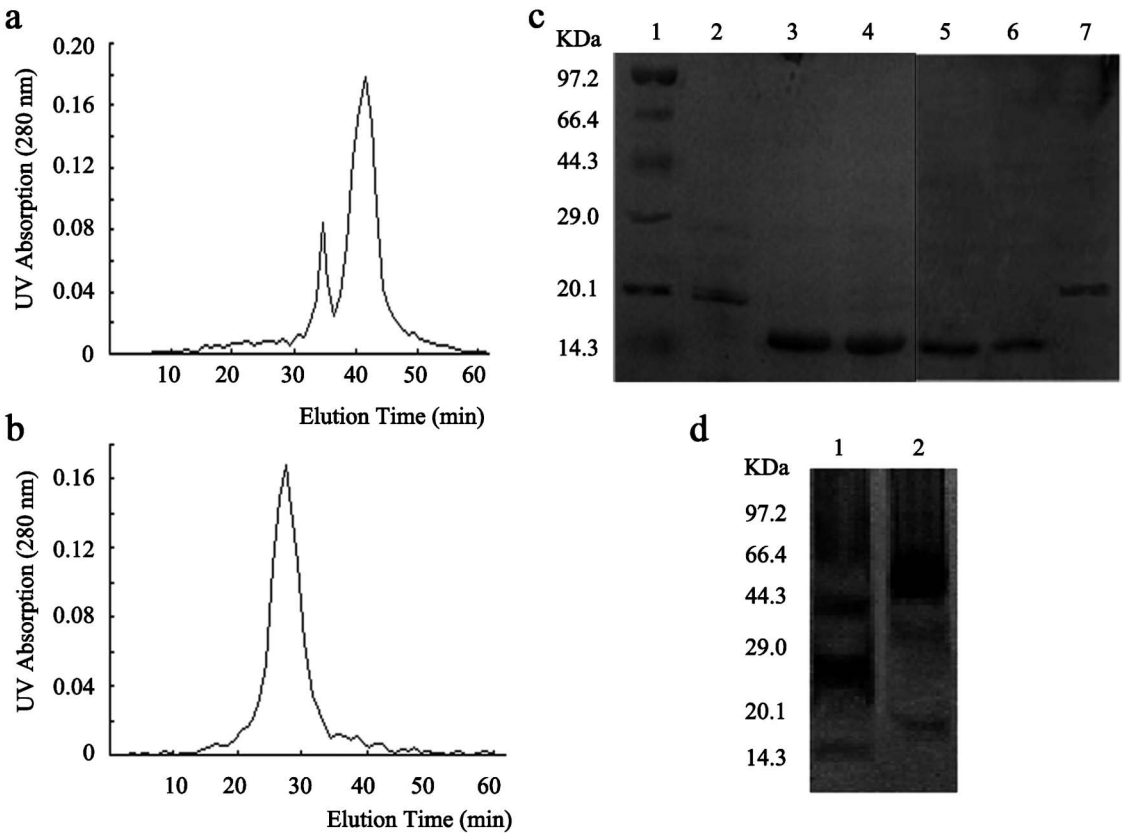


**Figure 2** Characterization of ILZ-hsGITRL proteins. (**a**) hsGITRL with two peaks was eluted from Sephadex G-100 column, with calculated molecular masses of ~28 and ~43 kDa, in accordance with dimer and trimer, respectively. (**b**) ILZ-hsGITRL from Sephadex G-100 appeared with a single peak of ~54 kDa, in accordance with a trimer. (**c**) Coomassie stain and western blotting of three peaks. Lanes 1, marker; lanes 2–4, Coomassie stained gel; lane 5–7, western blotting. Lanes 2 and 7, ILZ-hsGITRL trimer peak; lanes 3 and 5, hsGITRL trimer peak; lanes 4 and 6, hsGITRL dimmer peak. (**d**) Non-reducing SDS-PAGE of hsGITRL and ILZ-hsGITRL. Lane 1, hsGITRL; lane 2, ILZ-hsGITRL. These experiments were repeated more than three times with essentially the same results. hsGITRL, human soluble glucocorticoid-induced tumor-necrosis factor receptor ligand; ILZ-hsGITRL, isoleucine-zipper hsGITRL; SDS-PAGE, SDS–polyacrylamide gel electrophoresis.

$CD4^+$ T-cell proliferation, ILZ-hsGITRL showed significantly higher biological activity, especially at protein concentrations of 0.5–5.0 μg/ml ($P<0.05$). However, when the protein concentrations were lower, the extent of $CD4^+$ T-cell proliferation was not significant.

