國立交通大學

生物科技學系

博士論文

幽門螺旋桿菌熱緊迫蛋白 60 活化 β1 轉化生長因子訊息傳遞之機制

Helicobacter pylori Heat Shock Protein 60 Trigger SMAD Signal Pathway by Interacting with Transforming Growth Factor-β receptor II

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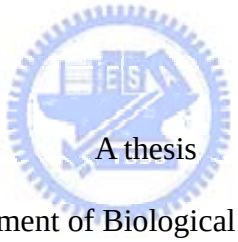

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摘　　要

幽門螺旋桿菌為普遍存在人體的致病菌。根據統計，全球約有一半左右的人口感染此菌。該菌會在胃部造成持續性的慢性感染，進而增加十二指腸潰瘍、胃癌或淋巴腫瘤之罹患率。在病原慢性感染的機制中，抑制宿主的免疫反應扮演了極為重要的角色；而在幽門螺旋桿菌的免疫抑制效應方面，目前雖然有若干的研究進行探討，然而其機制仍尚未完全被釐清。幽門螺旋桿菌熱緊迫蛋白 60 為該菌的黏附因子之一；而由於其能引發許多炎症細胞激素的產生，故該蛋白也常作為幽門螺旋桿菌疫苗研發的主要抗原。然而，近來相關的研究顯示，熱緊迫蛋白 60 具有調控免疫反應的功能。本研究的目的在探討幽門螺旋桿菌熱緊迫蛋白 60 的結構及其在免疫反應中所扮演的角色。首先，由氨基酸序列比對、圓二色光譜、以及蛋白質膠體電泳的結果顯示：該蛋白質可形成雙體或是四聚體，而這與目前已知的大腸桿菌熱緊迫蛋白 60 的結構極為不同。而在免疫功能的探討方面，我們發現幽門螺旋桿菌熱緊迫蛋白 60 和人類β1 轉化生長因子的蛋白質結構有部分的相似性，其中有一段相似區已知為β1 轉化生長因子與其第二型受器之結合位。後續的結果顯示：在 ELISA 與細胞的實驗中均可證實熱緊迫蛋白 60 可與β1 轉化生長因子受器結合。此種結合會啟動β1 轉化生長因子所調控的 SMAD 訊息傳遞路徑，開啟下游基因表現，並抑制周邊血球細胞與單核球細胞株 THP-1 的免疫活性。綜合以上的實驗數據，我們認為幽門螺旋桿菌熱緊迫蛋白 60 會藉由模仿β1 轉化生長因子的生物活性來抑制宿主的免疫系統，以利於該菌的持續性感染。

Helicobacter pylori Heat Shock Protein 60 Trigger SMAD Signal Pathway by Interacting with Transforming Growth Factor-β receptor II

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Abstract

Helicobacter pylori have explored multiple mechanisms to evade host immune surveillance for chronic infection. But either of them is restricted by certain bacterial strains containing potential virulent factors, or those immune-restrained functions only limit to some specific immunocytes. However, the long-term persistence of *H. pylori* suggests a more comprehensive and powerful factor(s) hinds behind to regulate host immune system. *Helicobacter pylori* heat shock protein 60 (HpHsp60) was previous identified as an adhesion molecule or a potent immunogen. This study aims to study the structure of HpHsp60s and evaluates their functions on host immune responses. Analyzing the structure of HpHsp60 via amino acid blast, circular dichroism and electrophoresis indicated most recombinant HpHsp60s form dimers or tetramers that are quite different than *E. coli* Hsp60 protein structure. Moreover, a novel property of HpHsp60 was found, which is, by mimicking TGF-β1, HpHsp60 could exert immune regulatory effects. With structural homology to the receptor binding site of TGF-β1, HpHsp60 could interact with TGF-β receptor II, trigger SMAD pathway, and inhibiting the immune functions of THP-1 monocytic cells and peripheral mononuclear cells (PBMCs). Our study provides a new hint that *H. pylori* may employ Hsp60 to surrender host immunity.

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大家都說，好不容易畢業了，終於海闊天空了呀！但我卻覺得背上的重擔開始真實了起來。不能再拿「我是學生」這個藉口胡作非為了呀！心裡默默的想著。一切，很真實，也很不真實。「一起為了光明的未來而努力」這樣的話果然我是說不出口的，想說的是，因為未來會遇到的一切都是未知，所以，認真的戰鬥吧，「不要輸給灰暗時代而來呼吸吧」。

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Overview

Heat shock proteins (HSP) were initially found through a joyful accident involving the overheating of a *Drosophia* salivary gland preparation on a microscope stage. This was first reported as: “A new puffing pattern induced by temperature shock and DNP in Drosophila” in 1962 (F., 1962). HSPs carry out pivotal housekeeping functions and are molecular chaperones that are important for the survival pf prokaryotic and eukaryotic cells. On the basis of their molecular size they have been divided into several families: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps (Jolly and Morimoto, 2000; Lindquist and Craig, 1988). Heat shock proteins are strongly conserved and show high homology among all organisms, and some mammalian family members have highly conserved microbial homologous, which results in immunological cross-recognition between mammalian and microbial homologous. HSPs play roles in various aspects. Although they were investigated as molecular chaperones at first, recent studies have revealed their involving in regulation of cell metabolism or decision of cell fate. It has been found that glucocorticoid receptor fails to mature to a transcriptionally active form in the absence of Hsp90 (Picard, et al., 1990). Hsp27 and Hsp70 are also found they can both inhibit caspase dependent apoptosis pathways. Hsp70 inhibits a caspase-independent form of death by interacting with lysosomal membrane and preventing the activity of hydrolytic lysosomal enzymes (Daugaard, et al., 2007).

Immune responses toward certain HSPs develop in almost all inflammatory diseases.

The first evidence of a role for HSPs as antigens in inflammatory responses was obtained in the 1980s in the rat model of heat-killed mycobacterial-induced adjuvant arthritis (van Eden, et al., 1988). It was found that T cells isolated from rats with disease were responding to mycobacterial Hsp60. Since then, immune reactivity to HSPs has been recognized in human or other experimental models of inflammatory diseases, Recently, several studies have shown that HSP-reactive T cells have an immunoregulatory phenotypes, indicating that HSPs --- and in particular, Hsp60 and Hsp70 --- constitute a group of autoantigens with the potential to trigger immunoregulatory pathways, which can suppress immune responses that occur in various inflammatory diseases. These results display one thing for certainty that HSPs have double-edged-sword effects on host immune systems, and both microbial and mammalian HSPs may possess these immune-regulation abilities. The effects of Hsp60 and other HSPs on host immunity will be reviewed in the followings.

1. The immunodominant activity of microbial Hsp60

Host immune responses to Hsp60 from bacteria, protozoa, fungi and helminthes have frequently been detected after infection with these organisms (Zugel and Kaufmann, 1999). During bacterial infections, the bacterial members of the Hsp60 and Hsp70 families are also common targets of humoral and cell-mediated immune responses in mammals (Kaufmann, 1990; Lydyard and van Eden, 1990). In fact, Hsp60 has been known as the common antigen of gram-negative bacteria before it was identified as a member of the Hsp60 family (Shinnick, et al., 1988; Thole, et al., 1988). Analysis of T-cell responses in mice immunized with *Mycobacterium tuberculosis* showed that ~10-20% of the *M. tuberculosis*-specific T cells responded to *M. tuberculosis* Hsp60 (Kaufmann, et al., 1987). Antibodies specific for Hsp60 of *Chlamydia trachomatis* have also been detected at high titers in the sera of patients infected with *C. trachomatis*. With prompting a high-titer antibody response, *C. trachomatis* Hsp60 has been implicated in devoting to the pathogenesis of *C. trachomatis*-mediated inflammation and conjunctival scarring (Dean, et al., 2008). Bulut and colleagues has identified the *C. trachomatis* Hsp60 can activate macrophages and endothelial cells through toll-like receptor 4 and MD2 to turn on NF-κB activation and promote inflammation (Bulut, et al., 2002). Antibodies and T cells specific for mycobacterial Hsp60 have also been detected in healthy humans not known to have been exposed to mycobacterial infections (Munk, et al., 1989). It revels

that Hsp60 can be an immunodominant antigen to evoke host immunity.

The occurrence of immunodominant immune responses to Hsp60 is likely to result from several factors that are related to their function. First, because they are essential in cell survival, HSP sequences are highly conserved between different bacterial species. So, each time the immune system encounters bacteria, its initial response might be targeted to the strongly conserved regions of the bacterial HSP that are recognized by memory cells arising from previous infections with unrelated bacteria. Second, during infection, the invading microorganism experiences stress (for example, a rise in temperature and exposure to reactive-oxygen species produced in host defense), and it upregulates expression of its own HSPs, making them easily available targets for the host immune cells. Third, HSP-specific responses might be immunodominant because, as molecular chaperones, HSPs associate with other, often unfolded, newly synthesized antigenic microbial proteins or with microbial proteins that are denatured or partially unfolded as a result of stress. As a consequence, B cells with cell-surface B-cell receptors specific for a (partially unfolded) microbial antigen can internalize the associated microbial HSP together with the microbial antigen, process them both, and present peptides derived from the two proteins in the context of MHC class II molecules for recognition by T helper (Th) cells.

2. Immune responses to mammalian HSPs

In spite of the existence of immune tolerance to self antigens, host-HSP-specific T and B cells have been found in association with chronic inflammation, as well as in healthy individuals (Cohen, 1991). Because HSPs are intracellular proteins, in the presence of cellular stress, self-HSPs are efficiently presented by major histocompatibility complex (MHC) class I molecules to cytotoxic T lymphocytes (CTLs). An early study showed that CTLs raised against mycobacterial Hsp60 also recognized macrophages that were subjected to various forms of cellular stress, in the absence of exogenously added antigen, indicating that endogenous self-Hsp60 is presented in the context of MHC class I molecules to the CTLs (Koga, et al., 1989). The CTL-mediated lysis of macrophages that were prompted by treatment with IFN-γ was inhibited when Hsp60 expression was suppressed Hsp60-mediated antisense oligonucleotides (Zugel, et al., 1995), thereby confirming the specificity of the CTLs for Hsp60.

HSP-derived peptides can also be presented by MHC class II molecule. In vitro evidence has revealed that stressed antigen-presenting cells (APCs) were recognized by CD4+ T cells that were generated against a highly conserved mycobacterial Hsp60 sequence (Anderton, et al., 1995). In addition, after transferring these T cells to model animals, it was shown to protect against adjuvant-induced arthritis and to produce IFN-γ, interleukin-4 (IL-4) and IL-10. Therefore, recognition of HSPs expressed by stressed cells

might induce a disease-suppressive regulatory phenotype in responding T cells. These findings indicate the existence of CD4+ T cells with the capacity to recognize self-HSP epitopes and may manipulate immune responses associated with disease progression under certain conditions.

3. Immunoregulatory roles of Hsp60

The studies on previous section have implicated the potential role for Hsp60 as an immunoregulator. Because the T cells which can specifically recognize mycobacterial Hsp60 release IL-4 and IL-10 in animal model, which are so-called immune-suppressive cytokines, to protect hosts against inflammatory damages. In fact, the first findings that immune responses to HSPs have a regulatory role in inflammatory diseases were shown for mycobacteria-induced adjuvant arthritis (van Eden, et al., 1988). The animals pre-immunized with mycobacterial Hsp60 administered in incomplete Freund's adjuvant (IFA) inhibited disease. Afterward many succeeding findings also showed the protective effects of microbial HSPs in various experimental disease models. So it is possible that immune responses to HSPs could be involved in the control of human chronic inflammatory diseases that have distinct, although as-yet-unknown, initiating autoantigens.

Hsp60 has been investigated and identified as immune targets in rheumatoid arthritis

(RA), type 1 diabetes, systemic lupus erythematosus, atherosclerosis, dermatomyositis, inflammatory muscle disorders, organ transplantation and cardiac surgery. Although there is no clear and complete data on human diseases, a picture is emerging in which expression of HSPs or immune reactivity to HSPs seems to be related to downregulation of inflammation, rather than with induction or propagation of inflammation. Recent findings have revealed that arthritic synovial-tissue samples from animals with experimentally induced arthritis and patients with RA or Juvenile idiopathic arthritis (JIA) expressed increased levels of HSP60 (Boog, et al., 1992; de Graeff-Meeder, et al., 1990). In an initial study, T cells from the synovial fluid and blood of patients with JIA or RA proliferated in response to mycobacterial HSP60 (De Graeff-Meeder, et al., 1991). However, in contrast to patients with RA or healthy control individuals, cells from patients with JIA had substantial proliferative responses to self-HSP60. Further studies showed that CD4+ T-cell responses to human HSP60 at the onset of JIA correlated with responses to mycobacterial HSP60 and with a favourable prognosis (Prakken, et al., 1996). So, these findings indicated that microbial-HSP-crossreactive responses of T cells to endogenous human Hsp60 might have a regulatory role in the course of self-remitting JIA.

Similar results are found in type 1 diabetes and atherosclerosis. Immunization with Hsp60 has been shown to protect against type 1 diabetes in non-obese (NOD) mouse model (Elias, et al., 1990). Cohen and colleagues showed that the Hsp60-derived peptide

p277 could bind human MHC class II molecules and induce proliferative T-cell responses in patients with type 1 diabetes (Abulafia-Lapid, et al., 1999; Cohen, 2002). In addition, while the rabbits and mice receiving immunization of mycobacterial Hsp60 increased disease in the presence of atherosclerosis-promoting genetic and dietary conditions (Wick, et al., 2004), more recent studies have shown that mycobacterial Hsp60, when administered orally or intranasally, has the potential to suppress induced atherosclerosis (Harats, et al., 2002; Maron, et al., 2002).

4. Potential mechanisms for microbial or endogenous Hsp60s of immune-regulation

The analysis of immune responses to Hsp60 in experimental models and in patients has indicated the capacity of HSPs to induce regulatory T-cell responses. It is possible for HSPs with unique characteristics that endow them with this regulation-inducing capacity. Such characteristics might be related to the interactions of HSPs with receptors of the innate immune system, such as TLRs, or to their stress inducibility or their molecular conservation, which results in immune crossreactivity between bacterial and mammalian homologoues.

4.1 Hsp60 might mediate immune-regulation through engagement of specific receptors

Mammalian and bacterial HSPs have been described to directly activate antigen presenting cells (APCs) probably through binding to cell-surface receptors such as CD14,

CD40, TLRs and scavenger receptors CD36, CD91 and lectin-type oxidized LDL receptor 1 (LOX1) (Binder, et al., 2004; Delneste, 2004). Such immune activation might contribute to the breaking of tolerance to autoantigens, such as Hsp70, leading to the induction of autoimmune disease (Millar, et al., 2003). In the previous studies, both human and microbial Hsp60 have been found to activate pro-inflammatory responses through TLR-2/4 (Takenaka, et al., 2004; Zhao, et al., 2007). However, recent researches have shown that mammalian Hsp60 has a direct innate effect on T cells through binding to TLR2 (Zanin-Zhorov, et al., 2003) and results in activation of T-cell adhesion and inhibition of chemokine-receptor expression. In addition, Hsp60 has recently been shown to modulate the expression of transcription factors involved in T_H1-versus T_H2-cell differentiation: Hsp60 downregulates expression of the T_H1-cell promoting transcription factor T-bet, as well as NF-κB and the intracellular-signaling molecule NFATp, whereas it upregulates the expression of T_H2 cell-promoting transcription factor GATA3. this results in decreased secretion of the pro-inflammatory cytokines TNF and IFN-γ and increased secretion of the regulatory cytokine IL-10 by Hsp60-activated T cells (Zanin-Zhorov, et al., 2005).

4.2 Central or peripheral selection of self-HSP-reactive T cells and lacking of co-stimulatory molecules generate HSP-specific regulatory T cells

The existence of self-HSP reactive T cells has been demonstrated in many different

studies, even in human umbilical cord lymphocytes (Fischer, et al., 1992). In the peripheral immune system, when the cells have left the central lymphoid organs, microbial HSP can be the full agonists for these T cells compatible with immuno-dominant character of these HSP. At the same time, when self-HSP is expressed in the periphery, under conditions of cell stress, the self HSP can act as a partial agonist producing a regulated or actively regulatory response in these T cells.

