國立交通大學

分子醫學與生物工程研究所

碩士論文

幽門螺旋桿菌熱休克蛋白 60 N 端之活性

The activities for the N-terminal domain of *H. pylori* heat shock protein 60

研 究 生：沈筱薇

指導教授：廖光文　教授

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研究生：沈筱薇　　Student：Hsiao-Wei Shen

指導教授：廖光文　　Advisor：Kuang-Wen Liao

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中文摘要

幽門螺旋桿菌的感染與許多上消化道的疾病相關，像是慢性胃炎、消化性潰瘍、黏膜相關淋巴組織淋巴癌 （MALT lymphoma）以及胃癌。感染幽門螺旋桿菌患者若無以抗生素加以治療，將轉變為慢性、持續性感染 ； 而上述疾病的發生則主要起因於該病原菌的慢性感染。並且該菌所分泌的熱休克蛋白 60 已被證實為一黏附分子可連結幽門螺旋桿菌以及人類胃上皮細胞，進而造成胃部疾病。

對於幽門螺旋桿菌熱休克蛋白 60 在免疫調節功能上，較多的研究是指出此蛋白可引發前發炎激素（pro-inflammatory cytokine）例如：干擾素-γ （IFN-γ）、腫瘤壞死因子-α（TNF-α）、白介素 6、8（IL-6、8）而引起發炎反應。特別是在氨基酸序列 300 到 435 的位置可引發大量 IL-8 的表現。在另一方面，近年來也有研究指出不同物種的熱休克蛋白 60 似乎在人體免疫系統可以抵禦病原菌的慢性感染。例如人類與日本血吸蟲的熱休克蛋白 60 可引發調節型 T 細胞（regulatory T cells）的增生達到免疫抑制的效果。然而，幽門螺旋桿菌熱休克蛋白 60 對於免疫調節的機制依然不清楚。在本篇實驗中，

我們利用人類周邊血液單核球細胞（human peripheral blood mononuclear cells, PBMCs）作為研究系統，探討幽門螺旋桿菌熱休克蛋白 60 之 N 端蛋白對其增生所造成之影響。研究結果顯示，幽門螺旋桿菌熱休克蛋白 60 之 N 端位置於 101-200 和 1-200 之蛋白能夠降低 PBMCs 之增生以及引發 TGF-β之產生。根據上述研究結果，幽門螺旋桿菌熱休克蛋白 60 可藉由接近 N 端的 101-200 之處引發 TGF-β並且達到免疫抑制的效果。

The activities for the N-terminal domain of *H. pylori* heat shock protein 60

Student: Hsiao-Wei Shen Advisor: Dr. Kuang-Wen Liao

Institute of Molecular Medicine and Bioengineering

National Chiao Tung University

Abstract

Helicobacter pylori can lead to variety of upper gastrointestinal disorders, such as chronic gastritis, peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer. Without treatment, *H. pylori* would become chronic infection in almost all of those patients. The expression of heat shock protein 60 by *H. pylori* (HpHSP60) has been shown as an adhesion molecule that interacts with host gastric epithelial cells.

For immunoregulation, many literatures showed that HpHSP60 can induce the secretions of pro-inflammatory cytokines, such as IFN γ, TNF-α, IL- 6, IL-8 and cause inflammation. Particularly, the sequence of HpHSP60 from 300 to 435 can induce dramatically IL-8 expression. Interestedly, researchers found that HSP60 in different species seems to be related to the regulation of immune responses in

chronic infection disease. Literatures indicated that HSP60 in human and *S. japonicum* can induce regulatory T cell (Treg) expression and suppress immunity. However, the feature of HpHSP60 for immunoregulation still remains unknown. In this study, the N-terminal domains of HpHSP60 were constructed and measured their activities on immune response. The results showed that the treatment with the sequence 101-200 ($HpHSP60_{101\text{-}200}$) or 1-200 ($HpHSP60_{1\text{-}200}$) of HpHSP60 to human peripheral blood mononuclear cells (PBMCs) decreased the proliferation rate. Furthermore, $HpHSP60_{101\text{-}200}$ and $HpHSP60_{1\text{-}200}$ could increase TGF-β secretion from THP-1 cells. Taken together, the sequence of HpHSP60 from 101 to 200 could induce the expression of TGF-β and which may have contributions to their immune suppressive activity.

致謝

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首先一定要感謝實驗室的大佬也就是我的指導教授廖光文博士，謝謝老師收留當時剛從別的實驗室離開的我，以及教導我做人處事的道理和做為一個研究人員該有的思維、態度。也感謝鄭添祿教授、吳彰哲教授、蔡女滿教授抽空參加口試，在口試當中也學習到利用不同角度和更嚴謹的方式看待科學實驗，學生受益良多。

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Contents

List of Figure and Legend

Figure and Legend

List of Appendix

Introduction

Helicobacter pylori (*H. pylori*) have been shown to play a pathogenic role in gastric diseases. It is now recognized as a cause of chronic gastritis, peptic ulcer, gastric cancer and MALT lymphoma (Algood and Cover, 2006). *H. pylori* Heat Shock Protein 60 (HpHSP60) has been first identified as an adhesion molecule that interacts with host gastric epithelial cells and mucin (Huesca *et al.*, 1996). Moreover, HpHSP60 has been indicated that it can cause inflammation by inducing releases of pro-inflammatory cytokines (Matsuura *et al.*, 2009). Recently, HpHSP60 for gastric tumor progression can promote the migration ability of cancer cells, increase in the angiogenic activity of endothelial cells, induction of pro-inflammatory cytokine in both gastric epithelial cells and monocytes. These results suggest that HpHSP60 is able to accelerate tumorigenesis by multiple mechanisms (Lin *et al.*, 2010b).

HSP60s are conserved proteins, even if they from different species are highly similar for their protein sequences (Ellis, 1992). For example, the alignment of amino acid sequence between *H. pylori*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *M. leprae* and human HSP60 was up

to 72% of similarity (Macchia *et al.*, 1993). In addition, they also play the similar role in inducing pro-inflammatory cytokines. The HSP60 of *Mycobacterial tuberculosis* was demonstrated to induce secretion of pro-inflammatory cytokine, IL-6, IL-8 and TNF-α from human monocytes (Friedland *et al.*, 1993). *Chlamydia pneumoniae* HSP60 can increase the level of IL-6 in the bronchialveolar lavage fluid to cause acute pulmonary inflammation via TLR4 in mice (Bulut *et al.*, 2009). Bulut Y. and his colleagues have proved the *Chlamydia trachomatis* HSP60 could promote inflammation through TLR4-mediated NF-κB pathway to result in the activation of macrophages and endothelial cells (Bulut *et al.*, 2002). The expressions of IL-6 and TNF-α for endothelial cells were also found that they can be induced by *E. coli* (Galdiero *et al.*, 1997) or human HSP60 (Kol *et al.*, 1999) (Vabulas *et al.*, 2001). Recently, *Helicobacter pylori* HSP60 has been shown that it can induce many expressions of pro-inflammatory cytokines such as TNF-α, IL-8, GRO, IFN-γ (Lin *et al.*, 2009a). This effect could be resulted from the TLR2 or 4 pathway for human monocytes (Takenaka *et al.*, 2004). Moreover, the IL-8 inductive activity of HpHSP60 has been explored by Lin and his colleagues, the effective domain is located between 300 and 435 a.a. (Lin

et al., 2005).

In addition to inflammation, HSP60 has been indicated recently that it has the capacity to regulate the host immunity. In 2006, Dr. Zanin-Zhorov, A. showed human HSP60 treatment can enhance the IL-10 and TGF-β secretions of Treg via TLR2 to inhibit the expressions of IFN-γ and TNF-α of CD4+CD25- T cells (Zanin-Zhorov *et al.*, 2006). Similarly, *Schistosoma japonicaum* HSP60 can also increase the numbers of $CD4^{+}CD25^{+}$ regulatory T cells to raise the expressions of IL-10 and TGF-β via TLR2 pathway (Wang *et al.*, 2009). In our published paper, HpHSP60 also show its potential to elevate the secretions of anti-inflammatory cytokine IL-10 and TGF-β in human monocytes (Lin *et al.*, 2009b). Therefore, HpHSP60 may be a dual-function protein for inflammation and immune suppression. However, the effect of HpHSP60 on immunosuppression is still unclear.