There was a similar potency between the activities of the two proteins in inducing IFN-γ secretion with the presence of 50 ng/ml anti-human CD3 mAb as a stimulator (Figure 3d); the observed effects were notable at protein concentrations of 1.0–5.0 μg/ml ($P<0.05$); when the protein concentrations were lower, the effects of IFN-γ secretion were not significant.

To compare in more detail the biological activity between hsGITRL and ILZ-hsGITRL, we treated $CD4^+$ T cells with purified proteins of hsGITRL and ILZ-hsGITRL, and examined the phosphorylation level of ERK1/2 (Figure 3e). Our data indicated that hsGITRL induced noticeable phosphorylation of ERK1/2 within 10 min, whereas the level of ERK1/2 phosphorylation was reduced little by little afterward. ILZ-hsGITRL induced more persistent ERK1/2 phosphorylation that was stronger than hsGITRL.

## DISCUSSION

Recent studies indicate that hsGITRL profoundly enhances the activity of $CD4^+$ T or THP-1 cells and stimulates the production of IFN-γ, IL-8, intercellular adhesion molecule-1 and other inflammation cytokines.[3,9–14] Interestingly, hsGITRL displays biological activity by forming multiple oligomerization.[16] The ILZ motif has been shown to form trimers in solution and many soluble TNF/TNF receptor superfamilies modified by an ILZ motif can form stable trimeric or multiple oligomerization states.[23]

In the current study, a few soluble proteins, including hsGITRL (amino acids E52–S177) without the ILZ motif, and ILZ-hsGITRL expressed in *E. coli* strain Rossetta (DE3), were found in solution, but two proteins were mainly in inclusion body. Because the Trax sequences have been characterized to facilitate the aim protein forming soluble states, the host *E. coli* expression strain Rossetta (DE3) can obviously improve production of the aim proteins containing scarce codons. However, the products would be improved by further optimizing condition.

The predicted calculated peptide molecular masses for pure hsGITRL and ILZ-hsGITRL were almost 14 and 18 kDa, respectively. In fact, each protein band migrated slightly higher than the predicted molecular mass because each GITRL protein contained Trx-Tag, His-Tag, S-Tag and cleavable enterokinase. After each soluble GITRL preparation was digested by recombinant enterokinase, the calculated peptide molecular masses for pure hsGITRL and ILZ-hsGITRL were consist with our predicted molecular. Moreover, we found that compared with hsGITRL, the ILZ-GITRL was purified with difficulty by a $Ni^+$-IMAC column in that the ILZ could affect connection of His-Tag with the $Ni^+$-IMAC column. In the phase of protein purification, it