In addition, at the site of inflammation HSP will be up-regulated in all (stressed) cells of which the majority will be tissue cells lacking co-stimulatory molecules. In the absence of co-stimulatory molecules T cells will adopt a state of anergy or regulation (Taams, et al., 1998). Through these mechanisms HSP-specific T cells can adopt a regulatory phenotype upon antigen recognition in the periphery of the immune system. It is also possible that this tendency of these cells to stay in a tolerant or regulatory state is further promoted by mucosal tolerance in the gut associated lymphoid system (GALT) for abundantly present microbial HSP from the gut microbiota will be dominated by tolerance fro the conversed sequences, as these are shared among the variety of bacterial species present.

5. Immune responses toward other HSPs

In addition to Hsp60, other families of HSPs also display contradictory effects on

immune responses. Hsp70 share almost the same characteristics as Hsp60 on the host immunity. Both mammalian and microbial Hsp70 were found to activate inflammatory responses (Cohen, 1991) while they also suppress NF-κB activation to establish an anti-inflammatory microenvironment at the same time (Bender, et al., 2007; Zheng, et al., 2008). Human Hsp70 levels were in positive correlation with monocyte TLR4, IL-6, and TNF-α expression, which are index for inflammation (Satoh, et al., 2006). Moreover, Campisi and colleagues have revealed that *E. coli* Hsp72 can stimulate rat splenocytes and macrophages to elevate nitric oxide (NO), TNF-α, IL-1β, and IL-6 to facilitate host recovery from bacterial inflammation (Campisi, et al., 2003). In contrast to Hsp70-mediated immune activation, they also display immune-regulatory effects to protect rats from aggravation of stroke or patients from obesity-induced insulin resistance (Chung, et al., 2008). As for Hsp90, there is a report indicating the inhibition of Hsp90 can attenuate inflammation in endotoxin-induced uveitis (Poulaki, et al., 2007).

6. TGF-β plays an important role in immune-regulatory responses

TGF-β is a regulatory cytokine with pleiotropic effects on cell proliferation, differentiation, migration, and survival that affect multiple biological processes, including development, carcinogenesis, fibrosis, wound healing, and immune responses (Blobe, et al., 2000). As for immune modulation, TGF-β has also performed multifaceted functions

on a variety of immune cells. TGF-β exerts the greatest impact on T lymphocytes. T cell proliferation, differentiation, and survival are all affected by TGF-β (Cerwenka and Swain, 1999; Gorelik and Flavell, 2002), and it also induces the FoxP3-expressing CD4+CD25+ Tregs and can therefore indirectly influence T cell activation.

TGF-β inhibition of T cell proliferation is influenced by the differentiation status of T cells and the integrated signals from cytokines and costimulatory molecules on T cells, which correlates with reduced TGF-β receptor II expression (Cottrez and Groux, 2001). In addition to influence T cell proliferation, TGF-β also regulates helper T (Th) cell differentiation, and it generally inhibits the acquisition of Th cell functions (Gorelik and Flavell, 2002). TGF-β-mediated inhibition of Th cell differentiation occurs even in the presence of IL-2, in which case T cell proliferation is largely unaffected (Sad and Mosmann, 1994). As for CD8+ cytotoxic T cells, TGF-β has been found to potently inhibit cytotoxic T cell (CTL) differentiation. Early studies showed the CD8+ T cells activated in the presence of TGF-β do not acquire CTL functions (Ranges, et al., 1987), which is likely due to TGF-β inhibition of perforin expression in activated CD8+ T cells (Smyth, et al., 1991). TGF-β also inhibits Fas-ligand (FasL) expression in T cell lines (Genestier, et al., 1999), thereby affecting the death receptor cytotoxic pathway of CD8+ T cells. As in Th1 cells, the presence of TGF-β greatly attenuates IFN-γ expression in CD8+ T cells (Ahmadzadeh and Rosenberg, 2005; Bonig, et al., 1999). Thus, TGF-β

inhibits the expression of multiple effector molecules of CTLs. Investigators have shown that TGF-β can convert CD4+CD25- T cells to Treg in vitro (Chen, et al., 2003; Fantini, et al., 2004; Horwitz, et al., 2003; Wan and Flavell, 2005; Zheng, et al., 2004). Overexpression of TGF-β in the islets of the pancreas also expands the Treg population that protects nonobese diabetic (NOD) mice from type 1 diabetes (Peng, et al., 2004). However, the function of TGF-β in Treg generation/maintenance under physiological conditions remains controversial and poorly understood.

7. The interaction between TGF-β and Hsp60-mediated immunregulation

Since Hsp60 are highly expressed in immune-dysregulated diseases such as RA or JIA (Boog, et al., 1992; de Graeff-Meeder, et al., 1990) and some pathogen infections, the pathogenic role they play in disease progression is becoming attractive. In the animal models of rheumatoid arthritis (RA), T cell responses to self Hsp60 protect against the induction of arthritis. The same results were shown in RA patients since human Hsp60-reactive synovial fluid (SF) T cells produced significantly lower amounts of IFN-γ and higher amount of IL-4 (van Roon, et al., 1997). In the other case, severe injury may lead to immunosuppression with various factors involvement such as hsp60 and hsp70. Locally released Hsps at the site of soft tissue trauma would impaired the capacity of mononuclear cells (MNCs) to release TNF-α and cause monocytopenia restricted to

wound fluids (Flohe, et al., 2007). Chlamydia Hsp60 was also associated with activating Th2 immunity and persistent chronic infection (Kinnunen, et al., 2001). Although the exact mechanisms of Hsp60-mediated immune inhibition are still unmasked, previous studies have revealed some hints. Klimartin and the colleagues proved that anti-IL-10 neutralizing antibodies could restore the suppression of TNF-α production of monocytes treated with human Hsp60 (Kilmartin and Reen, 2004). Furthermore, Adjuvant arthritis (AA) patients vaccinated with human Hsp60 or hsp65 genes were found to enhance both IL-10 and TGF-β1 releases (Quintana, et al., 2002). Together, these results shed us twilight about the immunoregulatory effects of Hsp60; however, there is must something new and comprehensive needing us to explore. *H. pylori* are important factors of gastric cancer, and host immunosuppression caused by them is viewed as one of the major effectors to maintain the persistent infection and carcinogenesis. Since the recent research has indicated that *H. pylori* Hsp60 (HpHsp60) is vital to pathogen survival and could not be knockout, this protein must play important character in *H. pylori* infection. In this study, we attempt to investigate the immunoregulatory effects of HpHsp60 from a brand-new aspect. Quite different from previous reports, we found HpHsp60 not only surrender host immunity by secreting immunosuppressive cytokines, they also directly imitate them. That is to say, HpHsp60 could mimic TGF-β to bind to its specific receptor, trigger TGF-β-specific SMAD pathway, switch on the SMAD-associated downstream

target genes and suppress immune activities of peripheral mononuclear cells (PBMCs) and monocytic cells. These evidences broaden the view of Hsp60-mediated immunoregulation and strengthen its role in pathogen survival and persistent infection.

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Characterizing the Polymeric Status of *Helicobacter pylori* Heat Shock Protein 60

Abstract

Helicobacter pylori heat shock protein 60 (HpHsp60) was first identified as an adhesion molecule associating with *H. pylori* infection. With highly homology among species, however; HpHsp60 has also been investigated as a potent immunogen and elicits host proinflammatory immune responses. Here we have analyzed the structure of HpHsp60 via amino acid blast, circular dichroism and electrophoresis and the results indicated most recombinant HpHsp60s form dimers or tetramers that are quite different than *E. coli* Hsp60. Treatment of human monocytic cells THP-1 with HpHsp60 was found to up-regulate a panel of cytokines including IL-1α, IL-8, IL-10, IFN-γ, TNF-α, TGF-β1, GRO and RANTES, while carboxymethylated HpHsp60s which were switched their oligomeric status could further enhance NF-κB-mediated IL-8 and TNF-α secretion in THP-1 cells comparing to intact HpHsp60s. The oligomeric status of HpHsp60s may participate in regulating host inflammation to facilitate *H. pylori* persistent infection.

Introduction

Helicobacter pylori (*H. pylori*) has been attributed with the pathogenic role in gastric disease. This organism is associated with pathogenesis of upper gastrointestinal disorders, including chronic gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Algood and Cover, 2006). The virulent factors such as CagA (Peek and Blaser, 2002), Vac A (Molinari, et al., 1997), BabA (Boneca, et al., 2003), or *rocF*-gene encoded arginase (Gobert, et al., 2001) have been demonstrated to play important roles in *H. pylori* colonization and persistent infection.

The expression of heat shock protein (Hsp) 60 (also known as chaperonin (Cpn) 60) by *H. pylori* was previously identified as an adhesion molecule to interact with host gastric epithelial cells and mucin (Huesca, et al., 1996). It was a little contradictory that even though Cpn60 proteins have significant sequence conservation among species, they own extreme immunogenicity (Young DB, 1987). As a potent antigen of *H. pylori*, Hsp60 could activate myeloid cells to produce proinflammatory cytokines including IL-1β (Maguire, et al., 2005), IL-6 (Lin, et al., 2005; Maguire, et al., 2005), and IL-8 (Maguire, et al., 2005; Zhao, et al., 2007), and it also stimulates KATO III human gastric epithelial cells to secrete IL-8 (Takenaka, et al., 2004).

Although the structure of *H. pylori* Hsp60s has not been investigated, previous work was established on the resolution of *Escherichia coli* Hsp60 (Braig, et al., 1994). *E. coli* Hsp60 is

a porous cylinder of 14 subunits consisting of two approximately 7-mer rotationally symmetrical rings stacked back-to-back. Due to the profound stability of the bacterial chaperonin system and their evolutionary conservation among species, both mammalian and prokaryotic Hsp60s have been assumed to function in a similar way. However, an increasing number of studies are showing that there are variations in structures and functions of other species' Hsps (Chen and Sigler, 1999; Shimamura, et al., 2004; Zahn, et al., 1996). Possibly, these differences reflect a special adaptation of specific homologues to their microenvironments and endogenous substrates.

We have cloned and expressed recombinant *H. pylori* Hsp60 (rHpHsp60) and analyzed the structure via sequence alignment, gel electrophoresis and circular dichrosim. Although it contains highly conserved amino-acid sequences and similar secondary structure compositions, the oligomerization of Hsp60s from *H. pylori* and *E. coli* are quite different. This difference has been reflected on the divergence of proinflammatory cytokine profiles produced by monocytic cell line THP-1 treated with Hsp60s. Blocking the thiol group within Cys residue of *H. pylori* Hsp60 altered its molecular conformation and the abilities of cytokine inductions and NF-κB activation. This would imply that HpHsp60 may regulate inflammatory responses via variant oligomer composition.

Materials & Methods

Cell culture

Human monocytic cell line THP-1 was obtained from Bioresource Collection and Research Center (HsinChu, Taiwan) and maintained in RPMI media supplemented with 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 μg/ml of Penicillin/Streptomycin and 10% heat-inactivated FBS (Biological Industries, Haemek, Israel) at 37℃, 5% CO_2.

Amino acid sequence analysis

The amino acid sequences of *H. pylori* and *E. coli* hsp60 were aligned using the Vector NTI Suite 9 software (Invitrogen, USA). Variable amino acid positions and their relative frequencies were identified from the alignment.

Expression and purification of *H. pylori* Hsp60

Genomic DNA of *H. pylori* as the template, the DNA fragments containing the gene of Hsp60 was amplified by polymerase chain reaction and cloned into T7 promoter-driven pET 30a (+) expression vector (Novagen, Darmstadt, Germany). The sequences of HpHsp60 were sequenced, verified and submitted to Genbank (accession No.: DQ674277). The proteins were then expressed in *Escherichia coli* (BL21 strain) and purified using HisTrap affinity (Ni-NTA) chromatography (General Electric, NY, USA) followed by sephadex G-25 column (General

Electric, NY, USA) to remove salts associated with proteins. The purity of HpHsp60 was determined by SDS-PAGE, western blot assay (detected by HRP conjugated goat polyclonal antibody to polyhistidine; Novus, CO, USA) and confirmed with mass spectrometry analysis.

Carboxymethylation of *H. pylori* Hsp60

For carboxymethylation, 5 mg of *H. pylori* Hsp60 were dissolved in 5 ml of 0.1 M Tris-HCl buffer (pH 8.6) containing 5.4 M urea and 1% v/v β-mercaptoethanol. After being flushed with nitrogen and incubated at room temperature for 2 h, the reaction mixture was added with 20 mg of iodoacetic acid and maintained at pH 8.6 by the addition of 0.1 M NaOH for another 2 h. In the end, the carboxymethylated (CM)-HpHsp60 was dialyzed in 1X PBS buffer (pH 7.4) to desalt.

Gel electrophoresis

Reducing SDS-PAGE was carried out on 10% using 1 mm thick slab gels with the BioRad MiniProtean™ 3 system (BioRad, CA, USA). The final volume of the resolving gel was 4.0 ml with 1.3 ml of 30% acrylamide solution, 1 ml of 1.5 M Tris-HCl (pH 8.8), 1.6 ml of distilled water, 40 µl of 10% SDS, 20 µl of 10% APS, and 3.6 µl of TEMED. Proteins mixed with 3X sample buffer (30% v/v glycerol, 37.5% v/v of 0.5 M Tris-HCl (pH6.8), 15% v/v β-mercaptoethanol (2-Me), 0.66% w/v, 0.000075% w/v Bromophenol blue) were

incubated in 95℃ for 15 minutes and then loaded into each well and electrophoresis was run. All procedures were followed as described above for non-reducing SDS-PAGE except for removing β-mercaptoethanol from the 3X sample buffer. Native-PAGE was performed as sequential processes on 7.5% native gels as SDS-PAGE except SDS was definitely excluded from 1X sample tracking dye, stacking/separating gels, and electrophoresis running buffer. Proteins analyzed by native gel were directly incubated with 1X sample tracking dye without boiling.

Circular dichrosim (CD) spectroscopy

CD measurements for the *H. pylori* Hsp60 were conducted on AVIV Model410 Circular Dichroism spectropolarimeter. Instrument optics and sample chamber were continuously flushed with 10 l/ml of dry N_2 gas. The purified proteins were dissolved in 0.1 M phosphate buffered saline (PBS) at pH 7.4 to obtain a protein concentration of 0.5 mg/ml. Spectra were recorded in the far-ultraviolet (UV) region (200-260 nm). Protein samples were measured at room temperature in a 1 mm path-length quartz cell (300 μl) by using a scan speed of 50 nm/min, a time response of 1 s, a bandwidth of 1 nm and average of 8 scans.

Human protein cytokine array

THP-1 cells (10^5/well) were seeded in 24-well culture plates with 1 ml growth medium

per well and incubated at 37℃ in a 5% CO_2 atmosphere for 2 hours. 10 μg HpHsp60s were then added into cells. After 16-h incubation, supernatants were harvested and assayed by human protein cytokine array (RayBiotech, GA USA) according to the manufacturer's instructions. Membranes were briefly blocked with a blocking buffer, and then 1 ml of medium from either HpHsp60-treated or untreated THP-1 cell culture was added and incubated at room temperature for 2 hours. After incubation, membranes were treated for 1 hour with biotin-conjugated anti-cytokine antibodies, reacted with horseradish peroxidase-sreptoavidin and then developed using ECL.

IL-8 and TNF-α production inducted by *H. pylori* Hsp60 treatments on THP-1 cells

THP-1 cells were seeded in the same condition described in previous description. Following, 10 μg naïve or CM-HpHsp60 proteins were added into the well for a 16-h incubation period. Supernatants were then harvested and cytokines were assayed for TNF-α and IL-8 by DuoSet® ELISA development systems and operated according to the manufacturer's specifications (R & D Systems, MN, USA).

NF-κB-dependent reporter gene expression assay

The plasmid containing a minimal human NF-κB promoter-driving hrGFP gene (pNF-κB-hrGFP) (Strategene, USA) was applied to investigate the NF-κB activity of *H.*

pylori Hsp60-treated THP-1 cell. Transfections of THP-1 cells with pNF-κB-hrGFP were carried out by electroporation method with Multiporator® (Eppendorf, Hamburg, Germany). THP-1 cells (3×10^6 cells/ml) were incubated with 75 μg of plasmids followed by electroporation (350 V, 80 μs, 1 pulse). The cells were harvested at 48 hours after electro-transfection and hrGFP expression was assayed by flow cytometer.

Statistical analysis

The results are presented as means ± SEM. Significant differences were evaluated with ANOVA . A *P* value of < 0.05 was considered statistically significant.