Currently, the literatures showed that the numbers of Treg will be elevated in *H. pylori*-associated gastritis and gastric cancer (Jang, 2010). As our previous results shown, although HpHSP60 can induce strong

releases of proinflammatory cytokines, it also increases the secretions of IL-10 and TGF-β for THP-1 cells (Lin *et al.*, 2009a) (Lin *et al.*, 2010a). In addition, HpHSP60 would cause the proliferative inhibition by interfering with the cell cycle but not inducing apoptosis in our unpublished results. As well-known, IL-10 and TGF-β are important factors for the increase of Treg cell (Toms and Powrie, 2001) and the Treg cell's activity is associated with the suppression of T-cell proliferation. Thus, we are interesting in which domain is involved in the inhibition of T-cell. Through the identification of this domain, it may not only find out the inhibitory domain for T-cell proliferation but also get the effective sequence for the increase in Treg cell.

Materials and methods

Preparation of recombinant HpHSP60

E. coli [BL21(DE3)] (Yeastern Biotech, Taipei, Taiwan) were transformed with pET-HpHSP60 and grew on LB plate containing kanamycin (30mg/ml) at 37°C for 16 hours. Then single colony was picked and inoculated in 100 ml LB medium containing kanamycin (30mg/ml). After 16 hours incubation at 37°C, the bacteria in the LB broth were refreshed in 900 ml LB with vigorous shaking. Assayed the OD value until OD600 reaches 0.6~0.8, then protein induction was performed by adding 1.25 ml of IPTG (800mM). After 4 hours incubation, harvested the bacteria by centrifugation at 5000 rpm for 15 min at 4 °C. Discarded the supernatant and resuspended the pellet with 30ml binding buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 40 mM imidazole, pH 7.4). Total lysates were sonicated for 15 min and then centrifuge at 12,000 rpm for 30 min to collect the supernatant which containing rHpHSP60. HisTrap™HP column (General Electric, NY, USA) (1cm) was used to purify rHpHSP60. All the buffers and protein samples needed to be filtered with 0.45μm syringe filter. After protein sample loading into the column, washed the column with 30X volume of binding buffer to

remove the unwanted proteins and then eluted the rHpHSP60 with 10X volume of elution buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 500 mM imidazole, pH 7.4). Eluted rHpHSP60 were collected and loaded into G-25 column (General Electric, NY, USA) to remove the unnecessary salt and replace the buffer with PBS (Phosphate Buffer Saline, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM $KHPO_4$, pH 7.4). Protein concentration was quantified by Coomassie Plus reagent (BSA was used as the standard). SDS-PAGE and western blotting with anti-His conjugated HRP (ROCKLAND, PA, USA) were used to confirm the purity of rHpHSP60.

The coupling of HpHSP60 and FITC

First, 2mg HpHSP60 should be in $NaHCO_3$ buffer, so the dialysis of HpHSP60 was with 1L DDW in 4℃ for 1 hour. After 1 hr, the dialysis bag was placed in 1M $NaHCO_3$ buffer (pH=9) for 2 hours and repeated this step again. Then the container was changed new $NaHCO_3$ buffer overnight in cool room. 0.01g FITC (SIGMA, MO, USA) was solved in 10 ml DMSO and the solution was shaken in dark for 90 minutes. In

order to separate FITC-HpHSP60 and un-coupling FITC, the solution was loaded into sephadex G-25 column and FITC-HpHSP60 was first through the column. Protein concentration was quantified by Coomassie Plus reagent (BSA was used as the standard).

The binding of proteins and cells

After counting cells number (inactive or LPS-activated THP-1 cells or AGS cells), the cells were washed with staining buffer (0.5% skim milk in 1X PBS) by centrifuging at 4000 rpm for 5 minutes. The cells were re-suspended with staining buffer and treated with protein (FITC-HpHP60 or EGFP-HpHSP60) on ice for 30 minutes. Finishing incubation, proteins were not bound to the cells were washed by staining buffer. Then the pellet was re-suspended by 1X PBS. The intensity of fluorescence was detected by flow cytometry (Franklin Lakes, NJ, USA).

Cloning and expression and purification of recombinant protein

For proteins expression, the fragment containing the gene of

interesting DNA (EGFP or $HpHSP60_{1-200}$ or $HpHSP60_{101-200}$ or $HpHSP60_{1-500}$ or $HpHSP60_{300-547}$ or $HpHSP60_{1-435}$) were amplified by polymerase chain reaction and cloned into vectors (pET30-HpHSP60 or pET100). The proteins were expressed in *Escherichia coli* (BL21 strain) and purified using HisTrap affinity chromatography followed by sephadex G-25 column. The purity of rEGFP-pET-HpHSP60 was determined by SDS-PAGE, western blot assay (detected by HRP conjugated rabbit polyclonal antibody to polyhistidine) (or detected by mouse anti-HpHSP60 polyclonal antibody and HRP conjugated goat anti-mouse IgG antibody; Jackson ImmunoResearch, PA, USA).

IL-8 secretion from cells

THP-1 cells grown on 24 well plates were stimulated with 1μg LPS, 10μg HpHSP60, EGFP-HpHSP60, βG or 5 μg HpHSP60, $HpHSP60_{1-200}$, $HpHSP60_{101-200}$, $HpHSP60_{300-547}$ at 37℃ in THP-1 growth medium (RPMI 1640 with 4.5g/L glucose, 10mM HEPES, 0.05mM 2-mercaptoethanol and 1mM sodium pyruvate) for 24 hours. Supernatants and cells were separated by centrifugation at 1200rpm for 5

minutes. Supernatants were assayed for IL-8 production, and IL-8 was measured by IL-8 ELISA development kit. (R&D system, MN, USA).

Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated from human whole blood by using Ficoll-Paque™Plus. Dilute human whole blood with equal volume of PBS. Added 6 ml Ficoll-Paque™Plus into the 15 ml centrifuge tube and loaded 8 ml of the diluted blood sample on Ficoll-Paque™ Plus reagent carefully. Then centrifuged the tubes at 400g for 40 min at 18°C. After centrifuge, removed the plasma layer and collect the PBMC carefully between the plasma layer and Ficoll-Paque™Plus solution. Washed the cell with 2X volume of PBS, centrifuged at 1,500 rpm for 15 min. Discarded the supernatant and added 10 ml of ACK lysis buffer (0.15M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) to lyse the red blood cells. Centrifuged at 1,500 rpm for 15 min to remove the supernatant and then washed the cell with 10ml PBS again. Counted the cell number and seeded the PBMCs into 96-well culture plate with RPMI1640 culture medium.

PBMC proliferation assay

To mimic the action occur when the immune cells encounter antigen, anti-CD3 antibody (kindly provided from Dr. Steve R. Roffler) (OKT3, an antibody that specifically binds to CD3 surface marker and transduces signals continually to activate T lymphocytes) was used to selectively activate T lymphocyte (1μg/ml, 30μl per well of 96-well culture plate, incubate in 37°C for two hours). Fresh blood samples from healthy donors were obtained and the PBMCs were isolated by Ficoll-Pague™ reagent. Then seeded the cells into anti-CD3 mAb pre-coated 96-well plates ($2*10^5$/well, triplicate for each treatment) and treated with different proteins of HpHsp60, $HpHSP60_{1\text{-}200}$, $HpHSP60_{101\text{-}200}$, $HpHSP60_{300\text{-}547}$ for four days incubation. Cell proliferation was verified by MTT assay. In briefly, 96-well was centrifuged at 1,500 rpm for 15 min to remove the supernatant. Added 100 μl MTT solution into each well and incubated the plate at 37°C for four hours. Then centrifuged again, removed the supernatant and dissolved the purple crystal with 100 μl DMSO each well. Shook the plate for 10 min then measured the OD value at 595nm. The proliferation index was calculated with following equation: proliferation

= (OD value of protein-treated PBMC) ÷ (OD value of PBMC treated without HpHsp60)*100%.

Statistical analysis

The results are presented as mean ± SD. Significant is calculated by T test. The P value < 0.05 is considered as statistically significant.

Results

The binding activities of HpHSP60 to cells

At first, the different dosages of FITC-HpHSP60 were treated with THP-1 cells. As the additional amounts of FITC-HpHSP60 were increased, the fluorescent intensities of cell surface were increase. From 0.5 to 10 μg/ml, the Ka value for FITC-HpHSP60 and THP-1 cell was calculated as 469.1 (Fig. 1a). In contrast, the Ka value for FITC-HpHSP60 and THP-1 cell was 387.6 from 15 to 25 μg/ml (Fig. 1b). Similarly, FITC-HpHSP60s were treated AGS cells and the Ka values respective were 134.8 for low dosages (from 2 to 15 μg/ml, Fig. 2a) and 54.5 for high dosages (from 30 to 150 μg/ml, Fig. 2b). In addition, LPS-activated THP-1 cells were also treated with FITC-HpHSP60s (2 μg/ml or 20 μg/ml) to monitor the surface binding comparing to the inactive THP-1 cells. The results showed the stronger bindings to active THP-1 cells were significantly observed (Fig. 3c and d) than inactive THP-1 cells (Fig. 3a and b). The results seem to indicate that different cells may have different receptors with different affinity to HpHSP60.