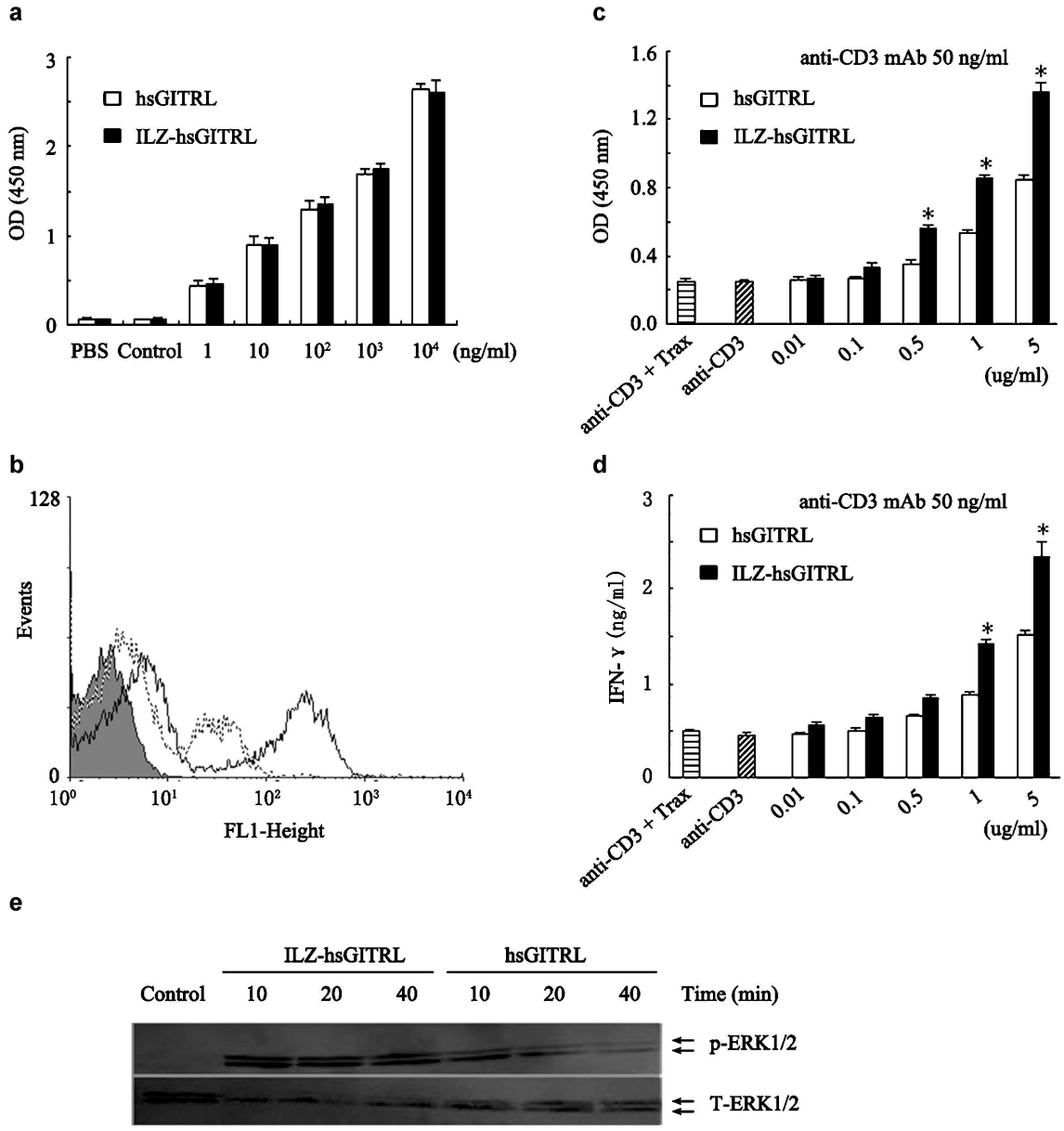


**Figure 3** ILZ-hsGITRL proteins possess stronger binding activities and costimulation. (**a**, **b**) Binding activities of hsGITRL and ILZ-hsGITRL with anti-hGITRL, were performed by ELISA (**a**) and FCM (**b**). $CD4^+$ T cells were stimulated with antihuman CD3 mAb for 24 h, and cultured with control proteins (gray histograms), hsGITRL (dotted lines) and ILZ-hsGITRL (solid lines) proteins, and stained with FITC-conjugated anti-hGITRL mAb. Data shown were the means (±SD) of triplicate cultures or representatives of three independent assays. (**c**) Comparing with hsGITRL, the trimer of ILZ-hsGITRL enhanced $CD4^+$ T cells' proliferative response to different protein concentrations, respectively. Data shown were the means (±SD) of triplicate cultures and representatives of three independent assays. (**d**) Levels of IFN-γ in the culture supernatants were measured by ELISA kit. Data shown were the means (±SD) of triplicate cultures. (**e**) $CD4^+$ T cells were stimulated with ILZ-hsGITRL or hsGITRL (5.0 μg/ml) in the presence of antihuman CD3 mAb. Cellular lysates were collected at indicated time points and the levels of ERK1/2 phosphorylation were analyzed by western blotting. The experiments were repeated three times with essentially the same results. ERK1/2, extracellular signal-regulated kinase-1/2; FCM, flow cytometry; hsGITRL, human soluble glucocorticoid-induced tumor-necrosis factor receptor ligand; IFN-γ, interferon-γ; ILZ-hsGITRL, isoleucine-zipper hsGITRL; mAb, monoclonal antibody.

was known that firstly, we could obtain the pure hsGITRL and ILZ-hsGIRTL with Trx-Tag, His-Tag, S-Tag and cleavable enterokinase; secondly, we purified the digested mixtures with $Ni^+$-IMAC, because the Trx-Tag, His-Tag and S-Tag could conjugate with the $Ni^+$-IMAC column, and the perforation peaks were pure hsGITRL and ILZ-hsGITRL proteins. When we detected the purity of hsGITRL or ILZ-hsGITRL protein with anti-His mAb (data not shown) and anti-hGITRL mAb by western blotting, the cleavable residual proteins consisting of Trx-Tag, His-Tag and S-Tag were not shown in the results, perhaps the cleavable residual proteins were extremely scant and not detected by western blotting.