Results

Analysis of amino acid sequences of *E. coli* and *H. pylori* Hsp60

The sequences of *E. coli* and *H. pylori* hsp60 proteins were retrieved from the NCBI Protein database (http://www.ncbi.nlm.nih.gov/sites/entrez? db=Protein). The accession numbers of *E. coli* and *H. pylori* Hsp60 proteins are **AAS75782** and **DQ674277** respectively. The protein sequence alignment showed that the positives and identity between HpHsp60 and *E. coli* Hsp60 are 73.0% and 59.9% (Fig. 1a). There is only one cysteine in the protein sequence of HpHsp60 (blue frame) while there is three cysteines in *E. coli* Hsp60 (red frame).

The results of circular dichroism measurements were provided for an overview of the secondary structures of *H. pylori* Hsp60 proteins. Spectra were recorded in 0.1 M phosphate buffered saline (PBS) at pH 7.4 with a protein concentration of 0.5 mg/ml (Fig. 1b). The quantitative evaluations of the data in terms of secondary structure components were shown in figure 2b. The CD spectra of *H. pylori* Hsp60 displayed a major α-helix conformation composed of 42.2% regular and 24.7% distorted forms, 15.3% β-sheet (with 3.5% regular and 11.8 distorted forms), 8.3% Turn and 9.7% Unordered structure (Fig. 1c).

Analysis for the effect of the disulfide bond on *H. pylori* Hsp60 structure

The *H. pylori* Hsp60 recombinant proteins (rHpHsp60) were analyzed by PAGEs with or without 2-ME treatments to reveal the potential structures. As seen in fig. 2a, most rHpHsp60

proteins were composed of monomer and only few could form the dimmer by intermolecular disulfide bonds. Results of native PAGEs indicated that there were three major forms for the rHpHsp60. According to the migration rate of molecular weight marker, recombinant *H. pylori* Hsp60 could form multiple oligomerization consisting of dimer, tetramer, and decamer (Fig. 2b). The results indicated that the cystein residue should not be involved in oligomerization of rHpHsp60 by intermolecular disulfide bonds.

Cytokine expression profiles of THP-1 cells induced by HpHsp60

Microarray membranes were scanned after reacted with the culture medium of THP-1 cells with or without HpHsp60 treatment (Fig. 3a) and the density of each spot was assessed by densitometry. The relative intensities of each cytokine profiles were respectively normalized to control spots on each membrane. The data showed conditioned medium from HpHsp60-treated THP-1 cells up-regulated several different target cytokines such as IL-1α (1.86 fold comparing to NC), IL-8 (11.67 fold), IL-10 (2.03 fold), TNF-α (1.86 fold), IFN-γ (1.79 fold), and TGF-β1 (1.5 fold). The chemokines such as GRO (2.24 fold) and RANTES (1.52 fold) were also elevated in response to HpHsp60 (Fig. 3b).

The effect of cysteine residue in *H. pylori* Hsp60 on the structure and the induction activities of proinflammatory cytokines

Although the cysteine residue contained within *H. pylori* Hsp60 did not involve in the formation of intermolecular disulfide bond, dissimilar distribution between *E. coli* and *H. pylori* Hsp60 is concerned if cysteine residues were relevant to any function. Therefore, the thiol group of cysteine residue within *H. pylori* Hsp60 was carboxymethylated and sequentially analyzed its status of oligomerization. The migration rate of CM-modified HpHsp60 became slower than the rHpHsp60 and the status of oligomeric structure changed when the only thiol group of HpHsp60 switched to the methyl group (Fig. 4a).

To determine whether the conformation change induced by carboxymethylation of Cys could cause the influence on the activities of HpHsp60, the proinflammatory cytokine releases of THP-1 cells induced by intact or CM-modified HpHsp60 were investigated. The results showed CM-modified HpHsp60 could elicit higher proinflammatory cytokine secretions, since IL-8 and TNF-α production were significantly elevated (Fig. 4 b,c, $p < 0.05$) comparing to those induced by rHpHsp60. It is known that activation of NF-κB pathway results in the transactivation of a multiple responsive genes that contribute to the inflammatory phenotype including IL-8 (Berger, 2002) and TNF-α (Simmonds and Foxwell, 2008) from monocytes, so the status of NF-κB activation is next investigated. CM-modified HpHsp60 was found to drive more NF-κB activation than rHpHsp60 did (Fig. 4d, $p < 0.05$).

Discussion

Hsp60s are among the most conserved proteins in all living organisms, either prokaryotic or eukaryotic. X-ray-determining structures from *E. coli* (Braig, et al., 1994) revealed that the Hsp60 oligomers are a porous cylinder of 14 subunits consisted of two approximately 7-fold rotationally symmetrical rings stacked back-to-back. Each Hsp60 subunit contains three structurally distinct domains: a large equatorial domain that constructs the foundation of the assembly at the waist and brings the ring together, an apical domain that forms the end of the cylinder, and a small intermediate domain that connects the two. However, although previous studies have been continuously disclosed that Hsp60s (or named as GroEL) from some prokaryotes or even yeast (Houston, et al., 1990) and pea chloroplast (Hendrix, 1979) have similar structures by electromicroscope analysis, no current evidence indicates that all Hsp60s shall possess the exact same pattern in response to truly complicated microenvironments of diverse species. The alignment in figure 1 between the Hsp60 amino acid sequences of *H. pylori* and *E. coli* shows over 70% positive sequences among them. It shows that the two Hsp60 genes have close relationship in evolution but still can not indicate they have the same activities or oligomerization. Furthermore, it has been shown that the ATP bound to GroEL is in direct contact with Cys-137 (Bochkareva and Girshovich, 1994), however, HpHsp60 did not own this cysteine residue. This revealed that even though there is high conservation between protein sequences, the Hsp60s coming from different species could have variant

activities or conformation. Thus, the oligomerization of *H. pylori* Hsp60 analyzed by native gel electrophoresis demonstrated that it is strictly different from *E. coli* Hsp60, since most HpHsp60 could form dimers and tetramers instead of heptameric *E. coli* Hsp60 (Fig. 2b).

Hsp60 class is one of the two families of chaperonins which have been identified, with members in the bacterial cytoplasm (GroEL) and in endosymbiotically derived mitochondria (Hsp60) and chloroplasts (Krishna, et al., 2007). Being chaperonins, Hsp60s are essential in mediating ATP-dependent polypeptide chain folding in a variety of cellular compartments. In addition to protein-folding assistants, Hsp60s are also designated as a potent immune regulator. Both mammalian and microbial hsp60s functions not only as active immunogens, but also regulate host innate or adaptive immunity (Tsan and Gao, 2004; van Eden, et al., 2005). The conserved sequences and multifaceted properties of Hsp60s imply that it is important for all species to help the organisms adapt to certain improper circumstances. Therefore the different oligomerization happened in certain species is reasonable.

It is known that hsp60s play important roles in triggering host immune responses. Several microbial homologues have been reported they can elicit pro-inflammatory responses. The responses comprise the induction of inflammatory mediators such as IL-6, TNF-α and NO (Tsan and Gao, 2004) and the activation of the T helper cells type 1 by promoting cytokines IL-12 and IL-15 (Pockley, et al., 2008).

The cytokine profile induced by *H. pylori* Hsp60 for monocyte stimulation was first

exposed by this study. Similar to Hsp60s derived from other species (Maguire, et al., 2005), *H. pylori* Hsp60 could stimulate THP-1 monocytes to produce IL-1α, IL-8, and TNF-α which manage the host inflammation. On the other hand, *H. pylori* Hsp60 also engages in IL-10 and TGF-β1 induction which potentially manipulates immune regulation and GRO and RANTES secretion associated with chemoattraction (Sager, et al., 1991), cellular communication, survival and differentiation (Wong and Fish, 2003). Although sharing ~70% homology with *E. coli* Hsp60, their physiological functions seem not exactly the same. The findings are supported by previous reports that although *H. pylori*, *Chlamydia* and human Hsp60s also share homologous amino acid sequences, their potency in cytokine inductions such as IL-1β, IL-6, and IL-8, and vascular endothelial cell adhesion protein expressions are quite different from each other (Maguire, et al., 2005).

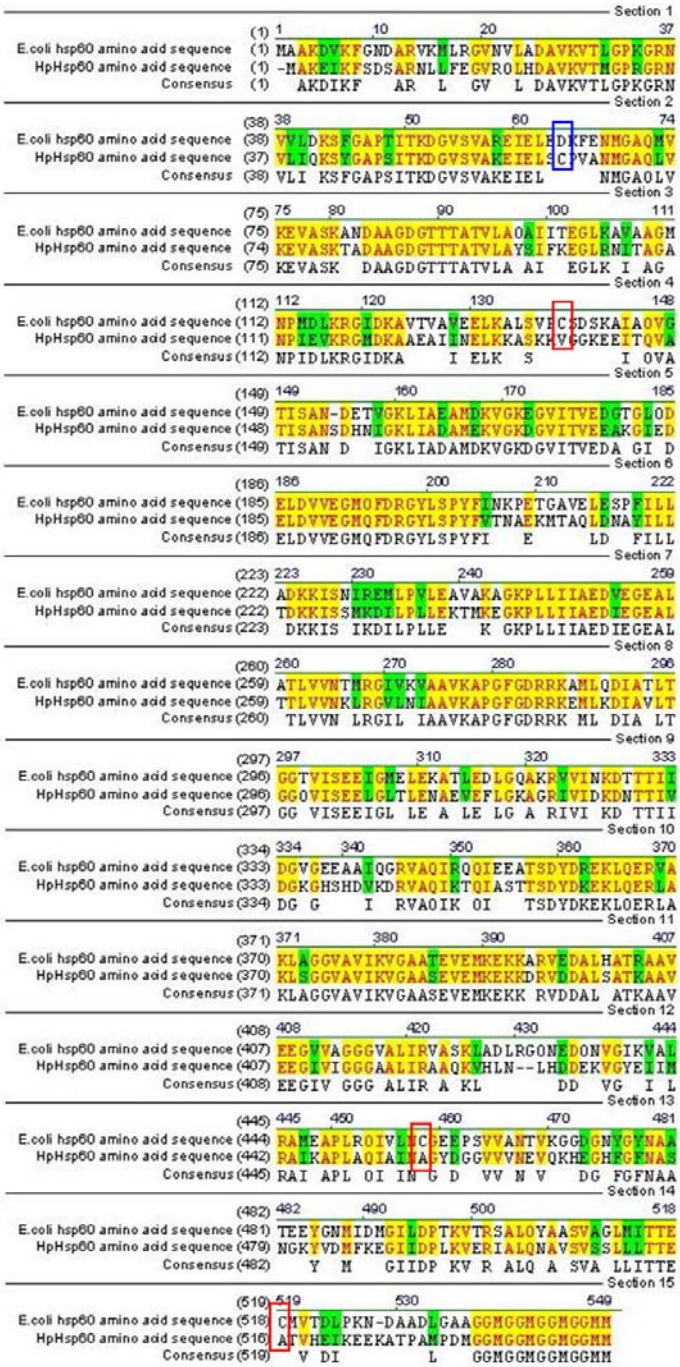
Although the single cystein residue within HpHsp60 was not involved in the formation of intermolecular disulfide bond, but it did contribute to HpHsp60 structural integrity and be relevant to proinflammatory cytokines IL-8 and TNF-α production. *H. pylori* Hsp60 was found to induce IL-8 release in both gastric epithelial cells (Takenaka, et al., 2004) and monocytes/macrophages (Zhao, et al., 2007) through the Toll-like receptor (TLR)-2/4-mediated signal pathway. By engagement with TLR-2/TLR-4, HpHsp60 activates mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and p38 and then induces the nuclear translocation of NF-κB to trigger TNF-α and IL-8

production (Zhao, et al., 2007). Our data was consistent with the previous reports that HpHsp60 could significantly stimulate THP-1 cells to release IL-8 and TNF-α via NF-κB activation; however, cystein blockage of HpHsp60 was found to strengthen this proinflammatory response. CM-modified HpHsp60 inspired the exacerbation of both NF-κB transcription factor/DNA binding activity and following-up cytokine secretions in monocytic cells. This phenomenon may disclose the importance for the oligomerization of HpHsp60 in inflammation. With proper structural composition, HpHsp60 could moderate host immune responses and may facilitate their survival and persistent infection within gastric microenvironments.

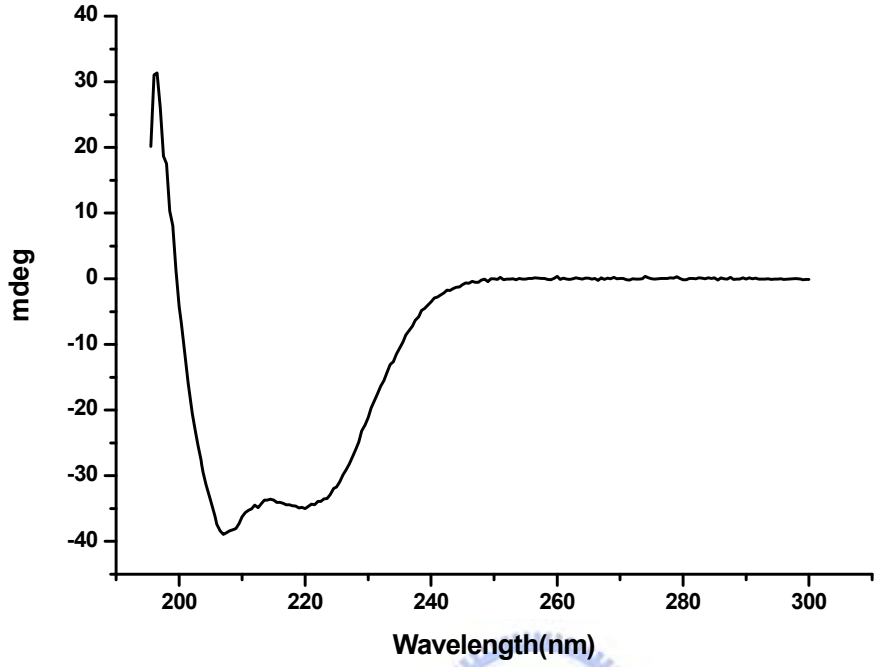
In this study, we have shown results for the oligomerization of HpHsp60. Quite different from other homologues, HpHsp60 could form dimers and tetramers and the oligomeric status which could affect the functions to activate NF-κB signaling and induce the releases of proinflammatory cytokines.

Figures and Legends

a.



b.


40
30
20
10
0
-10
-20
-30
-40
mdeg
200
220
240
260
280
300
Wavelength(nm)

c.

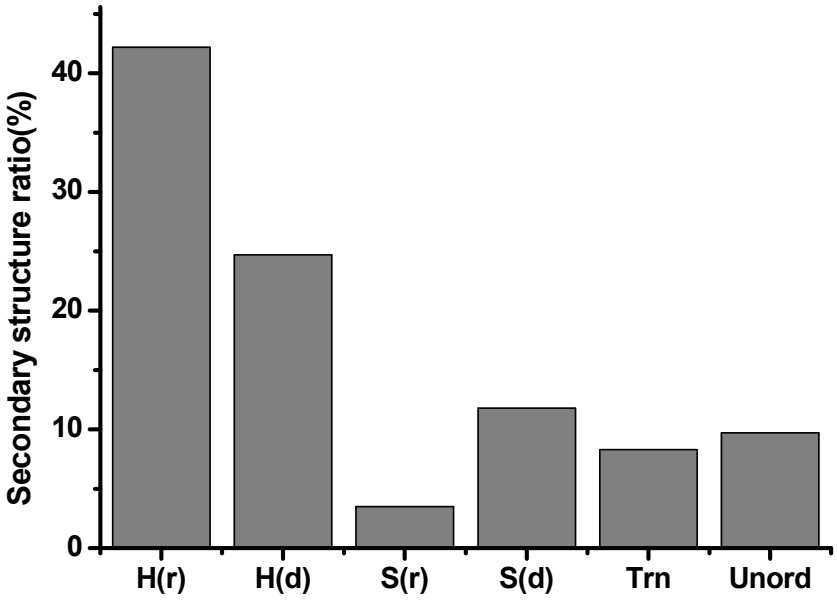
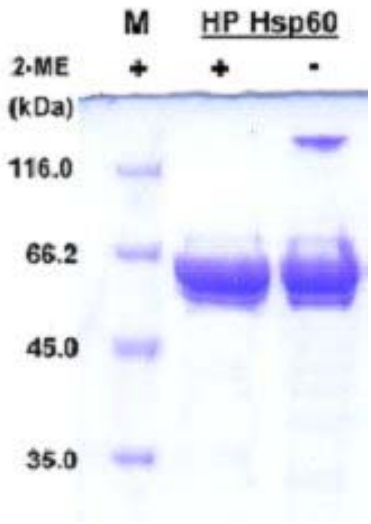

40
30
20
10
0
Secondary structure ratio(%)
H(r)
H(d)
S(r)
S(d)
Trn
Unord

Fig. 1 Primary and Secondary structure analysis of *H. pylori* Hsp60. (a) Sequence alignment of *H. pylori* and *E. coli* Hsp60s. The amino acid sequences of *E .coli* and *H. pylori* Hsp60s were retrieved from Pubmed database and analyzed by Vector NTI Suite 9 software. The blue and red frames indicated the cysteins within *H. pylori* and *E. coli* Hsp60s. CD spectra (b) and secondary structure estimation (c) of *H. pylori* Hsp60. Measurements were performed in 0.1 M phosphate buffered saline (PBS) at pH 7.4.

a.



b.