The N-terminal sequence of HpHSP60 for cell binding activity

EGFP-HpHSP60 was constructed by fusing enhance green fluorescence protein (EGFP) to N-terminal of HpHSP60 (Fig. 4a) and such fusion did not abolish the fluorescent activity (Fig. 4b). HpHSP60 proteins were formed as dimmer (154 K.D.) or tetramer (306K.D.) (Fig. 4c) but N-terminal fusion interfered EGFP-HpHSP60 to form tetramer (Fig. 4c). In addition, EGFP-HpHSP60 fusion proteins were also valued their activities to induce proinflammatory cytokines. The results showed these fusion proteins still keep the capabilities to trigger the releases of IL-8 (Fig. 5). However, the N-terminal fusion did not only interfere with the cell binding activities to surface of inactive THP-1 cell but also decrease the surface binding to active THP-1 cells (Fig. 6). Taken together, these results reveal that N-terminal fusion to HpHSP60 did not influence the activity to induce the expression of proinflammatory cytokine but interfere with its binding activity to cell surface.

The functions of HpHSP60

To further investigate the function of N-terminal sequence of HpHSP60, certain truncated HpHSP60 were constructed. Figure 7 showed the structures of these transgenes. All transgenes were expressed by BL-21 *E. coli* system but only two recombinant proteins could be significant inducted by IPTG ($HpHSP60_{1-200}$ and $HpHSP60_{101-200}$), their molecular weights were about 25 K.D. and 17 K.D. (Fig. 8), respectively. In contrast, $HpHSP60_{1-500}$ (57 K.D.), $HpHSP60_{1-435}$ (50 K.D.) and $HpHSP60_{300-547}$ (17 K.D.) were not inducted significantly by IPTG (Fig. 8).

Sequentially, we examine whether these truncated recombinant proteins still remain their activities to induce the releases of proinflammatory cytokines. The results showed that $HpHSP60_{300-547}$ could still induce IL-8 expression as intact HpHSP60, but $HpHSP60_{1-200}$ and $HpHSP60_{101-200}$ lost this ability, which indicated that the domain to induce IL-8 release is not located near N-terminal sequence of HpHSP60.

According to our unpublished results that HpHSP60 could inhibit the proliferation of T cells in human PBMC. Therefore, whether N-terminal sequence of HpHSP60 would influence this activity was monitored. Figure10 indicated that $HpHSP60_{1-200}$ and $HpHSP60_{101-200}$ could inhibit T

cells' proliferation as HpHSP60. However, $HpHSP60_{300-547}$ could not inhibit T cells' proliferation at all. These results indicated the domain respond to suppression of T-cell proliferation is located near amino acid 101 to 200. In addition, the N-terminal domain of HpHSP60 could induce TGF-β secretion such as $HpHSP60_{1-200}$ and $HpHSP60_{101-200}$ but $HpHSP60_{300-547}$ lost this activity (Fig. 11).

Discussion

In this study, we proposed that the domain located near the N-terminal sequence of HpHSP60 involved in the inhibitory activity to anti-CD3 antibody-induced T-cell proliferation. This domain is not participated in the activities relative to induction of inflammatory cytokines.

As the additional HpHSP60s were increasing, HpHSP60s bound to cell surface by different association constants (Fig. 1 and 2). Thus, we proposed that there are two or more HpHSP60 receptors existed on the cell surface. Although HpHSP60 has been showed that it could bind to TLR2 or TLR4 to induce IL-8 release (Takenaka *et al.*, 2004). However, Dr. Takenaka *et al*. indicate that TLR2 play an important role in the secretion of HpHSP60-induced IL-8 via the signaling pathway involving NF-κB activation but TLR4 play the minor or no effect on this phenomenon. According their experiments, they proposed that receptors other than TLR2 and TLR4 may also recognize HpHSP60 (Takenaka *et al.*, 2004). In 2007, Zhao *et al*. also indicate that TLR2 but not TLR4 can trigger HpHSP60-induced IL-8 expression via the mitogen-activated protein (MAP) kinase pathway associated with activation of NF-κB

(Zhao *et al.*, 2007). Whereas they also find the IL-8 releases only were partially decreased using siRNA-TLR2. So they suggested there were other receptors for HpHSP60. Thus, HpHSP60 has at least two kinds of receptors on the THP-1 cell, a human monocytic cell is reasonable.

In 2005, Lin, *et al*. have identified that the activity of HpHSP60 to induce the IL-8 secretion is resulted from the region 300-435 a.a. (Lin *et al.*, 2005). Subsequently, the sequence of this peptide was compared with the HSP60 sequences of other species such as *C. pneumoniae*, *M. leprae*, human, *M. tuberculosis* or *E. coli* by Vector NTI Suite 9 software. Their similarities were about 65% and the positions were near 320-460 a.a.. In addition, these HSP60s all can induce proinflammatory cytokine releases (Lin *et al.*, 2009a). Except of inflammation, human HSP60 has been shown it has other activity for immunoregulatory function of T cell by induction of anti-inflammatory cytokine such as IL-10 (Wieten *et al.*, 2007). Vaccination with human $HSP60_{31\text{-}50}$ (KFGADARALMLQGVDLLADA) increases the secretions of IL-10 and TGF-β which are immunomodulatory cytokines capable of inhibiting experimental adjuvant arthritis (AA) (Francisco J. Quintana, 2003). IL-10 is an anti-inflammatory cytokine (Quinn *et al.*, 2000) and TGF-β can

enhance the production of regulatory T cell (Yoshimura *et al.*, 2010). In this study, figure 11 also showed that the sequence of HpHSP60 from 101 to 200 could induce the expression of TGF-β but the sequence from 300 to 547 did not. Thus, the information indicates that HSP60s have other functional sequences, except of the domain to result in inflammation. So, HSP60 may engage the receptor which is not responsive to inflammation by these immunoregulatory sequences. For example, CXCL12 can direct the production of Treg cells via CXCR4 (Karin, 2010) and it also controls these processes of cell adhesion, survival and tumor progression via CXCR7 (Mahabaleshwar *et al.*, 2008).

Furthermore, our results showed that the fluorescent intensity of active THP-1 cells bound with EGFP-HpHSP60s was lower than FITC-HpHSP60s (8.8 versus 563.8) (Fig. 3 and 6). The decrease of fluorescence was not resulted from the difference between the fluorescent degree of FITC and EGFP, because the fluorescence of FITC was only higher 3 fold than EGFP. It may be due to N-terminal fusion of EGFP interfere the cell binding activity of HpHSP60, which imply that there is at least one cell binding domain locating near the N-terminal sequence with cells. According to our results, this study showed HpHSP60 can

provide the different functions via different receptors. HpHSP60 and $HpHSP60_{300\text{-}547}$ could induce dramatically IL-8 releases but lose of the sequence from 300-435 a.a. ($HpHSP60_{1\text{-}200}$ and $HpHSP60_{101\text{-}200}$) would abolish this activity (Fig. 9). In contrast, lose of the sequence from 101 to 200 a.a ($HpHSP60_{300\text{-}547}$) would abolish the activity to suppress T cell proliferation (Fig. 10). Thus, we supported the N-terminal domain near 101-200 a. a. of HpHSP60 could inhibit the proliferation of T cell.

According the literature (Lin *et al.*, 2009a), HpHSP60s were formed as dimmers or tetramers but EGFP-HpHSP60s only were dimmers in native gel (Fig. 4). The information implied that N-terminal sequence of HpHSP60 involved in the polymerization of HpHSP60. Taken together, we proposed that the N-terminal sequence of HpHSP60 have multiple functional domain in which it can trigger TGF-β release to cause the proliferative suppression for T-cell and also involved in the formation of structure. This study first explored the immunoregulatory activity of HpHSP60 is came from the domain located the sequence from 101 to 200. This finding may be useful to further study for the chronic infection of *H. pylori*.