In recent experiments, hsGITRL exists as a mixture of monomers, dimmers, trimers and superclusters. Gel filtration and crosslinking studies indicate that hGITRL exists as dimers and trimers in solution and can also form a supercluster.[16,17] In our study, both Sephadex G-100 analyses and non-reducing SDS-PAGE revealed that ILZ-hsGITRL might form high-order superclusters, and the trimer was predominant and more stable in solution. hsGITRL might also form

multiple oligomerization, and the dimer was predominant and stable in solution. hsGITRL or ILZ-hsGITRL proteins had a disparate result between Sephadex G-100 filtration and non-reducing SDS-PAGE, and the different experimental conditions might have an effect on the hGITRL's C-terminus conformation, as the flexible C-terminus was critical for trimer formation, which was confirmed in a previous study. These data indicated that hsGITRL or ILZ-hsGITRL proteins existed as a mixture of monomers, dimers, trimers and superclusters in solution. The dimer of hsGITRL was more stable than the trimer, which was consistent with a previous report.[17] However, the trimer of ILZ-hsGITRL was more stable than other states in solution. These observations support our hypothesis that compared with hsGITRL, the ILZ-hsGITRL can more easily form trimers and superclusters binding to receptors. Although soluble hGITRL had shown a considerably weak affinity for related receptor, the trimerization of ILZ-hsGITRL markedly enhanced its receptor-binding activity. However, in the hsGITRL/ILZ-hsGITRL binding assay by ELISA, the difference of OD values between ILZ-hsGITRL and hsGITRL was not notable. However, the binding activities of hsGITRL with hGITRL on the activated peripheral blood mononuclear cells were significantly lower than ILZ-hsGITRL by flow cytometric analysis. Compared with other tumor-necrosis factor superfamily members, the hsGITRL protein loosely and unstably assembled into open trimers in solution displayed a lower receptor affinity in previous reports.[16,17] Thus, it was possible that the ELISA system might affect the conformation of hsGITRL and ILZ-hsGITRL. We concluded that the ILZ facilitated hsGITRL to form stable trimer states, and the multiple oligomerization structures of ILZ-hsGITRL would be completely certified by the X-ray crystal structure and other studies.

Recent studies reveal that soluble GITRL becomes a stronger costimulatory molecule when engineered to form the multiple oligomerizations.[16,17] The multiple trimer, hGITRL, is required for a stronger ERK activation in $CD4^+$ T-cell proliferation activity. Compared with hsCD40L, ILZ-hsCD40L exerts more potent activation that is required to efficiently trigger CD40-mediated signaling.[24] The GITRL stimulation induces the expression of proinflammatory cytokines and the phosphorylation of ERK1/2.[27–29] The plasmacytoid dendritic cells promote natural killer cell cytotoxic activity and IFN-$\gamma$ production through GITRL–GITR signaling pathway.[30] These studies clearly demonstrate GITRL-mediated signaling as a means of regulating immunological effects of the GITRL–GITR pathway. In this study, the expressed hsGITRL and ILZ-hsGITRL proteins were biological activities, they increased $CD4^+$ T-cell proliferation and IFN-$\gamma$ secretion by different protein concentrations, possibly due to multiple oligomerizations formed in higher concentrations. Moreover, ILZ-hsGITRL stimulation induced higher level of ERK1/2 phosphorylation that was more constant than hsGITRL, which was consistent with previous reports.[16] The ILZ-hsGITRL proteins showed significantly higher biological activity than the hsGITRL proteins; therefore, it was possible that the ILZ motif-enhanced hsGITRL formed multiple oligomerizations.

In summary, we had successfully expressed human soluble trimer protein with the ILZ motif (ILZ-hsGITRL) in the current study. Their biological activities had been confirmed by binding analysis with peripheral blood mononuclear cells, inducing $CD4^+$ T-cell proliferation, IFN-$\gamma$ secretion and ERK1/2 activation. The activity of ILZ-hsGITRL binding to hGITR on peripheral blood mononuclear cells was notable higher than hsGITRL. Both hsGITRL and ILZ-hsGITRL in inducing $CD4^+$ T-cell proliferation and IFN-$\gamma$ secretion depended on the protein concentrations. The level of ERK1/2 phosphorylation by ILZ-hsGITRL stimulation was more persistent and higher than that of hsGITRL stimulation.

Moreover, the two proteins displayed similar and different physical structures by Sephadex G-100 filtration and non-reducing SDA-PAGE, respectively. The ILZ-hsGITRL proteins were prone to fold into trimers, whereas the hsGITRL existed as a mixture of monomers, dimmers and trimers in solution, and the dimmers of hsGITRL were predominant. The crystal structure of ILZ-hsGITRL awaits to be determined in the future.

The hsGITRL trimers with biological activity will have important clinical implications as novel therapeutic strategies in the treatment of human diseases.

## ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (Grant No. 30871193 and No. 30972748), Natural Science Foundation of Jiangsu Province (Grant No. BK2004405), Natural Science Foundation of Jiangsu Province Educational Commission (Grant No. 08KJB320002), Research Foundation of Jiangsu Province Health Department (Grant No. H200952), and Top Talent Program of Jiangsu University and SCI-Tech Innovation Team of Jiangsu University.

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