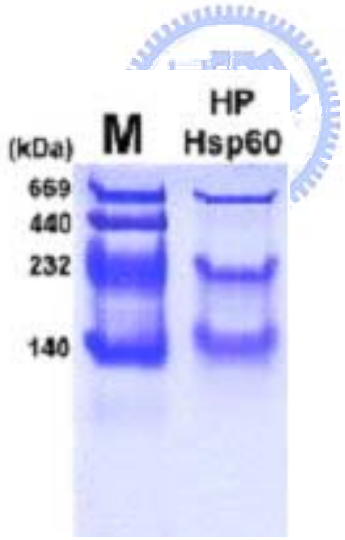
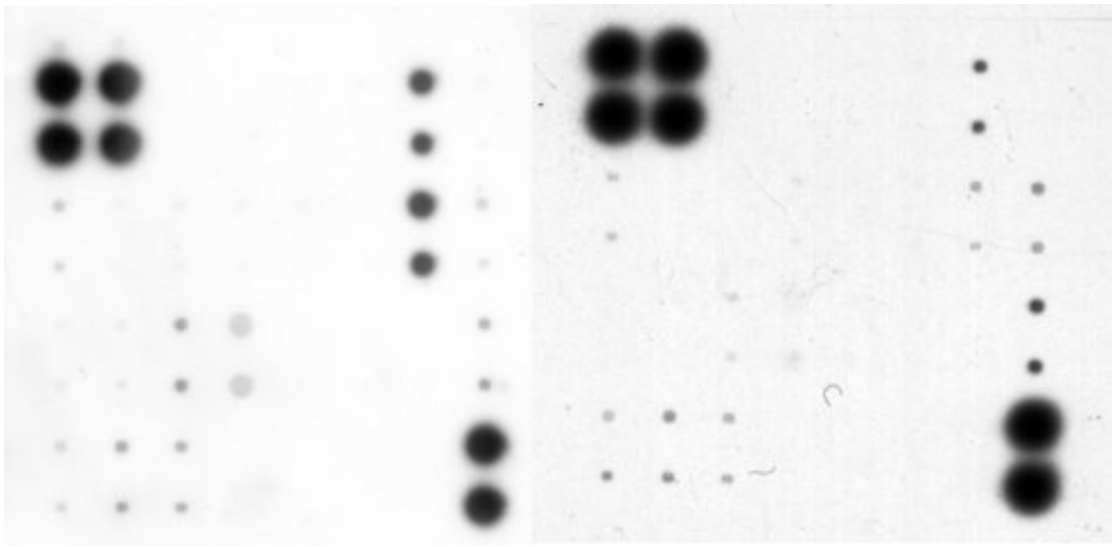


Fig. 2 Electrophoresis profile of HpHsp60. (a) Reducing and non-reducing (w/o 2-Me) SDS-PAGE. (b) Native-PAGE. M: Marker.

a.



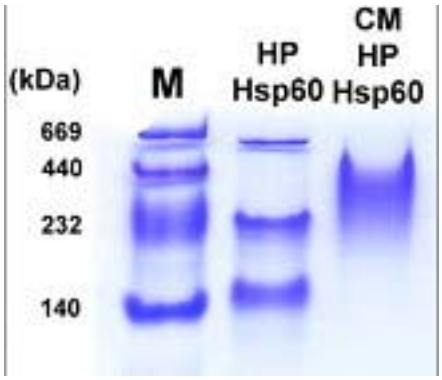
HpHsp60 treated **NC**

b. Human inflammatory cytokine levels elevated from HpHsp60-treated THP-1 cells

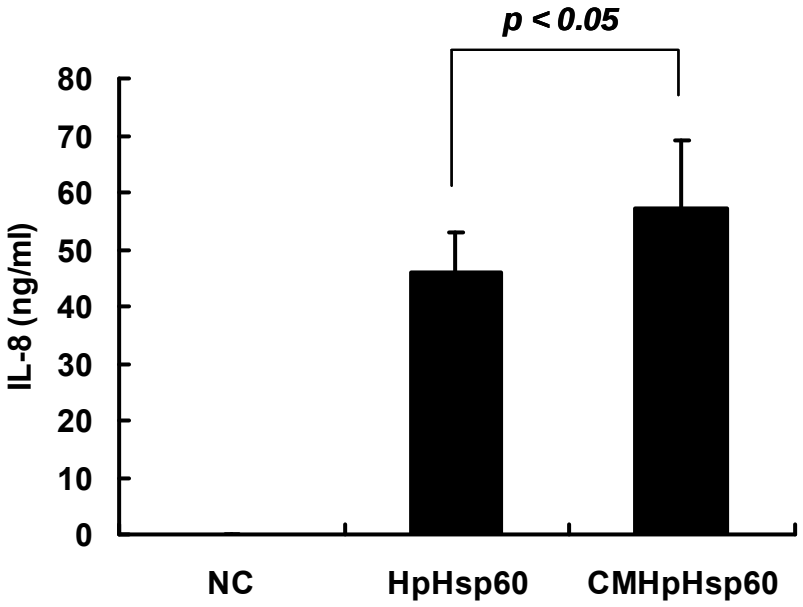
	Human Cytokine Protein	Fold Increase
IL-8	Interleukin-8	11.67 fold
GRO	Growth-related oncogene	2.24 fold
IL-10	Interleukin-10	2.03 fold
TNF-α	Tumor necrosis factor-α	1.86 fold
IL-1α	Interleukin-1α	1.86 fold
IFN-γ	Interferon-γ	1.79 fold
RANTES	Regulated on activation normal T cell expressed and secreted	1.52 fold
TGF-β1	Transforming growth factor-β1	1.5 fold

Fig. 3 Human cytokine up-regulated from THP-1 monocytic cells treated with HpHsp60. Human protein-array analysis was used to determine the relevance in the release of HpHsp60-stimulated THP-1 cells. (a) Data from the array analyzed with filter 1, treated with HpHsp60 (Left) and filter 2, medium control (Right). (b) The intensities of the relative expression level of cytokines were quantified by densitometer (GE, USA). The values from scans were normalized based on the intensity of control spots on the filter corners, and the level increase for specific cytokines were shown.

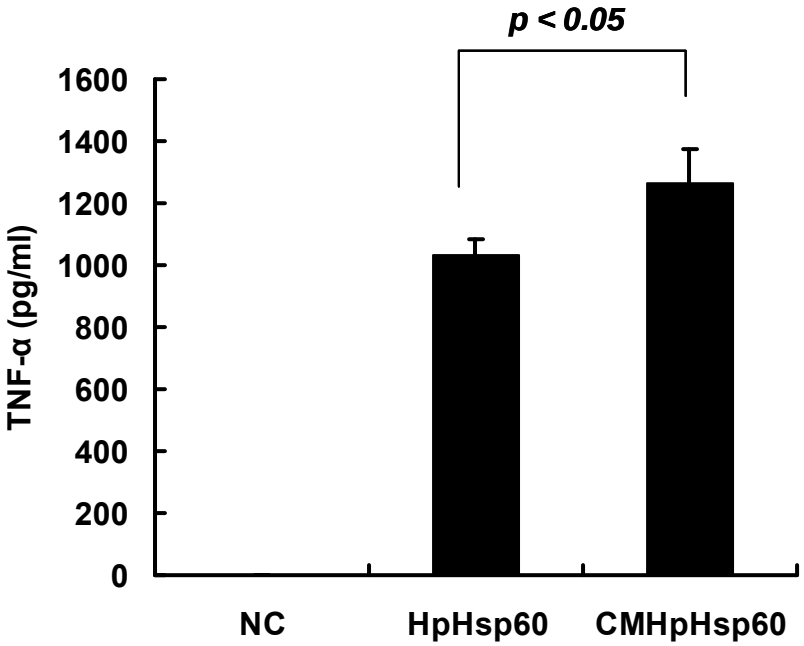
a.


(kDa)
M
HP Hsp60
CM HP Hsp60
669
440
232
140

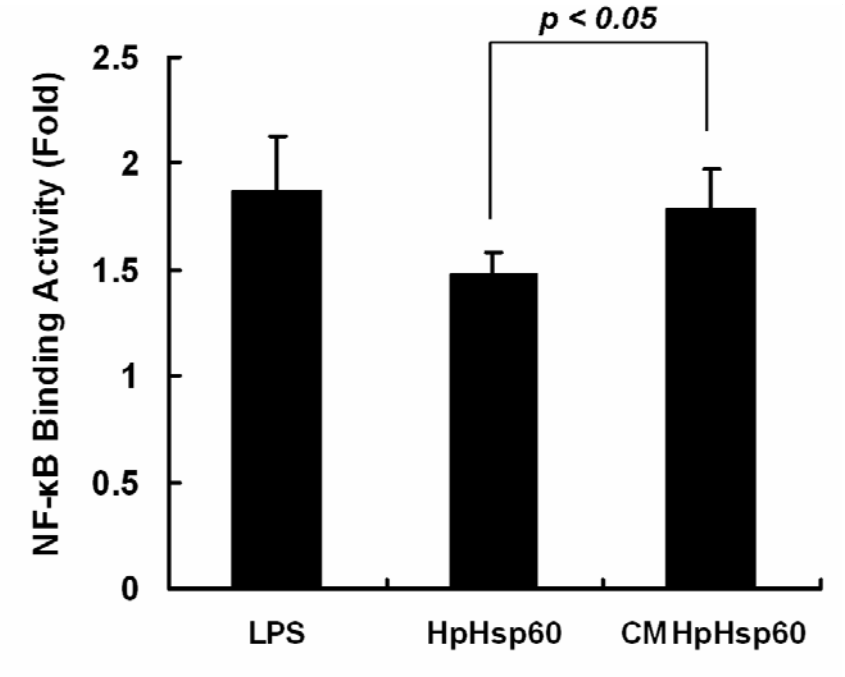
b.


p < 0.05
IL-8 (ng/ml)
80
70
60
50
40
30
20
10
0
NC
HpHsp60
CMHpHsp60

c.


p < 0.05
1600
1400
1200
1000
800
600
400
200
0
TNF-α (pg/ml)
NC
HpHsp60
CMHpHsp60

d.


p < 0.05
2.5
2
1.5
1
0.5
0
NF-κB Binding Activity (Fold)
LPS
HpHsp60
CM HpHsp60

Fig, 4 The effects of rHpHsp60 versus CM-HpHsp60s on proinflammatory cytokine induction and NF-κB transcriptional activity. (a) Native-PAGE analysis of HpHsp60 and carboxymethylated (CM) HpHsp60. (b) & (c) THP-1 cells were incubated with rHpHsp60 (10 μg/ml) for 16 hours at 37℃ in 5% CO_2 and conditioned media were harvested to be determined for the IL-8 (b) and TNF-α (c) levels with sandwich ELISA. Data are expressed as means ± SEM for three replicates from one of three representative experiments. (*, $P <$ 0.05.) (d) THP-1 cell were transiently transfected with NF-κB-dependent reporter plasmid (pNF-κB-hrGFP) and cotreatment with the oligomeric or monomeric HpHsp60s. The fluorescence was analyzed with flow cytometer.

Acknowledgments

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receptor-triggered pathway in cultured human gastric epithelial cells. *Microbiology* **150,** 3913-22.

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Helicobacter pylori Heat shock protein 60 trigger SMAD signal pathway by interacting with Transforming growth factor-β receptor II

Abstract

Helicobacter pylori have explored multiple mechanisms to evade host immune surveillance for chronic infection. But either of them is restricted by certain bacterial strains containing potential virulent factors, or those immune-restrained functions only limit to some specific immunocytes. However, the long-term persistence of *H. pylori* suggests a more comprehensive and powerful factor(s) hinds behind to regulate host immune system. *Helicobacter pylori* heat shock protein 60 (HpHsp60) was first identified as an adhesion molecule associating with *H. pylori* infection. With highly homology among species, however; HpHsp60 has also been investigated as a potent immunogen and elicits host proinflammatory immune responses. In this study, we have revealed a novel property of HpHsp60 which is, by mimicking TGF-β1, HpHsp60 could also exert immune regulatory effects. With structure homology to the receptor binding site of TGF-β1, HpHsp60 could interact with TGF-β receptor II, trigger SMAD pathway, and inhibiting the functions of THP-1 monocytic cells and peripheral mononuclear cells (PBMC). These immune suppressions could be reversed by specific TGF-β1 inhibitors. Our study provides a new hint that *H. pylori* may employ Hsp60 to surrender host immunity.

Introduction

Transforming growth factor (TGF) -β has been known to regulate a variety of immune cell activities. As a potent immune-regulator, TGF-β is associated with both innate and acquired immunosuppresion. Impaired proliferation and cytotoxicity of natural killer (NK) cells and deactive oxidative burst of monocytes are found in TGF-β-mediated damage of innate immunity (Kehrl, 1991). As for acquired immunity, TGF-β exerts extensively surrendering effects including restraining dendritic cell (DCs) maturation and antigen presentation, T cell proliferation, differentiation, and effector functions, and B cell survival and activation (Gorelik and Flavell, 2002; Li, et al., 2006). These findings showed TGF-β as a pleiotropic immuno-suppressive factor.

With diverse and vigorous capabilities, TGF-β plays a pivotal role in regulating immune system and therefore is implicated in the pathogenesis of several chronic infections. TGF-β has been indicated to promote microbial persistence by interfering monocyte/macrophage functions. Chronic infections such as *Cryptococcus neoformans* (Shao, et al., 2005) , *Candida albicans* (Letterio, et al., 2001), *Leishmanial amazonesis* (Barral-Netto, et al., 1992), *Trypanosoma cruzi* (Silva, et al., 1991), hepatitis B virus (HBV) (Yoo, et al., 1996), Human Immunodeficiency Virus (HIV) (Poggi and Zocchi, 2006), and *Mycobacterium tuberculosis* (Roberts, et al., 2007) are all found to produce amounts of TGF-β which might decreased chemokine production and oxidative burst to reduce phagocytosis of macrophages and

peripheral blood monocytes (Barral-Netto, et al., 1992; Letterio, et al., 2001; Shao, et al., 2005), or suppress the ability of interferon (IFN) -γ to activate intracellular inhibition of the parasite within macrophages (Silva, et al., 1991). Previous reports have indicated that *Helicobacter pylori*, a persistent pathogen which is highly associated with the development of human peptic ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (Algood and Cover, 2006), could also induce TGF-β production in either antral mucosa of *H. pylori*-positive patients (Li and Li, 2006; Stromberg, et al., 2003) or mononuclear cells in the duodenal lamina propria of both duodenal-ulcer patients and asymptomatic carriers with *H. pylori* infection (Stromberg, et al., 2003). However, the effects of TGF-β in *H. pylori* pathogenesis are not well elucidated.