Figure and Legend

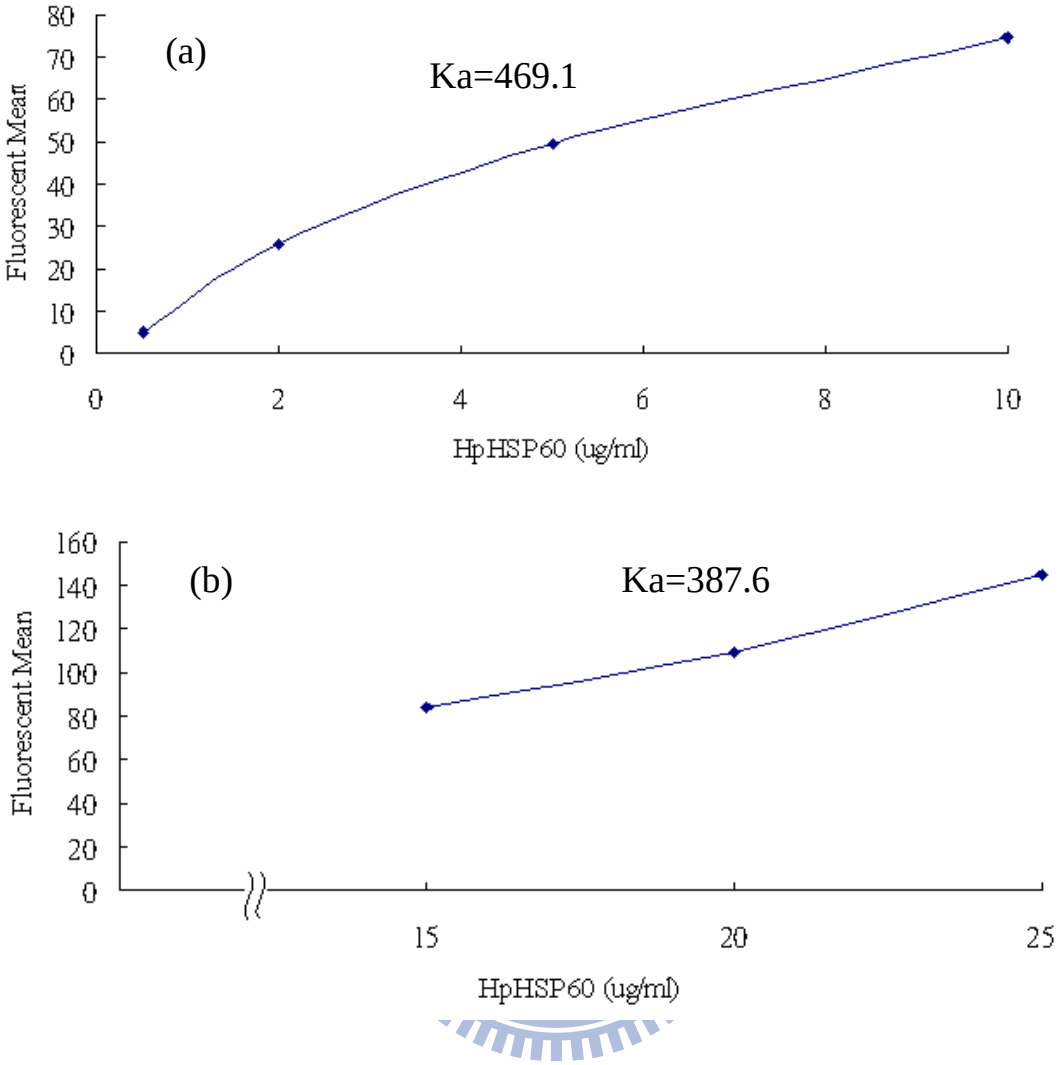


Figure 1 HpHSP60 could bind to THP-1 cells with different affinities. THP-1 cells ($2x10^5$) were treated with FITC-HpHSP60 (0.5 to 25 μg/ml) for 30 minutes. Fluorescence means of FITC-HpHSP60 bound on THP-1 cells detected by flow cytometry were plotted. (a) The binding affinity (Ka=469.1) of FITC-HpHSP60s (0.5 to 10 μg/ml) bound to THP-1 cells. (b) The binding affinity (Ka=387.6) of FITC-HpHSP60 (15 to 25 μg/ml) bound to THP-1 cells. Association constant (Ka) $= \frac{\Delta Fluorescence \cdot Mean}{\Delta Protein \cdot Concentration}$. The higher Ka value stands for the better binding affinity. N=1

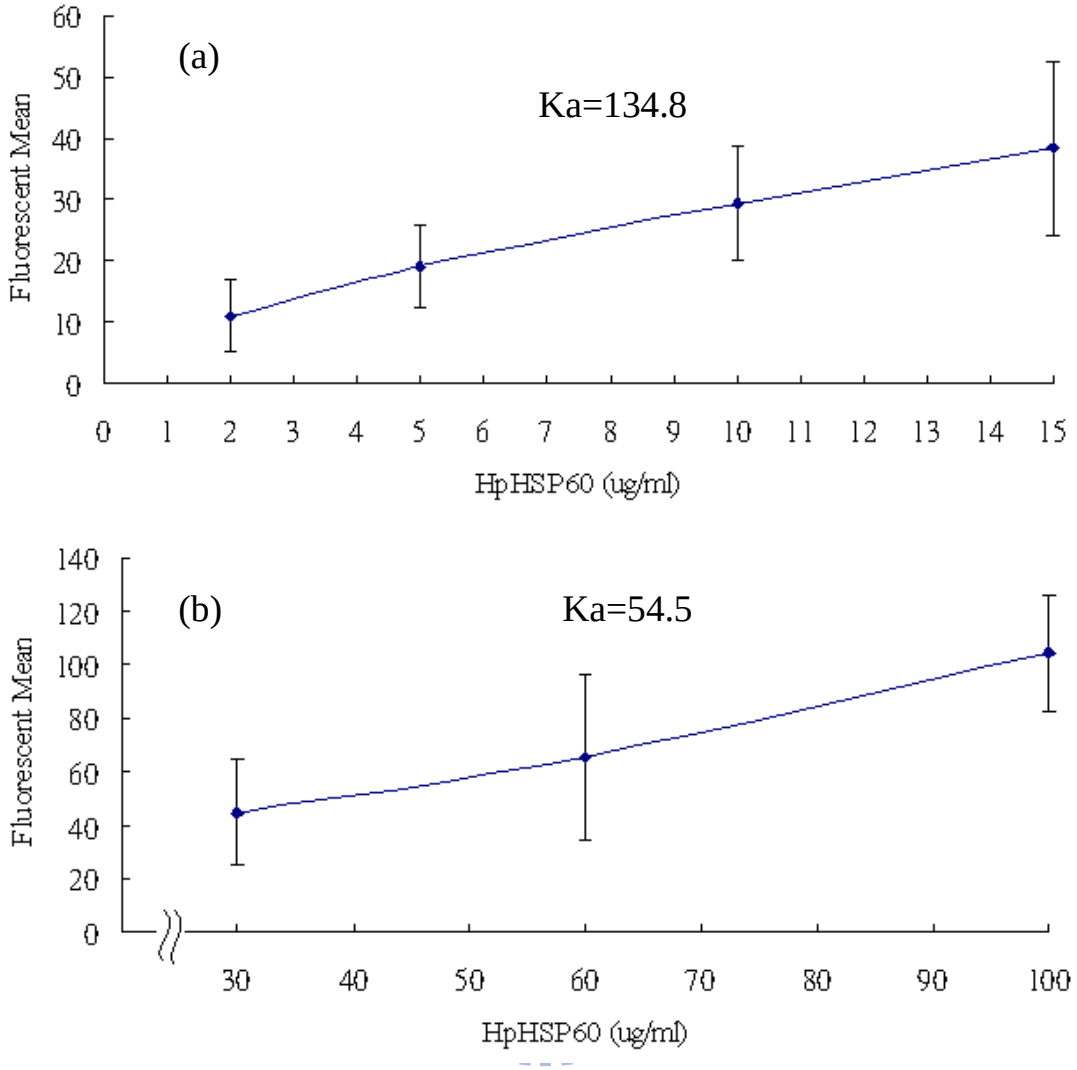


Figure 2 HpHSP60 could bind to AGS cells with different affinities. AGS cells ($2x10^5$) were treated with FITC-HpHSP60 (2 to 150 μg/ml) for 30 minutes in 250 μl. Fluorescence means of FITC-HpHSP60 bound to AGS cells detected by flow cytometry were plotted. (a) The binding affinity (Ka=134.8) of FITC-HpHSP60 (2 to 15 μg/ml) bound to AGS cells. (b) The binding affinity (Ka=54.5) of FITC-HpHSP60 (30 to 150 μg/ml) bound to AGS cells. Association constant (Ka) = $\frac{\Delta Fluorescence \cdot Mean}{\Delta \Pr otein \cdot Concentration}$

N=3

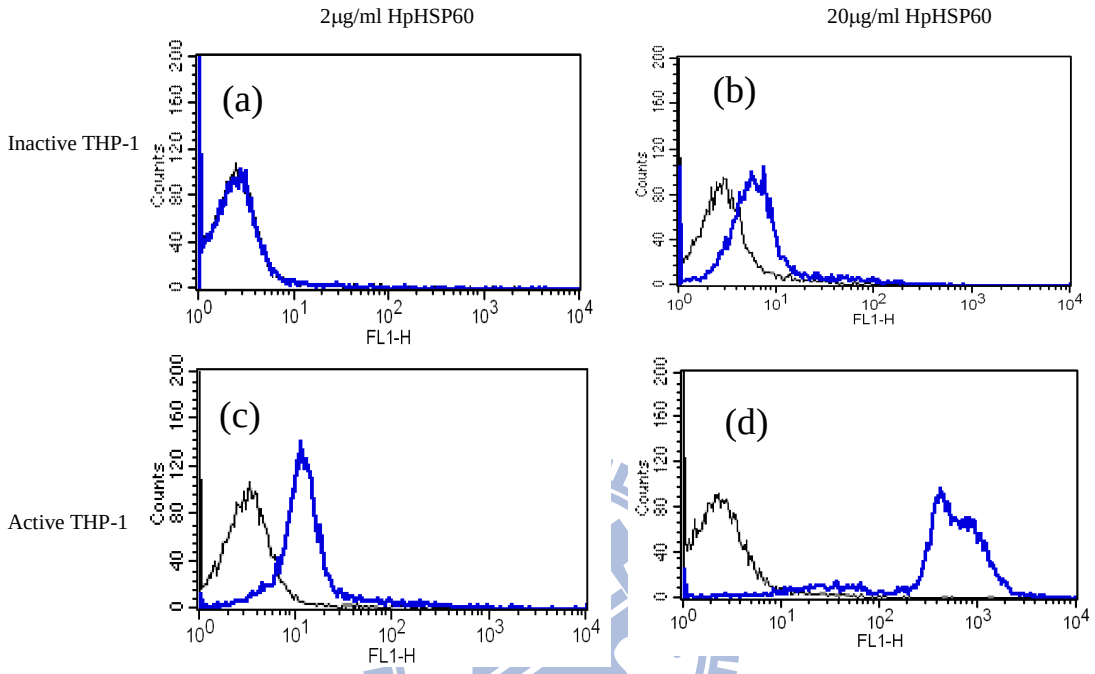


Figure 3 The binding intensities of HpHSP60 for LPS-activated THP-1 cells were dramatically increased than inactive THP-1 cells. (a) inactive THP-1 cells, (c) active THP-1 cells ($1x10^6$) were incubated with 2 mg/ml HpHSP60 in 500 ml. After 30 minutes co-incubation, the fluorescence mean of FITC-HpHSP60 was measured by flow cytometry. In the same way for 20 mg/ml HpHSP60s with (b) inactive THP-1 cells, (d) active THP-1 cells. The fluorescence mean was (a) 3.44, (b) 11.81 for FITC-HpHSP60 binding to inactive cells and (c) 16.59, (d) 563.75 for active cells.

— cell alone, — FITC-HpHSP60 with cells.

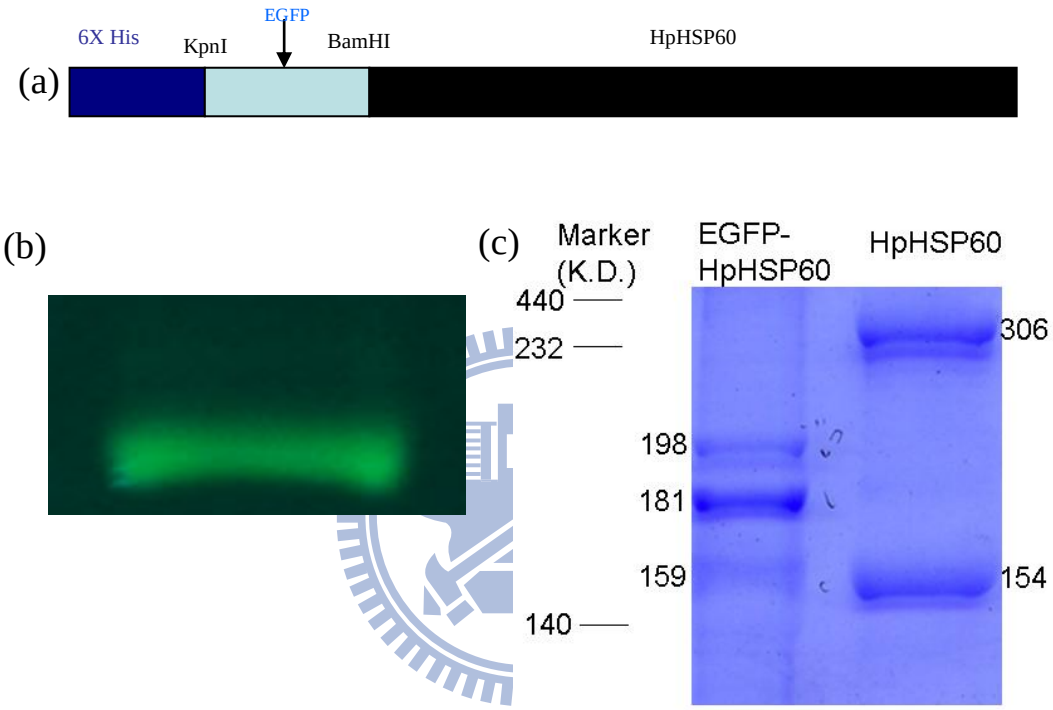


Figure 4 Electrophoresis profile of EGFP-HpHSP60. (a) Enhance green fluorescence protein (EGFP) was fused at KpnI and BamHI restriction sites on pET30-HpHSP60. (b) The fluorescence of EGFP-HpHSP60. (c) The protein structures were calculated by R_f value. Therefore, EGFP-HpHSP60 were dimmers and HpHSP60 were dimmers or tetramers.

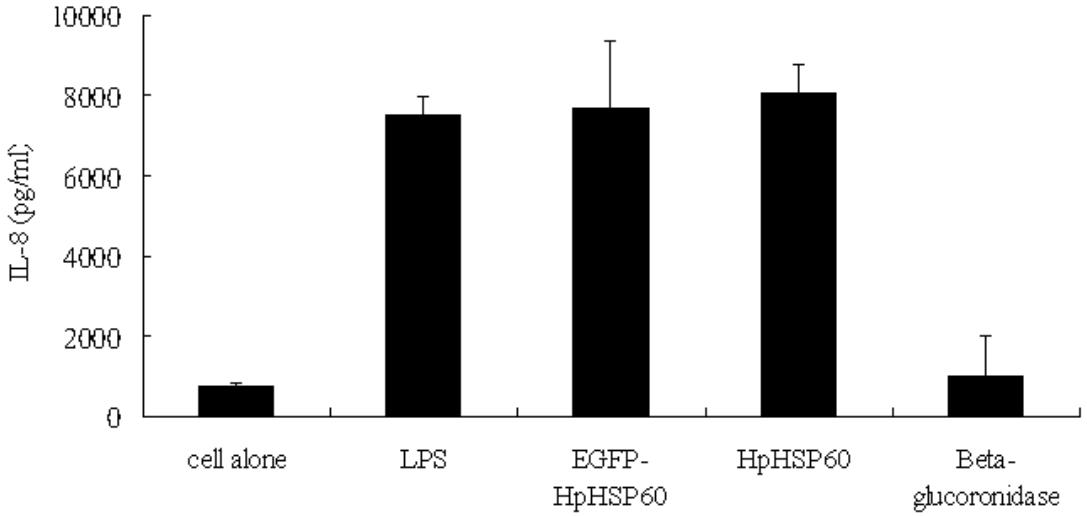


Figure 5 EGFP-HpHSP60 induced the secretion of pro-inflammatory cytokine IL-8. THP-1 cells were treated with 10μg EGFP-HpHSP60 or HpHSP60 for 24 hours in 5% CO_2 at 37℃ incubator. Positive control: THP-1 cells were treated with 1 μg/ml LPS. Negative control: THP-1 cells were treated with 10 μg β-glucuronidase. After 24 hours, the supernatants were collected and measured for IL-8 expression. EGFP-HpHSP60 triggered THP-1 cells to secrete IL-8 significantly (~8000 pg/ml) as well as HpHSP60 and LPS. N=3