The expression of Hsp60s or immune reactivity to Hsp60s seems to be related to downregulation of immune responses, rather than with induction or propagation of inflammation (Harats, et al., 2002; Maron, et al., 2002; Zanin-Zhorov, et al., 2005; Zanin-Zhorov, et al., 2003). The previous study revealed that CD4+ T-cell responses to human Hsp60 at the onset of Juvenile idiopathic arthritis (JIA) correlated with responses to *mycobacterial* Hsp60 and with a favourable prognosis (Prakken, et al., 1996). Immunization with Hsp60 also has been shown to protect against type 1 diabetes in non-obese (NOD) mouse model (Elias, et al., 1990). The analysis of immune responses to Hsp60 in experimental models and in patients has indicated the capacity of Hsps to induce regulatory

T-cell responses. Human Hsp60 was found to downregulate T cell migration (Zanin-Zhorov, et al., 2003) and inhibit the secretion of proinflammatory cytokines by activated T cells (Zanin-Zhorov, et al., 2005). Further analysis disclosed that Hsp60s could enhance CD4+CD25+ regulatory T cell (Tregs) function via Toll-like receptor (TLR) 2 engagement. Hsp60-treated Tregs inhibited target T cells both by cell-to-cell contact and by release of TGF-β and IL-10 (Zanin-Zhorov, et al., 2006). Recent study also showed immuninization of Hsp60 or the peptide Hsp60 in mice induced TGF-β and IL-10 secreting CD4+CD25+Foxp3+ Tregs to dampen the development of the immune responses in atherosclerosis (van Puijvelde, et al., 2007). Together, Hsp60s may construct a microenvironment for immunosuppression by manipulating immune-regulatory effectors such as Tregs or TGF-β; however, the underlying mechanisms are attractive to be investigated.

In this study, we sought to elucidate the role of *H. pylori* Hsp60 (HpHsp60) in host immune regulation. Although HpHsp60 was defined as an adhesion molecule (Huesca, et al., 1996) or inflammatory inducers (Lin, et al., 2005; Maguire, et al., 2005; Zhao, et al., 2007) in previous works, we have identified their role in immunosuppression now. By binding to TGF-β receptor II, HpHsp60 could trigger TGF-β1-specific SMAD pathway, turn on the SMAD transcriptional activities, activate TGF-β1-regulated downstream genes, and mediate immune functions of human peripheral blood mononuclear cell inhibition. This TGF-β1 functional mimicry may make HpHsp60 an important factor contributing to *H. pylori*

persistent infection.

Materials & Methods

Cell culture

All cell lines applied in this study were obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). Gastric epithelial cancer cells AGS were cultured in RPMI media supplemented with 2 mM L-glutamine, 50 μg/ml of Penicillin/Streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (Biological Industries, Haemek, Israel) at 37℃, 5% CO_2. HEK293 cells were cultured in DMEM media supplemented with 2 mM L-glutamine, 50 μg/ml of Penicillin/Streptomycin and 10% heat-inactivated FBS at 37℃, 5% CO_2. Monocytic cells THP-1 were maintained in RPMI media supplemented with 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 μg/ml of Penicillin/Streptomycin and 10% heat-inactivated FBS.

Amino acid sequence analysis

The amino acid sequences of *H. pylori* and *E. coli* hsp60 were aligned using the Vector NTI Suite 9 software (Invitrogen, USA). Variable amino acid positions and their relative frequencies were identified from the alignment.

Protein structure alignment

The protein structure of human TGF-β1 (PDB ID 1KLC) was retrieved from RSCB

Protein Data Bank (www.rcsb.org/pdb). Because *H. pylori* Hsp60 structure has not resolved now, $(PS)^2$ protein structure prediction server (http://ps2.life.nctu.edu.tw/) was used to predict HpHsp60 protein structure. 3D protein structure comparison between human TGF-β1 and HpHsp60 were performed by Combinatorial Extension (CE) method (http://cl.sdsc.edu/).

Cloning and expression of *H. pylori* Hsp60

The DNA fragments containing the gene of Hsp60 was amplified by polymerase chain reaction and cloned into T7 promoter-driven pET 30a (+) expression vector (Novagen, Darmstadt, Germany). The sequences of HpHsp60 were sequenced, verified and submitted to Genbank (accession No.: DQ674277). The proteins were then expressed in *Escherichia coli* (BL21 strain) and purified using HisTrap affinity (Ni-NTA) chromatography (General Electric, NY, USA) followed by sephadex G-25 column (General Electric, NY, USA) to remove salts associated with proteins. The purity of HpHsp60 was determined by SDS-PAGE, western blot assay (detected by HRP conjugated goat polyclonal antibody to polyhistidine; Novus, CO, USA) and confirmed with mass spectrometry analysis.

Dot blot

Recombinant *H. pylori* Hsp60 and human TGF-β1 were spotted on a nitrocellulose membrane. Samples were incubated for 10 min at room temperature and the membrane was

blocked with blocking solution (5% milk in PBS-0.05% Tween 20 (PBST)) for 2 h and washed in PBST. All incubation steps were carried out at room temperature. Both recombinant proteins were detected with either rabbit anti-*H. pylori* Hsp60 serum (1:10000 dilution in blocking solution,) or rabbit anti-human TGF-β1 polyclonal Ab (1:500) (Santa Cruz Biotechnology, INC, CA, USA) followed by 1: 10000 diluted goat peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co, MO, USA). Each antibody was incubated with the membranes for 1 h and then washed with PBST. Immunoreactivities were visualized using enhanced chemiluminescence kits (Pierce, Rockford, IL, USA).

Assessment of HpHsp60 binding to human TGF-β receptor II by ELISA

To analyze the interaction between HpHsp60 and human TGF-β receptor II (TGF-βRII), an ELISA-based approach with two kinds of experimental designs (to detect HpHsp60 or TGF-β1) were used and separately described below. Briefly, recombinant human TGF-βRII was prepared at a concentration of 5 μg/ml in coating buffer (1X PBS, pH 7.4). Microtiter plates (Costar Co. NY, USA) were coated with TGF-βRII by adding 100 μl of the appropriated protein in the assay wells and incubating overnight (at room temperature). Wells were then washed three times with PBST, blocked with 300 μl of 5% skim milk in PBST for 1 h, and washed three times with PBST. HpHsp60 were diluted in PBS to the concentration of 1 μg/ml, 100 μl of proteins was added to each well and the plates were incubated for 2 h. Wells

were then washed three times with PBST to remove unbound HpHsp60. Bound HpHsp60 was detected by incubation with 100 μl of mouse antiserum against HpHsp60 (diluted 1:10000 in PBST) for 1 h, followed by incubation for 1 h with peroxidase-conjugated goat anti-mouse IgG 1:10,000. Binding was shown using TMB solution (KPL, MD, USA) and absorbance at A490 was measured. Control wells were coated with PBS. In another setting, TGF-β1 signaling inhibitor SB431542 (Inman, et al., 2002) (Sigma Chemical Co, MO, USA) was used to compete the binding of HpHsp60 to TGF-βRII, 1 h incubation of 100 μl SB431542 (50 μM) was added to each well before or after HpHsp60 binding to investigate the effects on HpHsp60/TGF-βRII interaction. Visualization procedures were then followed as described above. For TGF-β1 and HpHsp60 competition assay, 100 μl of proteins containing 0.17 μg TGF-β1 (Peprotech Inc., NJ, USA) and different amounts of HpHsp60 (with HpHsp60/TGF-β1 ratios including 0, 10, 50, and 100) were mixed and added to each well for 2 h incubation, bound TGF-β1 was detected by biotinylated chicken anti-human TGF-β1 antibody (1:180) (R&D Systems, MN, USA) for 1 h, followed by Strptavidin-HRP (1:200) (R&D Systems, MN, USA) incubation. SB431542 was also used to evaluate the binding pattern of TGF-β1 to TGF-βRII. Bound proteins were calculated as follows: the A490 mean of triplicate tests minus the mean of triplicate control wells. Experiments were done in duplicate.

TLR2, TLR4, and TGF-βRII expression on HEK293, AGS, and THP-1 cells

For TLR phenotyping of AGS, HEK293, and THP-1 cells, cells were first incubated with anti-TLR-2 or anti-TLR-4 mAbs (Serotec, Oxford, UK) diluted in staining buffer composing of 0.05% NaN_3 and 1% of BSA in PBS for 1 hour at 4℃, followed by FITC-conjugated goat anti-mouse Ig secondary antibody (Serotec, Oxford, UK) incubated for 30 minutes at at 4℃. For TGF-βRII phenotype analysis, fluorescein-conjugated mouse anti-TGF-β receptor II Ab (R&D Systems, MN, USA) was used. Fluorescence related to immunolabeling was then measured by flow cytometer.

Cloning of human TGF-β receptor II

Human TGF-β receptor II gene was kindly provided by Dr. Chih-Sheng Lin in Department of Biological Science and Technology, National Chiao Tung University and amplified by means of PCR using following primers: forward primer: 5'-CACCATGGGTCGGGGGCTCA-3'; reverse primer: 5'-AGACTCGAGCTATT TGGTAGTGTTTAGGGAG-3'. The resulting PCR product was cloned directly into the eukaryotic expression vector pcDNA3.1D (pcDNA3.1 Directional TOPO Expression Kit, Invitrogen) and sequenced.

TGF-β receptor II transfection in HEK293 and AGS cells

HEK293 and AGS cells (4×10^5 cells/ml) were seeded in 6-well plates on the day before transfection. Lipofectamin™ 2000 (Invitrogen, CA, USA) was applied to carry out gene delivery according to the operating manual. Briefly, 10 μl of Lipofectamin™ 2000 were diluted in 250 μl Opti-MEM® (Invitrogen, CA, USA) for 5 min incubation, 3 μg of pTGF-β receptor II diluted in 250 μl Opti-MEM® was then added into Lipofectamin™ 2000 diluent for 20 minute incubation at room temperature to allow complex formation. Meanwhile, wash cells in 6-well plates with PBS to remove antibiotics containing growth medium. The formed DNA/ Lipofectamin™ 2000 complex was then added into cells for 16 hours at 37℃ in a 5% CO_2 incubator. Sequentially, the medium was replaced by HEK293 or AGS growth medium and the transfectants were incubated for 24 hours. Cells then were harvested and stained with fluorescein-conjugated mouse anti-TGF-β receptor II Ab (diluted 1:100 in PBS containing 5% BSA and 0.05% NaN_3) (R&D Systems, MN, USA) to confirm the gene expression. Meanwhile, cells were also incubated with HpHsp60 (2.5 μg for 2.5×10^5 cells), bound HpHsp60 was detected with mouse antiserum against HpHsp60 (1:5000) for 30 min, followed by incubation for 30 min with fluorescein-conjugated goat anti-mouse IgG (1:10,000). The fluorescence was assayed by flow cytometer.

SiRNA silencing of TGF-β receptor II expression in THP-1 cells

siRNA oligonucleotides to the human TGF-β receptor II were purchased from Qiagen

(CA, USA) and transfected by using HiPerFect (Qiagen, Valencia, CA). Cells were transfected according to HiPerFect transfection reagent manual protocol with 5 nM per well of siRNA oligos at 24-h intervals. Forty-eight hours after transfection, cells were harvested and detected with anti-TGF-β receptor II Ab or HpHsp60 binding by flow cytometer.

SMAD2/3 phosphorylation detected by western blot

THP-1 cells were seeded in 24-well culture plates with 1 ml of cell suspension (5×10^5/ml) seeded and incubated either with TGF-β1 (1 ng/ml), HpHsp60 (10 μg/ml), TGF-β1 and SMAD2/3 specific inhibitor SIS3 (10 μM) (Sigma Chemical Co, MO, USA), HpHsp60 and SIS3, or SIS3 alone. Cell lysates were collected at 30', 60', 90' and 120 min to be performed on 10 % SDS-PAGE with the BioRad MiniProtean™ 3 system (BioRad, CA, USA). The proteins were electroblotted onto a nitrocellulose membrane; Un-phosphorylated and phosphorylated SMAD2/3 were detected with either goat anti-SMAD2 (1:500) or anti-SMAD2-p antibodies (1:500) (Santa Cruz Biotechnology, INC, CA, USA) followed by rabbit peroxidase-conjugated anti-goat IgG (1:2000) (Abcam, Cambridge, UK), and β-actin was detected with a mouse anti-β-actin antibody (1:5000) (Novus Biologicals, CO, USA) followed by goat peroxidase-conjugated anti-mouse IgG (1:10000) (Jackson ImmunoResearch Laboratories, INC., PA, USA). The incubation and visualization procedures were described as above.

SMAD-dependent reporter gene expression assay

The plasmid containing a minimal human SMAD 2/3/4 promoter-driving hrGFP gene (pSMAD 2/3/4-hrGFP) (SABioscience, MD, USA) was applied to investigate the SMAD 2/3 activity of *H. pylori* Hsp60-treated HEK293 cells. Transfections of HEK293 cells with pSMAD 2/3/4-hrGFP were carried out by Lipofectamin^{TM} 2000 according to the operating manual described above. After 16 hours of transfection, change medium to growth medium and treat cells with TGF-β1 (1 ng/ml), HpHsp60 (10 μg/ml), TGF-β1 and SMAD2/3 specific inhibitor SIS3 (10 μM), HpHsp60 and SIS3, or SIS3 alone. The cells were harvested aat 24 hours after treatment and hrGFP expression was assayed by flow cytometer.

RNA purification and real-Time PCR

Total THP-1 cellular RNA was extracted with Trizol reagent (Invitrogen, NY, USA) and was reverse-transcribed into cDNA using the Superscript RT kit (Invitrogen, NY, USA). Human MMP9, P21 and Interleukin-6 (IL-6) gene expression were detected by quantitative real-time PCR. The PCR reactions were performed in a 25 μl reaction mixture with two gene-specific primers (10 μM; 1 μl of each primer). The primer sequences of MMP9, P21, IL-6 and β-actin were as follows: MMP9: 5'-GTGC TGGGCTGCTGCTTTGCTG-3'(sense), 5'-GTCGCCCTCAAAGGTTTGGAAT -3' (antisense); P21:

5’-GATGTCCGTCAGAACCC-3’(sense), 5’-CCCTCCAGTGGTG TCTC-3’ (antisense); β-actin: 5’-TTGCCGACAGGATGC AGAA-3’ (sense), 5’-GCC GATCCACACGGAGTACT-3’(antisense); cyber green mixture 12.5 μl (Ampliqon, Denmark), cDNA 1 μl and distilled water 9 μl. The cycling conditions were: 50 ℃ for 2 min, 95 ℃ for 10 min, 40 cycles of 95 ℃ for 15 s, 60 ℃ for 1 min. All the reactions were performed with two negative controls

Peripheral blood mononuclear cell (PBMCs) preparation

PBMCs were isolated from the blood of healthy human donors by density separation over Ficoll-paque^{TM} plus (General Electric, NY, USA). Mononuclear cells at the interface were carefully transferred into a Pasteur Pipette, then treated with ACK hemolysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in distilled water) and washed twice in PBS. Cells were cultured in RPMI media supplemented with 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 μg/ml of Penicillin/Streptomycin and 10% heat-inactivated FBS. To evaluate the effects of HpHsp60s on PBMC proliferation, PBMCs (2×10^5 /well) were seeded in 96-well plate which is pre-coated with anti-CD3 monoclonal antibody (1 μg/ml, 30 μl /well, at 37°C for 2 hours) and incubated with HpHsp60 (10 μg/ml) for 96 hours at 37℃, 5% CO_2. Subsequently, MTS reagent (Promega, WI, USA) was applied for cell proliferation measurement according the operating manual and the OD value was obtained at 490 nm with

ELISA reader. For assaying Interferon (IFN)-γ production, PBMCs (10^6 /well) were seeded in 24-well plate coated with anti-CD3 mAb and co-incubated with HpHsp60 (10 μg/ml) for 24 hours at 37℃, 5% CO_2. Supernatants were then collected and assayed for IFN-γ secretion by ELISA kit (R&D Systems, MN, USA).

Surface marker expression of THP-1 cells in the presence of HpHsp60

To define the effects of HpHps60 on the surface marker expressions of THP-1 cells, HpHsp60 (10 μg/ml) or HpHsp60 plus SB431542 (1 μM) were incubated with THP-1 cells (5 × 10^5 /ml) seeded in 24-well plate. After 16 h treatment, cells were harvested and incubated with PE-conjugated CD86, FITC-conjugated CD40, CD80, HLA-ABC, and HLA-DR antibodies (BioLegend, CA, USA) for 1 hour at 4°C. Fluorescence related to immunolabeling was then measured by flow cytometer.