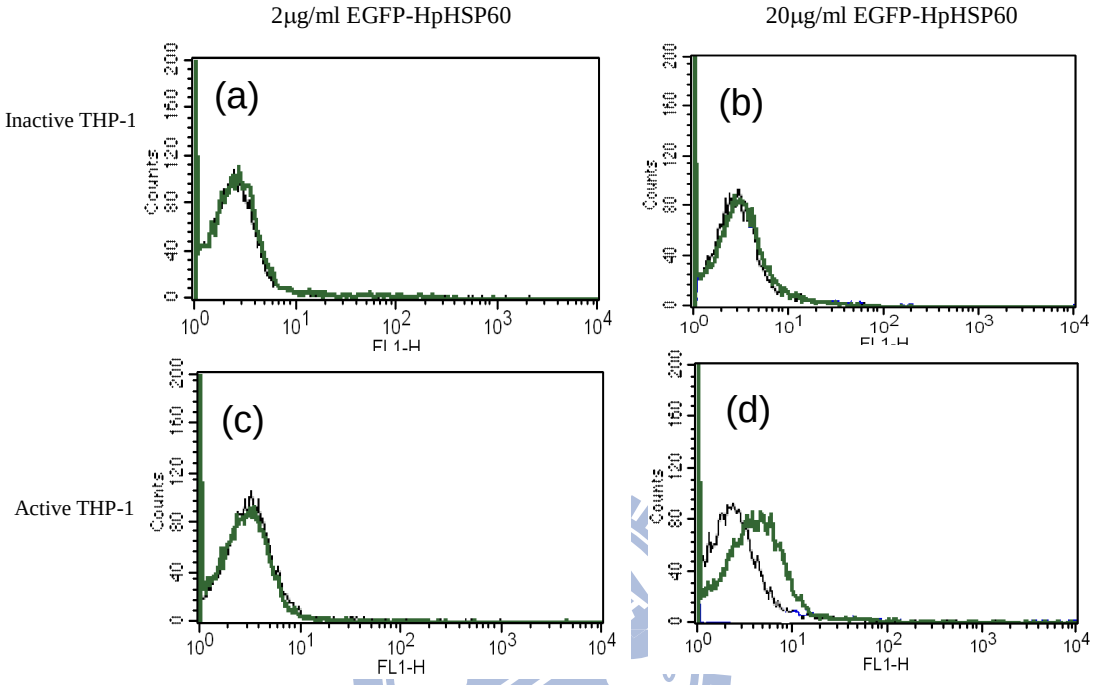


Figure 6 EGFP interrupted LPS-activated THP-1 cells to bind to HpHSP60. (a) inactive THP-1 cells, (c) active THP-1 cells ($1x10^6$) were incubated with 2 mg/ml EGFP-HpHSP60 in 500 μl. After 30 minutes co-incubation, the fluorescence mean of EGFP-HpHSP60 was measured by flow cytometry. In the same way, 20 mg/ml EGFP-HpHSP60s treated with (b) inactive THP-1 cells or (d) active THP-1 cells. The fluorescence mean was (a) 3.44、(b) 3.64 for EGFP-HpHSP60 binding to inactive cells and (c) 3.46、(d) 8.77 for active cells.

—cell alone, — EGFP-HpHSP60 with cells.

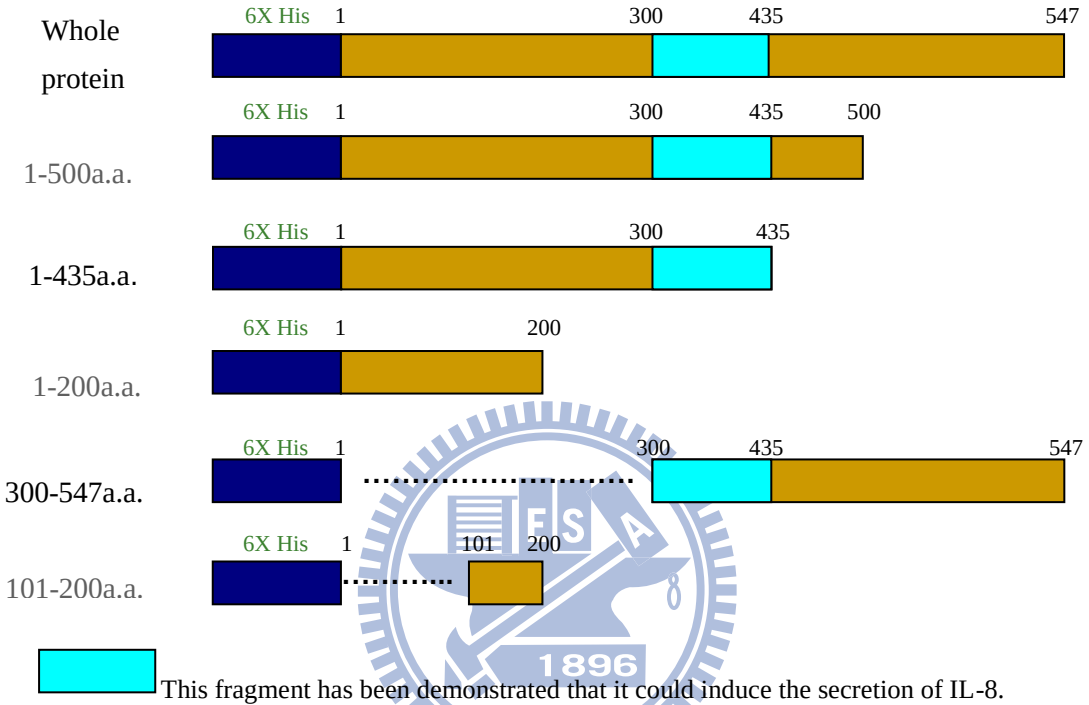


Figure 7 The different domains of HpHSP60. This figure simply showed that the different sequence of HpHSP60.

Position (a.a.)	M.W.	No induction	Induction
1-500	~57K.D.		
1-435	~ 50K.D.		
1-200	~ 25K.D.		
300-547	~ 30K.D.		
101-200	~ 17K.D.		

Figure 8 The protein expressions of the different fragments. Different fragments were constructed by Directional TOPO Expression Kit (invitrogen). Proteins were inducted by IPTG (1mM) in BL21 *E.coli* system. The induction of $HpHSP60_{1\text{-}200}$ and $HpHSP60_{101\text{-}200}$ were observed.

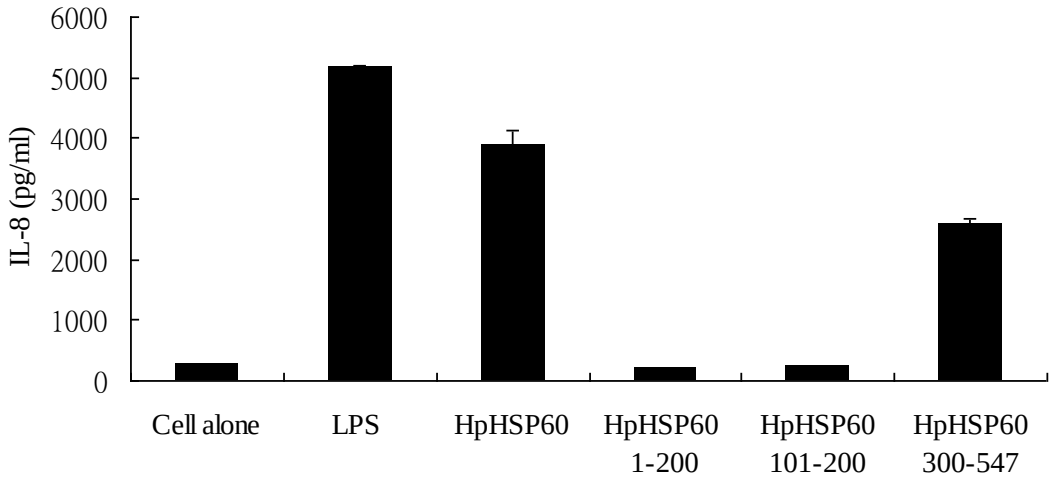


Figure 9 The IL-8 secretion of THP-1 cells after treating with HpHSP60 or different fragment proteins. THP-1 cells ($2x10^5$) were treated with 10 μg different proteins (HpHSP60, $HpHSP60_{1-200}$, $HpHSP60_{101-200}$ or $HpHSP60_{300-547}$) for 24 hours in 5% CO_2 at 37℃ incubator. Positive control: 1 μg/ml LPS were treated to THP-1 cells. After 24 hours, the supernatants were collected and measured IL-8 expression. Truncated $HpHSP60_{1-200}$, $HpHSP60_{101-200}$ could not induce dramatically IL-8 secretion. N=3

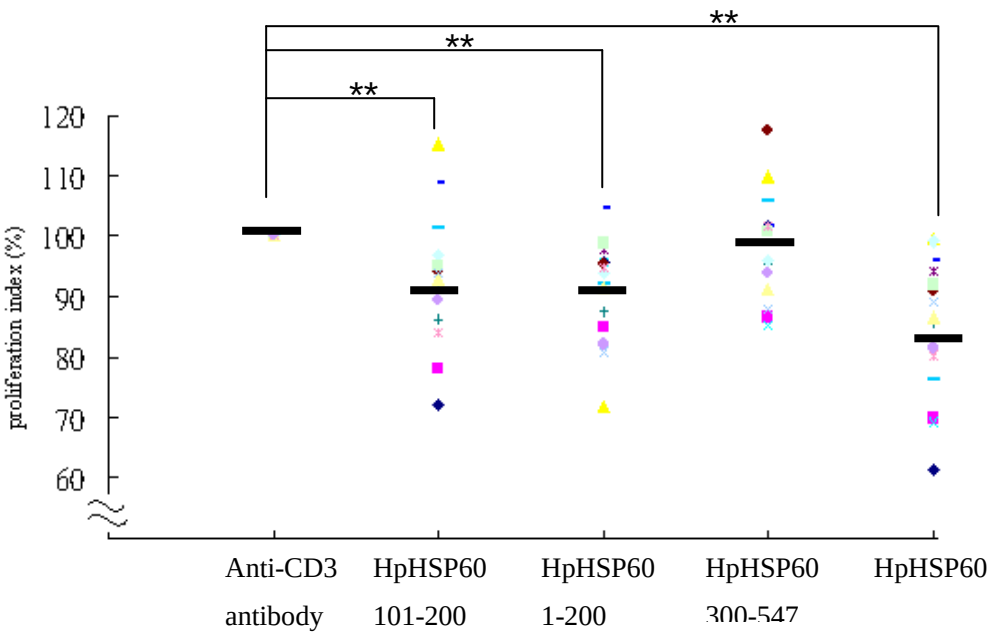


Figure 10 Treatment with HpHSP60 and different fragment proteins to PBMCs decreases the proliferation in the present of anti-CD3 antibody. PBMCs were isolated from fifteen healthy donors and seeded in 96 well, which was coated with anti-CD3 antibody (1μg/ml). Different proteins were co-cultured with PBMCs for four days. After four days incubation, MTT assay was used to detect the proliferation of PBMCs. PBMCs treated with anti-CD3 antibody was identified as 100%. HpHSP60, $HpHSP60_{101-200}$ and $HpHSP60_{1-200}$ could inhibit PBMCs proliferation significantly. ** $P< 0.01$. N=15

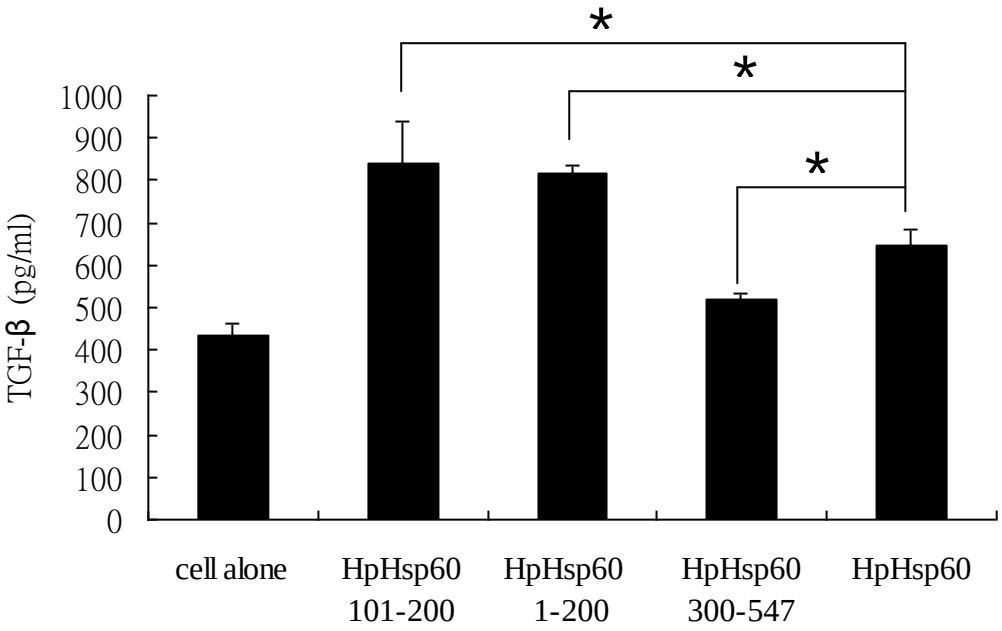
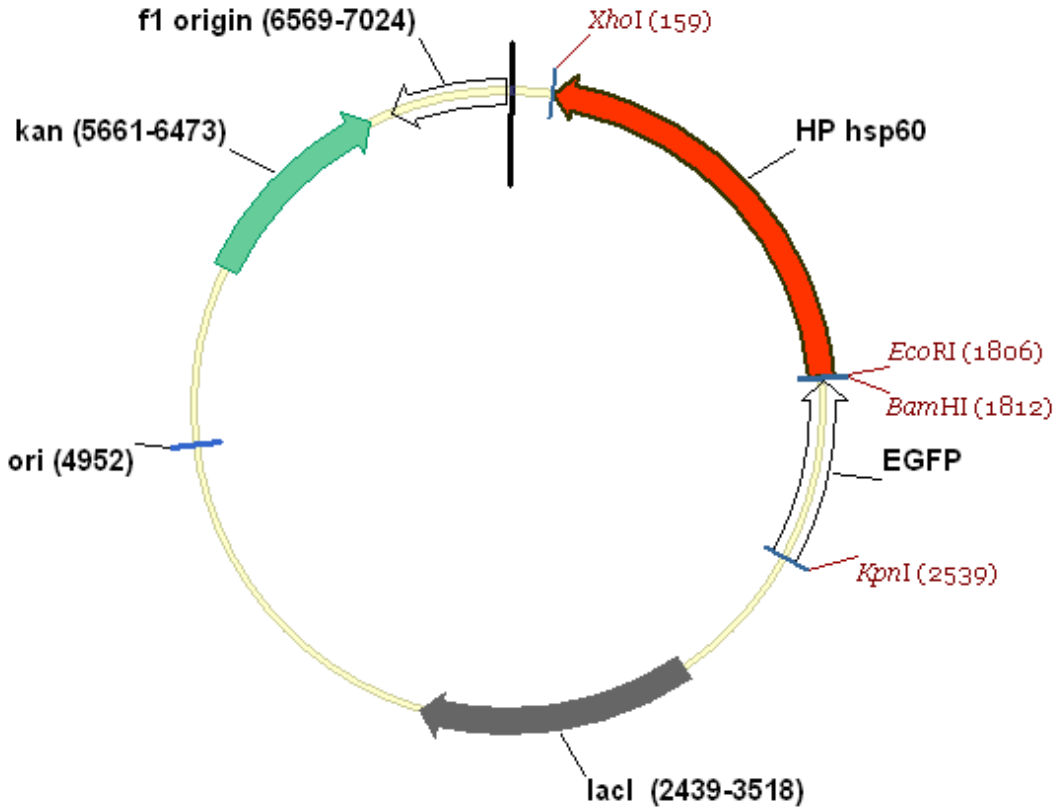


Figure 11 The TGF-β secretion of THP-1 cells after treating with HpHSP60 or different fragment proteins. THP-1 cells ($1x10^5$) were treated with 10μg different proteins (HpHSP60, $HpHSP60_{1-200}$, $HpHSP60_{101-200}$ or $HpHSP60_{300-547}$) for 24 hours in 5% CO_2 at 37℃ incubator. Negative control: only cells. After 24 hours, the supernatants were collected and measured TGF-β expression. $HpHSP60_{1-200}$, $HpHSP60_{101-200}$, HpHSP60 could significantly induce TGF-β secretion. *P<0.05, **P<0.01. N=1