Antigen uptake

For studying phagocytosis of THP-1 cells, Fluorescein-conjugated *E. coli* (Molecular Probe, CA, USA) was used. Briefly, 10 μl of FITC-labeled *E. coli* were added to a 200 μl suspension of THP-1 cells followed by 2-h incubation at 37°C. Phagocytosis was stopped by cooling on ice for 10 minutes.100 μl of quenching buffer made of trypan blue dye (0.16 mg/ml in PBS) was added to samples to exclude bacterial clusters and fluorescence from

bacteria adhering to the THP-1 surface. Samples were then washed twice with cooled PBS. Aggregation artifacts of bacteria were excluded by incubating cells with propidium iodide (50 μg/ml) before analyzed by flow cytometer.

Statistical analysis

The results are presented as means ± SEM. Significant differences were evaluated with one way ANOVA. A *P* value of < .05 was considered statistically significant.

Results

HpHsp60s share part of structural similarity with human TGF-β1

Our preliminary results revealed something interesting, which is, a polyclonal anti-human TGF-β1 antibody could also recognize HpHsp60 protein. This phenomenon was later confirmed by repetitive experiments containing different kinds of anti-human TGF-β1 antibodies and the results indicated that there may be certain epitope(s) of HpHsp60 could interact with antibodies we applied. HpHsp60 identified by polyclonal anti-human TGF-β1 antibody were shown in Fig. 1a. Meanwhile, the mouse anti-HpHsp60 polyserum was also found to be able to detect human TGF-β1 (Fig. 1b). The intersection between these two protein/antibody recognitions soon became an appealing issue, and we then sought to investigate the conformational similarity between HpHsp60 and human TGF-β1. Amino acid sequence alignment was first performed. The sequences of human TGF-β1 and *H. pylori* hsp60 proteins were retrieved from the NCBI Protein database (http://www.ncbi.nlm.nih.gov/). The protein sequence alignment showed that the positives and identity are 20.4% and 11.1% (Fig. 2) to disclose little amino acid similarity between them. However, the structural alignment gave rise to noticeable information. Although not fully matched, it seemed that HpHsp60 and human TGF-β1 both possessed one loop structure (From the amino acid sequence of TGF-β1 Thr56 to Cys77; HpHsp60: from Als344 to Val 369) with similar conformation (Fig. 3). This structural overlap between these two proteins might be

biologically meaningful because the amino acid residues 69~73 of human TGF-β1 has been known to involve in TGF-β1 anchorage to its receptors (Hinck, et al., 1996).

HpHsp60 can bind to human TGF-β receptor II

Because the similar region between HpHsp60 and human TGF-β1 contained TGF-β receptor binding site, we sought to explore the interaction between HpHsp60 and TGF-β receptor. Because TGF-β1 is a dimeric ligand and binds to a type II receptor, which recruits a type I receptor dimer forming a hetero-tetrameric complex with the ligand (Wrana, et al., 1992), we then investigate the binding of HpHsp60 to TGF-β receptor II (TGF-βRII). The results indicated HpHsp60 could adhere to TGF-βRII coated on the ELISA plate (Fig. 4a), and the binding was in a dose-dependent manner (Fig. 4b). Human TGF-β1 was next applied to unmask if HpHsp60/ TGF-βRII interaction could be competitive. Different dosages of HpHsp60 were mixed with 0.17 μg of TGF-β1 to achieve HpHsp60/TGF-β1 amount ratios as 0, 10, 50, and 100, the protein mixture were then incubated with TGF-βRII coated-ELISA plates. Detection of TGF-β1 revealed that HpHsp60 could interfere the binding of TGF-β1 to its naïve receptor in a dose-dependent tendency (Fig. 4c). However, the interference of HpHsp60 to TGF-β1/receptor binding could not happen if HpHsp60 was treated after TGF-β1 pre-incubating with TGF-βRII-coated wells (Fig. 4d), this implied that the binding of TGF-β1/ TGF-βRII may be more efficient than that of HpHsp60/ TGF-βRII. Furthermore, we

also explored if the two proteins share the same binding domain of TGF-βRII. The TGF-β1 signaling inhibitor SB431542 was found to be able to reduce TGF-β1 binding when it was pre-incubated with TGF-βRII-coated wells (Fig. 4e); however, it could not interfere with the HpHsp60/ TGF-βRII interaction (Fig. 4f). The different results exposed by SB431542 suggested HpHsp60 did not directly interact with the TGF-β1 binding site of the receptor.

The binding of HpHsp60 and its correlation with TGF-βRII expression on cell level was evaluated in HEK293, AGS, and THP-1 cells to be confirmed. HpHsp60 was reported to induce IL-8 both in human monocytes and gastric epithelial cells via TLR2 and TLR4 (Takenaka, et al., 2004; Zhao, et al., 2007), here we attempted to compare the expression of TLR2 and TLR4 with HpHsp60 bindings to investigate the consistency. As shown in Fig. 5, the HpHsp60 binding was not consistent with TLR2 or TLR4 expression. Since HEK293 cells were reported not to present TLR2 or TLR4 (Latz, et al., 2002) on their cell surface, HpHsp60 could still silently adhere to HEK293 cells. Furthermore, THP-1 cells, with highly TLR2 and TLR4 expression; however, the binding pattern of HpHsp60 obviously did not fit it. On the contrary, when we compared the TGF-βRII expressive profiles of the three cell line with their HpHsp60 binding counterparts, the extremely similar pattern could be found in HEK293, AGS or THP-1 cells (Fig. 5). Subsequently, due to the surface expression of naïve TGF-βRII on both HEK293 and AGS cells are only a small quantity, pTGF-βRII genes were transfected into the two cell lines to determine whether the overexpression of TGF-βRII could enhance

the HpHsp60 binding. Both cell lines transfected with pTGF-βRII gene expressed TGF-βRII on their cell surface which could be confirmed by fluorescein-conjugated anti-TGF-βRII antibody; meanwhile, we could also see the transfectants increased the HpHsp60 binding amounts comparing to mock cells (Fig. 6). Moreover, for THP-1 cells with higher TGF-βRII expression, we applied siRNA to specifically dampen the receptor mRNA and the results showed silencing of TGF-βRII did reduce the binding of HpHsp60 to THP-1 cells (Fig. 7). Together, the results in either ELISA-based methods or cellular assays have demonstrated that HpHsp60 could bind to TGF-βRII.

Binding of HpHsp60s to human TGF-β receptor II triggers SMAD2/3 phosphorylation and transcriptional activation

The next issue we sought to validate is if the binding of HpHsp60s to human TGF-βRII is biological relevant. Mature TGF-β1 binds to TGF-βRII in turn activates TGF-βRI, upon activation, TGF-βRI phosphorylates intracellular mediators called SMAD proteins which are central in most actions of TGF-β1 (Derynck, et al., 1998; Derynck and Zhang, 2003). In this study, SMAD2/3 phophorylation was observed at 30 minutes after treatment of HpHsp60 to THP-1 cells, and this phophorylation could be blocked in the presence of SMAD 2/3 specific phosphorylation inhibitor SIS3 (Fig. 8). This implied that HpHsp60 could also trigger TGF-β1 specific SMAD signal pathway. Subsequently, we evaluate the effects of HpHsp60 on SMAD

response elements contained mini promoter-driven GFP reporter (pSMAD 2/3/4-hrGFP) activities. Both HEK293 and AGS cells transfected with pSMAD 2/3/4-hrGFP showed higher expression of SMAD-driven fluorescence in the presence of HpHsp60; however, reduced responses were found when SIS3 was co-incubated with HpHsp60 for these transfectants (Fig. 9a, b, $p < 0.01$).

HpHsp60s induce TGF-β1-mediated downstream gene expression

Binding of HpHsp60s to TGF-βRII on cell surface subsequently activates TGF-β1 downstream SMAD 2/3 signaling pathway. The genes regulated by SMAD-related signal transduction were then evaluated. Since TGF-β1 is a pleiotropic cytokine, we chose three genes including P21, MMP9, and IL-6 referring to the cell cycle, migration, and immune functions regulated by TGF-β1. As shown in Fig. 10, THP-1 treated with HpHsp60 significantly enhanced the P21 and MMP9 mRNA expression, while co-incubated with SIS3 blocked the HpHsp60-mediated gene up-regulation (Fig 10a, b).

HpHsp60s inhibit IFN-γ production and proliferation of PBMCs and surface marker expression and phagocytotic activity of THP-1 cells

The immunesuppressive activities of HpHsp60s were evaluated on human PBMCs and monocytic cell line THP-1. Treatment of HpHsp60s to PBMCs from healthy donors

decreased the proliferation rate in the presence of anti-CD3 antibody stimulation (Fig. 11a). The IFN-γ production of HpHsp60-incubated PBMCs was also down-regulated (Fig. 11b). Since PBMCs is the mixture of immune cells, we then analyzed the effects of HpHsp60s on specific cell types. Monocytic THP-1 cells were chosen to simulate the responses from monocytes. Flow cytometric analysis revealed a phenotype of THP-1 cells, *i.e.* positive for MHC I , MHC II, and CD40, but negative for CD80 and CD86 (Fig. 12a). While HpHsp60-treated cells reduced the expression levels of MHC I, MHC II, and CD40, administration of an inhibitor of TGF-β1 type 1 receptor kinases, SB431542, restored this situation (Fig. 12a). For the ability of antigen capture, quantification of uptake by flow cytometer showed that THP-1 cells exhibited strong phagocytosis of *E.coli*-FITC and HpHsp60 inhibited the phagocytosis activities of THP-1 cells. The blockage of TGF-β1 signaling cascades made THP-1 cells regain its capability (Fig. 12b). Collectively, these data suggest that, HpHsp60 can weaken both the surface antigen expression and engulfing abilities of mococytic cells through TGF-β1 signal transduction.

Discussion

In this study, we have demonstrated HpHsp60s could bind to human TGF-βRII and induced a series of TGF-β1-mediated signal transduction, gene activation, and immunesuppression. The partial structure homology was first predicted by CE algorithm, and this homological between HpHsp60 and TGF-β1 contained the amino acid residues involving in TGF-β1/ TGF-βRII binding sites. The algorithm-based results were proven by evaluating the binding of HpHsp60s to TGF-βRII immobilized on ELISA plate or naïve as well as TGF-βRII-transfected cells. Furthermore, silencing of TGF-βRII expression on THP-1 cells was found to significantly reduce HpHsp60 binding to cells.

Collectively, our results clearly manifested that HpHsp60s could interact with human TGF-βRII. This HpHsp60s/ TGF-βRII engagement was relevant since it could trigger SMAD 2/3 phosphorylation and activate the Samd-specific gene transcription to turn on MMP9 and P21 mRNA expression. It seemed that HpHsp60s partially mimicked TGF-β1 to interact with its naïve receptor and trigger the downstream signaling cascades; moreover, HpHsp60s could even “borrow” the immunesuppressive effects from TGF-β1. Treatment of HpHsp60s in PBMCs and monocytic THP-1 cells significantly reduced their immune functions, and the inhibition could be reversed by TGF-β1 signaling inhibitor SB435142. Together, our data revealed that HpHsp60 caused immunesuppression as TGF-β1 did by triggering SMAD signal pathway.

Microbial Hsp60s coming from bacteria, protozoa, fungi, and helminthes are immunodominant since immune responses to them are frequently detected after infection (Zugel and Kaufmann, 1999). Mice immunized with *Mycobacterium tuberculosis* showed that ~10-20% of the *M. tuberculosis*-specific T cells responded to *M. tuberculosis* Hsp60 (Kaufmann, et al., 1987). Sera from *Chlamydia trachomatis*-infected patients also revealed high titers of antibodies specific for *C. trachomatis* Hsp60 (Matthews, et al., 1998).With regard to *H. pylori* Hsp60s, they were previously reported to induce a serious of proinflammatory cytokines including IL-1β, IL-6, and IL-8 (Gobert, et al., 2004; Lin, et al., 2005; Maguire, et al., 2005; Takenaka, et al., 2004). Therefore, it is no doubt for HpHsp60s to be an immunodominant vaccine candidate (Kamiya, et al., 2002).

Opposite to the role of immune-activation, Hsp60s coming from pathogens also serve as immunomodulators (Kinnunen, et al., 2001; Zanin-Zhorov, et al., 2006). In patients infected with *C. trachomatis,* microbial Hsp60 responding T cells produced IL-10 and shifted to Th2 immune responses (Kinnunen, et al., 2001). PBMCs in periodontitis patients treated with mycobacterial Hsp60 reduced the level of IFN-γ production and cell proliferation (Petit, et al., 1999). Furthermore, the previous studies have implicated the potential role for Hsp60 as an immunoregulator. Because the T cells which can specifically recognize *mycobacteral* Hsp60 release IL-4 and IL-10 in animal model to protect hosts against inflammatory damages (van Eden, et al., 1988). Collectively, microbial Hsp60s seem to possess double-edged effects on

host immunity. It might depend on the amounts of Hsp60s present within hosts and the how long the infection lasts. Hsp60s could be immune-potent when they are in the early stage of infections while repetitive exposure of microbial Hsp60s to host immune systems has generated tolerance to surrender immunity. Although the immune regulation of microbial Hsp60s have been observed in some cases, the mechanisms are not clearly elucidated. Our present results now disclose for the first time that HpHsp60s trigger SMAD signal pathway by binding to human TGF-β receptor II, and this may explain why Hsp60s play an important role in immuneregulation.

Recent study has shown human Hsp60s acts as a costimulator of human Tregs to downregulate CD4+CD25- or CD8+ target T cells by inhibiting target T cell proliferation and IFN-γ and TNF-α production. Both cell-to-cell contact and secretion of TGF-β and IL-10 were involved Hsp60-treated Tregs suppressing target T cells (Zanin-Zhorov, et al., 2006). TGF-β1 is an important immunoregulatory cytokine in regulating T cell activation and tolerance (Li, et al., 2006). It is not only an effective molecule secreted by Tregs to surrender inhibit T cells activation, but also the key factor to the generation of Tregs (Bommireddy and Doetschman, 2007). TGF-β1 could induce *Foxp3* expression in T cells and cause Th-to-Treg cell conversion and subsequent expansion through SMAD signaling (Fantini, et al., 2004). TGF-β1 also induces *Ctla4* expression in naïve T cells, which, in turn, is responsible for the induction of *Foxp3* in inducible Treg cells (Zheng, et al., 2006). Since human Hsp60 was

proved a costimulator for Tregs, *H. pylori* Hsp60s may also exert their influence on Tregs activation. Hsp60s are highly conserved proteins among different species, and the homology between amino acid sequences of human and *H. pylori* Hsp60s are ~50% (data not shown). In fact, our results have provided the strong hint for HpHsp60s to be Tregs activators. By mimicking the functions of TGF-β1, HpHsp60s could switch on SMAD pathway that is pivotal to the *Foxp3* expression and Tregs generation and subsequently protect *H. pylori* from host immune attack.

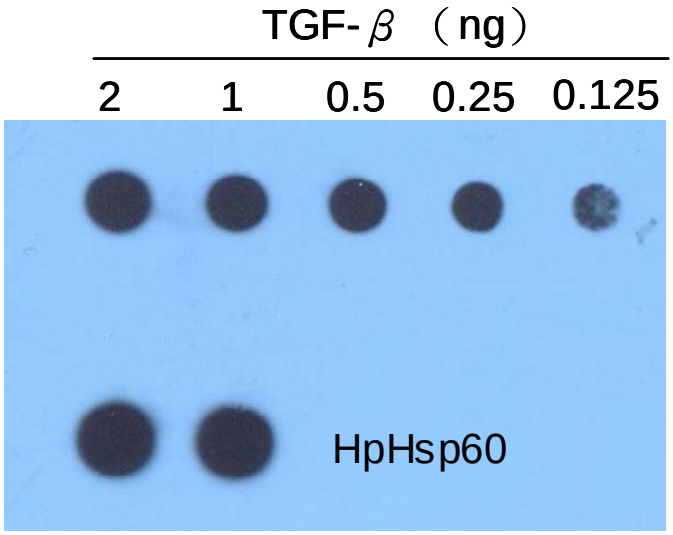
TGF-β1 has been implicated in the persistent pathogen infections such as *Cryptococcus neoformans* (Shao, et al., 2005), *Candida albicans* (Letterio, et al., 2001), *Leishmanial amazonesis* (Barral-Netto, et al., 1992), *Trypanosoma cruzi* (Silva, et al., 1991), hepatitis B virus (HBV) (Yoo, et al., 1996), HIV (Poggi and Zocchi, 2006), and *Mycobacterium tuberculosis* (Roberts, et al., 2007). So far, TGF-β1 induced by persistent microbes focused on dampening the functions of macrophages/monocytes, natural killer (NK) cells, and Tregs. Rats infected with pulmonary cryptococcosis were found increased TGF-β1 in epitheloid cells to reduce Ab and serum-mediated phagocytosis of *Cryptococcus neoformans* by rat alveolar macrophages and peripheral blood monocytes, which is associated with chemokine production and oxidative burst (Shao, et al., 2005). *L. amazonesis* (Barral-Netto, et al., 1992) and *T. cruzi* (Silva, et al., 1991) infection also led to macrophage deactivations by TGF-β1 induction. Opposite to fungi or parasite infections, Tat proteins from HIV-1 induced TGF-β1

production to enhance NK cells apoptosis (Poggi and Zocchi, 2006) and *M. tuberculosis* was observed to increase both TGF-β1 and Tregs generation (Roberts, et al., 2007).

The persistence of *H. pylori* infection revealed both adaptation to colonizing microenvironment and escape from host immunesurvaillence. Surrendering immune functions by *H. pylori* have been reported in various studies; however, the role for Hsp60s has not been defined until now. Binding to TGF-βRII, activating SMAD-mediated gene expression and exerting immunesuppression in both PBMCs and THP-1 monocytes signify the importance of HpHsp60s in *H. pylori* infection. Since TGF-β1 is closely related with chronic infection, HpHsp60s may potentially involve in modulating host immune responses, which, instead of inducing TGF-β1, by directly mimicking TGF-β1. Together, we proposed that HpHsp60s might be applied by *H. pylori* to construct an immune privilege microenvironment.

Figures and Legends

a.



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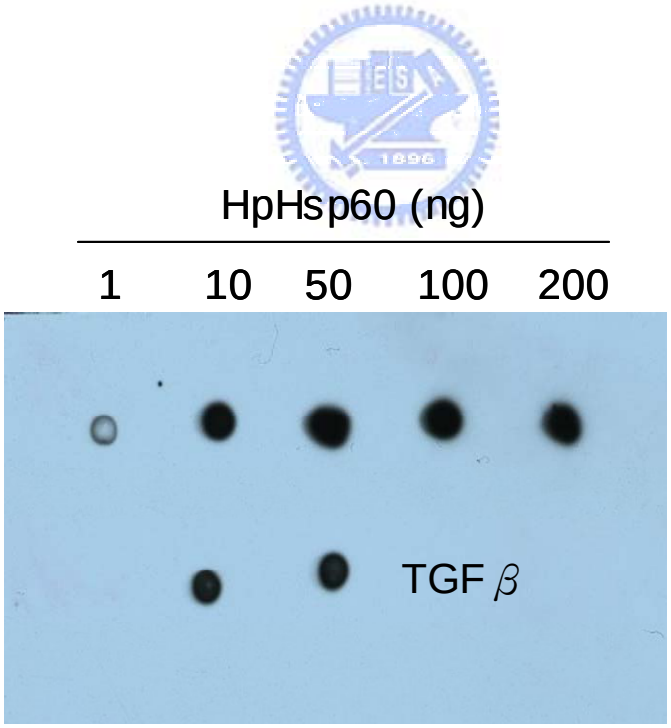


Fig. 1 Dot blot for investigating the intersection between HpHsp60/human TGF-β1 protein/antibody interaction.

(a) 0.125, 0.25, 0.5, 1 & 2 ng of TGF-β1 and 2 μg of HpHsp60 were spotted on the nitrocellulose membrane followed by detection by rabbit anti-human TGF-β1 polyclonal Ab (1:500) (Santa Cruz Biotechnology, INC, CA, USA) followed by 1: 10000 diluted goat peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co, MO, USA). (b) 1, 10, 50, 100, & 200 ng of HpHsp60 and 10 ng of TGF-β1 were spotted on the nitrocellulose membrane followed by detection by rabbit anti-HpHsp60 polyserum (1:10000) and goat HRP-conjugated anti-rabbit IgG. (1:10000).

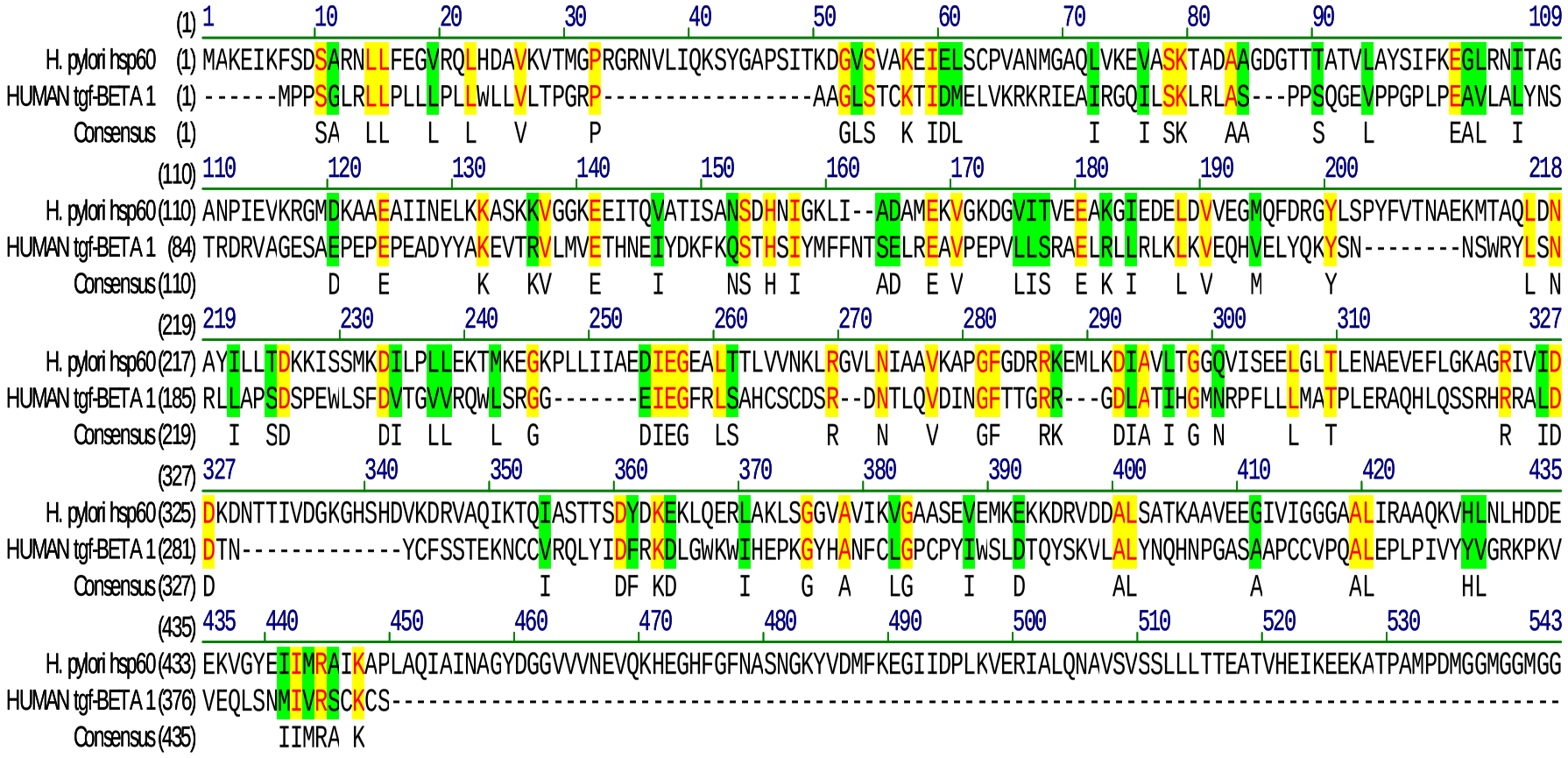

(1) 1 10 20 30 40 50 60 70 80 90 109
H. pylori hsp60 (1) MAKEIKFSDSARNLLFEGVRQLHDAVKVTMGPRGRNVLIQKSYGAPSITKDGVSVAKEIELSCPVANMGAQLVKEVASKTADAAGDGTTTATVLAYSIFKEGLRNITAG
HUMAN tgf-BETA 1 (1) ------MPPSGLRLLPLLLPLLWLLVLTPGRP----------------AAGLSTCKTIDMELVKRKRIEAIRGQILSKLRLASPPSQGEVPPGPLPEAVLALYNS
Consensus (1) SA LL L L V P GLS K IDL I I SK AA S L EAL I
(110) 110 120 130 140 150 160 170 180 190 200 218
H. pylori hsp60 (110) ANPIEVKRGMDKAAEAIINELKKASKKVGGKEEITQVATISANSDHNIGKLI--ADAMEKVGKDGVITVEEAKGIEDELDVVEGMQFDRGYLSPYFVTNAEKMTAQLDN
HUMAN tgf-BETA 1 (84) TRDRVAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKFKQSTHSIYMFFNTSELREAVPEPVLLSRAELRLLRLKLKVEQHVELYQKYSN--------NSWRYLSN
Consensus (110) D E K KV E I NS H I AD E V LIS E K I L V M Y L N
(219) 219 230 240 250 260 270 280 290 300 310 327
H. pylori hsp60 (217) AYILLTDKKISSMKDILPLLEKTMKEGKPLLIIAEDIEGEALTTLVVNKLRGVLNIAAVKAPGFGDRRKEMLKDIAVLTGGQVISEELGLTLENAEVEFLGKAGRIVID
HUMAN tgf-BETA 1 (185) RLLAPSDSPEWLSFDVTGVVRQWLSRGG-------EIEGFRLSAHCSCDSR--DNTLQVDINGFTTGRR---GDLATIHGMNRPFLLLMATPLERAQHLQSSRHRRALD
Consensus (219) I SD DI LL L G DIEG LS R N V GF RK DIA I G N L T R ID
(327) 327 340 350 360 370 380 390 400 410 420 435
H. pylori hsp60 (325) DKDNTTIVDGKGHSHDVKDRVAQIKTQIASTTSDYDKEKLQERLAKLSGGVAVIKVGAASEVEMKEKKDRVDDALSATKAAVEEGIVIGGGAALIRAAQKVHLNLHDDE
HUMAN tgf-BETA 1 (281) DTN------------YCFSSTEKNCCVRQLYIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVLALYNQHNPGASAAPCCVPQALEPLPIVYYVGRKPKV
Consensus (327) D I DF KD I G A LG I D AL A AL HL
(435) 435 440 450 460 470 480 490 500 510 520 530 543
H. pylori hsp60 (433) EKVGYEIIMRAIKAPLAQIAINAGYDGGVVVNEVQKHEGHFGFNASNGKYVDMFKEGIIDPLKVERIALQNAVSVSSLLLTTEATVHEIKEEKATPAMPDMGGMGGMGG
HUMAN tgf-BETA 1 (376) VEQLSNMIVRSCKCS----------
Consensus (435) IIMRA K

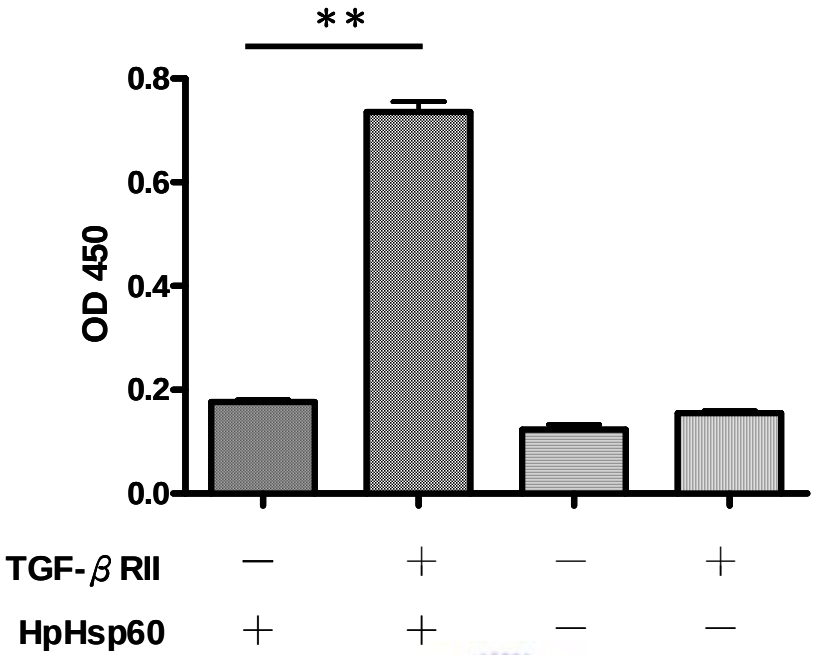
Fig.2 Amino acid alignment of HpHsp60 and human TGF-β1.

The amino acid sequences of HpHsp60 and human TGF-β1 were retrieved from Pubmed database and analyzed by Vector NTI Suite 9 software. Amino acids painted with green and yellow colors indicated the positive and identity respectively.

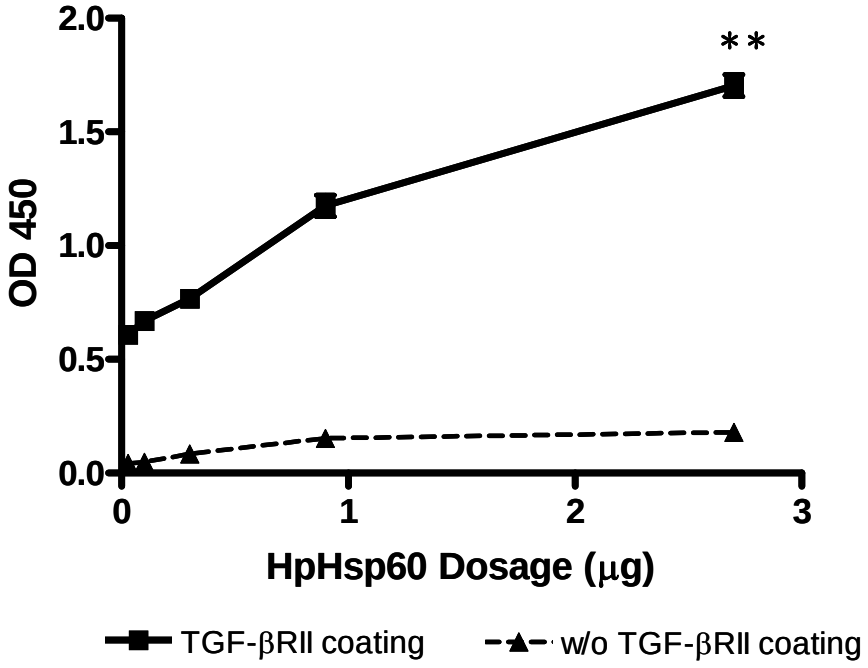
Fig. 3 Structural alignment of HpHsp60 and human TGF-β1

The NMR-resolved human TGF-β1 protein structural was retrieved from PDB (Accession No. 1KLC) and the structure of HpHsp60 was predicted by $(PS)^2$ protein structure prediction server. Structural alignment was performed by Combinational extension method. The blue and red frames indicated the structures of human TGF-β1 and HpHsp60 respectively.

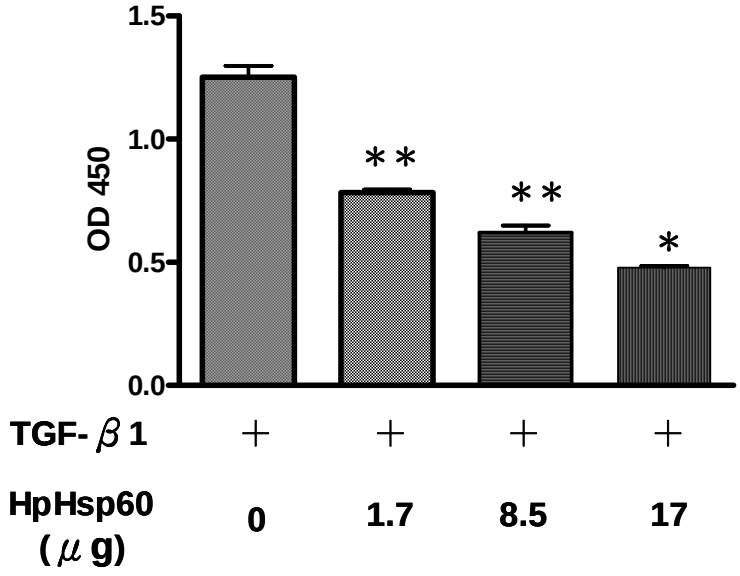
a.