Appendix

1. pET30-EGFP-HpHSP60

1.1 Map



1.2 pET30-EGFP-HpHSP60 DNA sequence

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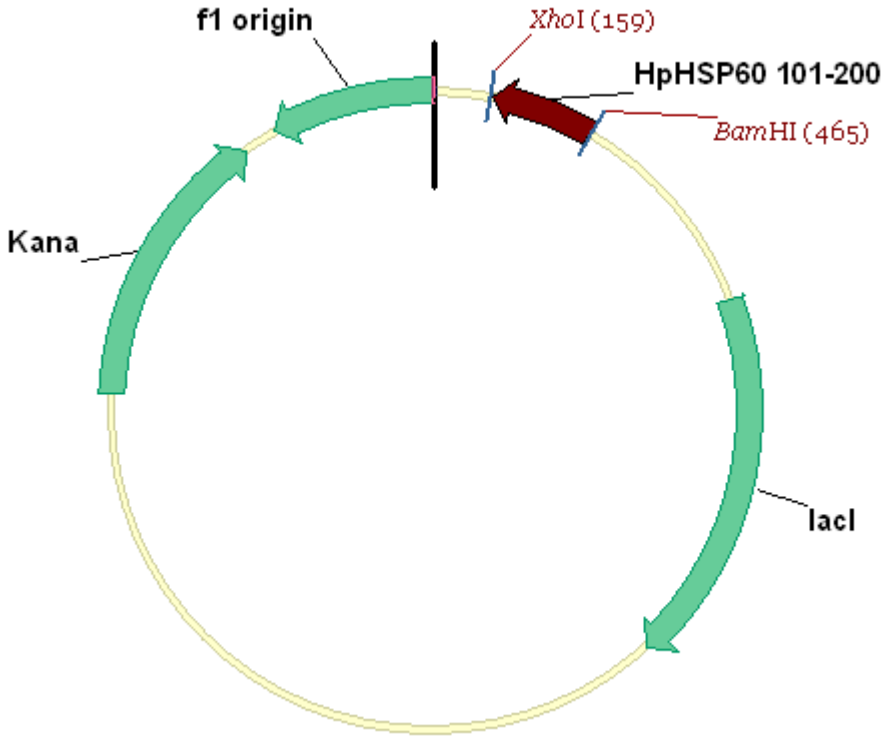
2. pET30-HpHSP60 $_{101\text{-}200}$

2.1 Map



2.2 pET30-HpHSP60 $_{101\text{-}200}$ DNA sequence

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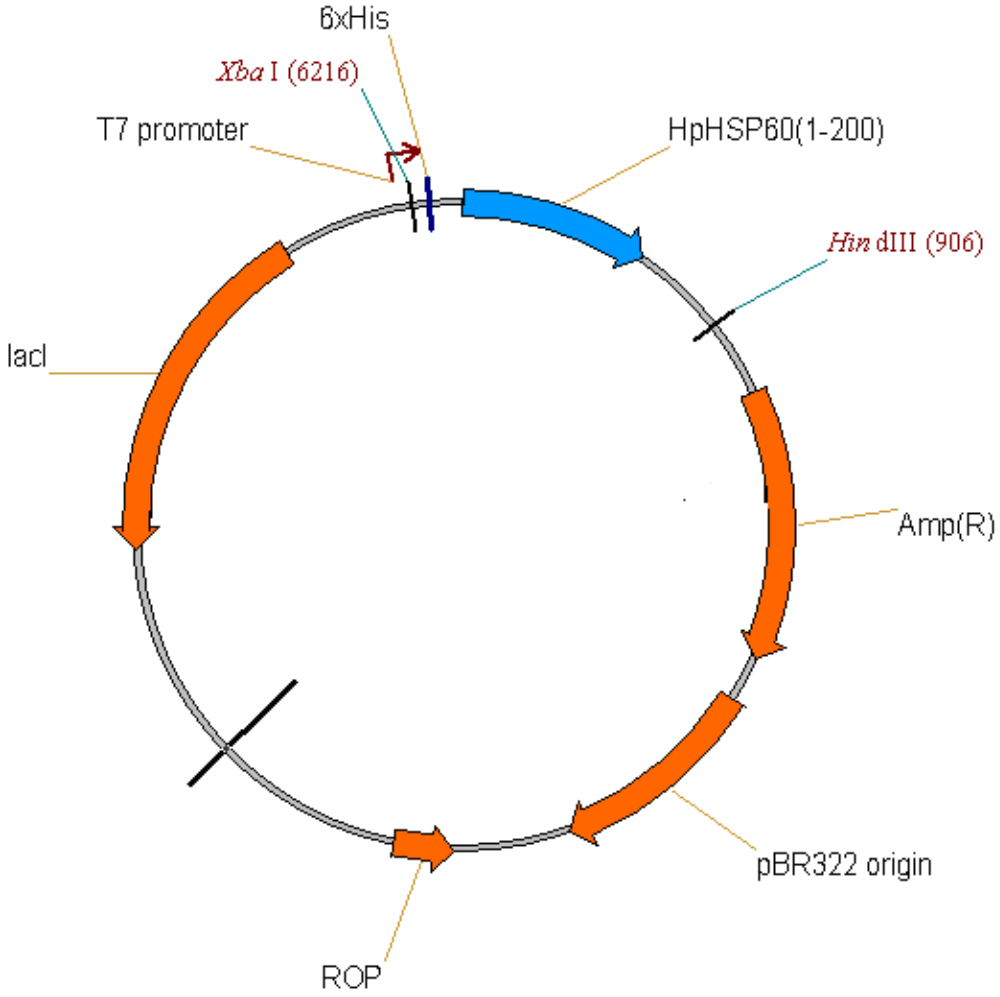
3. pET100-HpHSP60 $_{1\text{-}200}$

3.1 Map



3.2 pET100-HpHSP60 $_{1\text{-}200}$ DNA sequence

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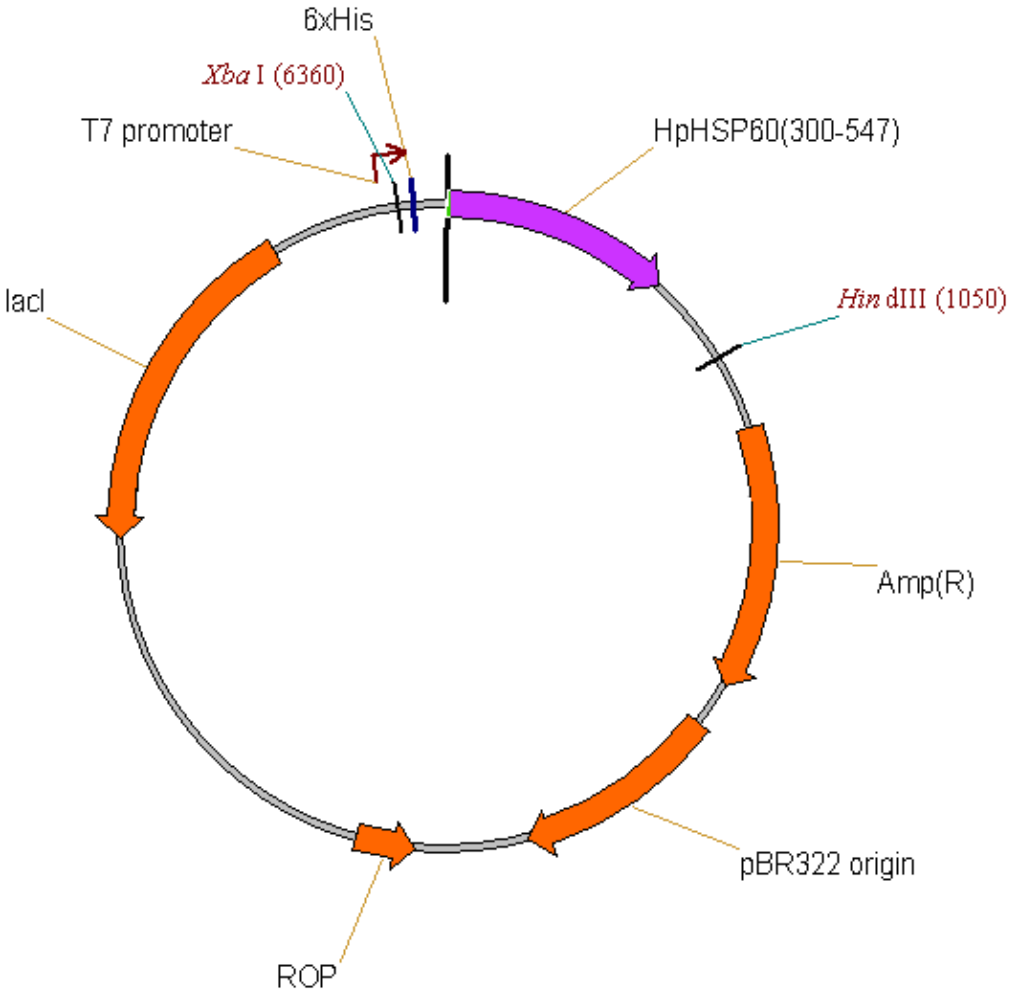
4. pET100-HpHSP60 $_{300-547}$

4.1 Map



4.2 pET100-HpHSP60 $_{300-547}$ DNA sequence

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6421 tcatcatcat ggtatggcta gcatgactgg tggacagcaa atgggtcggg atctgtacga

6481 cgatgacgat aaggatcatc ccttcacc

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