**
0.8
0.6
0.4
0.2
0.0
OD 450
TGF-β RII
HpHsp60
−
+
−
+
+
+
−
−

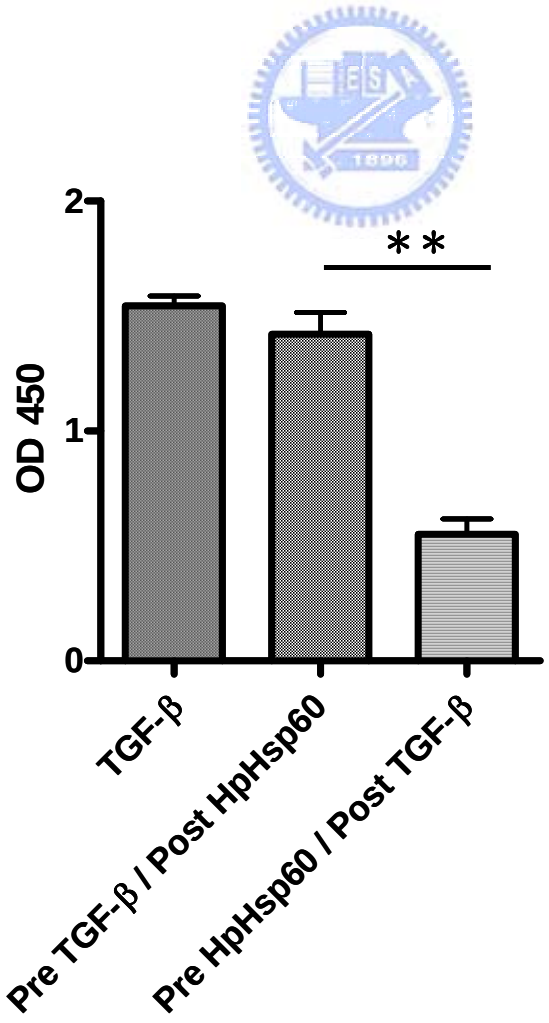
b.


2.0
1.5
1.0
0.5
0.0
OD 450
**
0
1
2
3
HpHsp60 Dosage (μg)
TGF-βRII coating
w/o TGF-βRII coating

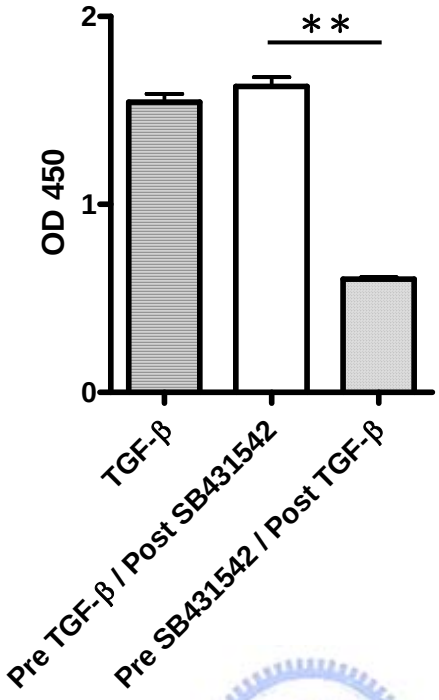
c.


1.5
1.0
0.5
0.0
OD 450
**
**
*
TGF-β1 + + + +
HpHsp60 (μg) 0 1.7 8.5 17

d.


2
1
0
OD 450
**
TGF-β
Pre TGF-β / Post HpHsp60
Pre HpHsp60 / Post TGF-β

e.


**
2
1
0
OD 450
TGF-β
Pre TGF-β / Post SB431542
Pre SB431542 / Post TGF-β

f.

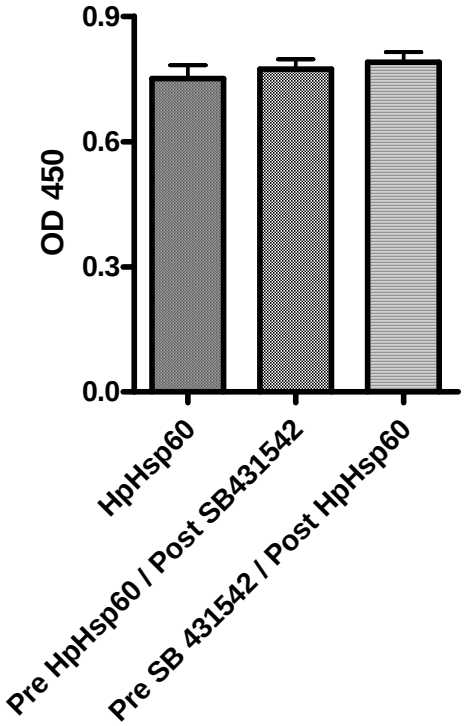

0.9
0.6
0.3
0.0
OD 450
HpHsp60
Pre HpHsp60 / Post SB431542
Pre SB 431542 / Post HpHsp60

Fig. 4 HpHsp60 binding to human TGF-β receptor II.

(a) HpHsp60 was incubated with human TGF-βRII-coated wells. Attached HpHsp60 to the coated proteins were detected by mouse antiserum against HpHsp60 and peroxidase-conjugated goat anti-mouse IgG. (b) A variety of HpHsp60 dosages containing 0.03, 0.1, 0.3, 0.9, & 2.7 μg were incubated with human TGF-βRII-coated wells and detected the HpHsp60 binding pattern. (c) Proteins with different HpHsp60/TGF-β1 ratios including 0, 10, 50, and 100 were mixed and added into human TGF-βRII-coated wells, bound TGF-β1 was detected by biotinylated chicken anti-human TGF-β1 antibody (1:180) (R&D Systems, MN, USA) and Strptavidin-HRP (1:200) (R&D Systems, MN, USA). (d) Either HpHsp60 or TGF-β1 was pre-incubated with TGF-βRII-coated wells followed by TGF-β1 or HpHsp60 incubation and bound TGF-β1 was detected. (e) Either SB431542 or TGF-β1 was pre-incubated with TGF-βRII-coated wells followed by TGF-β1 or SB431542 incubation and bound TGF-β1 was detected. (f) Either HpHsp60 or SB431542 was pre-incubated with TGF-βRII-coated wells followed by SB431542 or HpHsp60 incubation and bound HpHsp60 was detected. Results are the average of duplicate experiments. (**, $P < 0.01$).

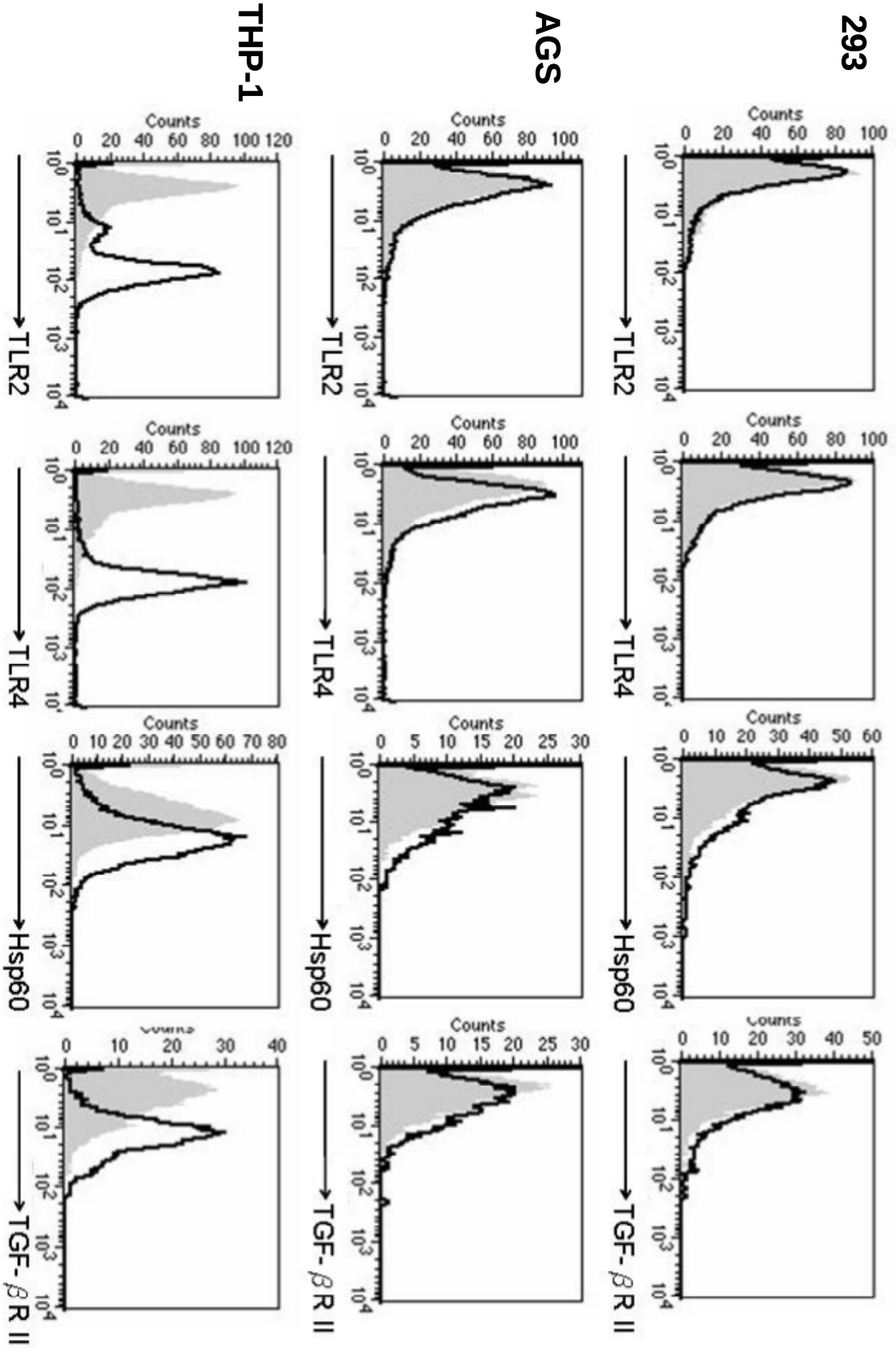

293
AGS
THP-1
TLR2
TLR4
Hsp60
TGF-βR II
Counts

Fig. 5 HpHsp60 binding patterns and TLR2, TLR4, TGF-βRII expression profiles of HEK293, AGS, and THP-1 cells.

HEK293, AGS, and THP-1 cells were detected by anti-TLR2, anti-TLR4 or anti- TGF-βRII Ab and the fluorescence related to immunolabeling was then measured by flow cytometer.

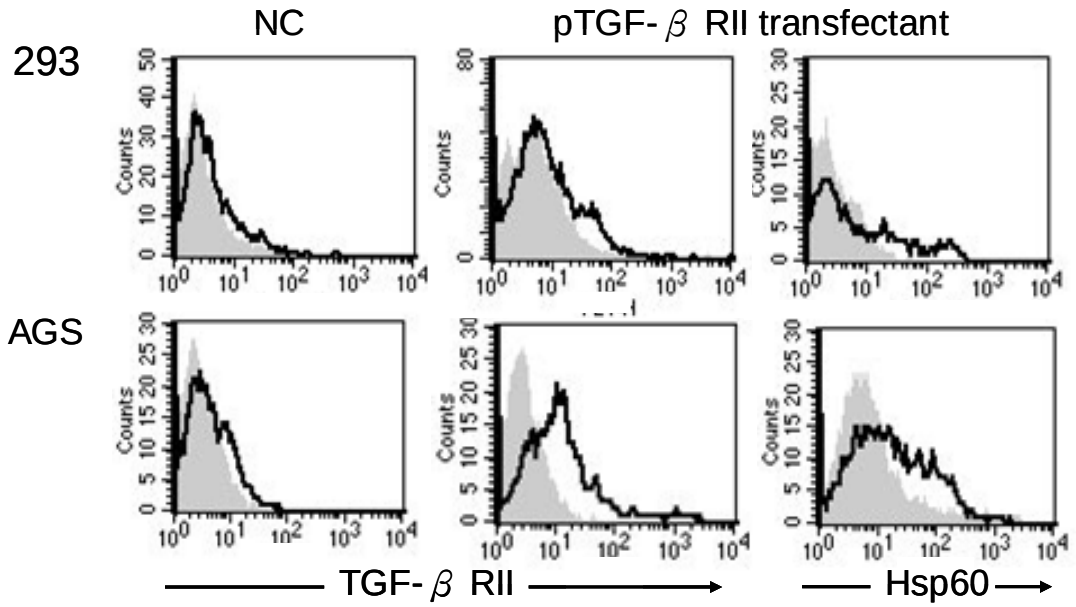


Fig. 6 TGF-βRII expression and HpHsp60 binding pattern of pTGF-βRII transfected HEK293 and AGS cells.

HEK293 and AGS cells were transfected with pTGF-βRII gene and the TGF-βRII expression was confirmed by anti-TGF-βRII antibody detection. The binding of HpHsp60 to HEK293 or AGS transfectants were also verified by mouse antiserum against HpHsp60 and fluorescein-conjugated goat anti-mouse IgG. The fluorescence was assayed by flow cytometer.

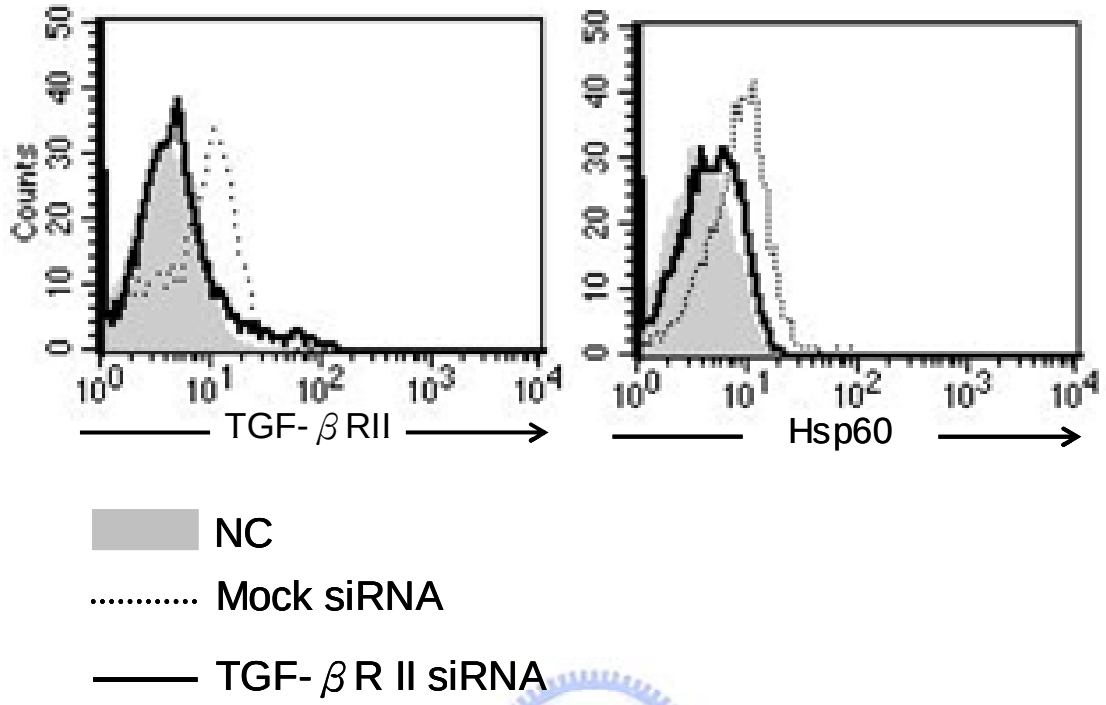


Fig. 7 TGF-βRII expression and HpHsp60 binding pattern of TGF-βRII siRNA-treated THP-1 cells.

THP-1 cells transfected with siRNA specifically silencing TGF-βRII expression was confirmed by anti-TGF-βRII antibody detection. The binding of HpHsp60 to THP-1 transfectants were verified by mouse antiserum against HpHsp60 and fluorescein-conjugated goat anti-mouse IgG. The fluorescence was assayed by flow cytometer.

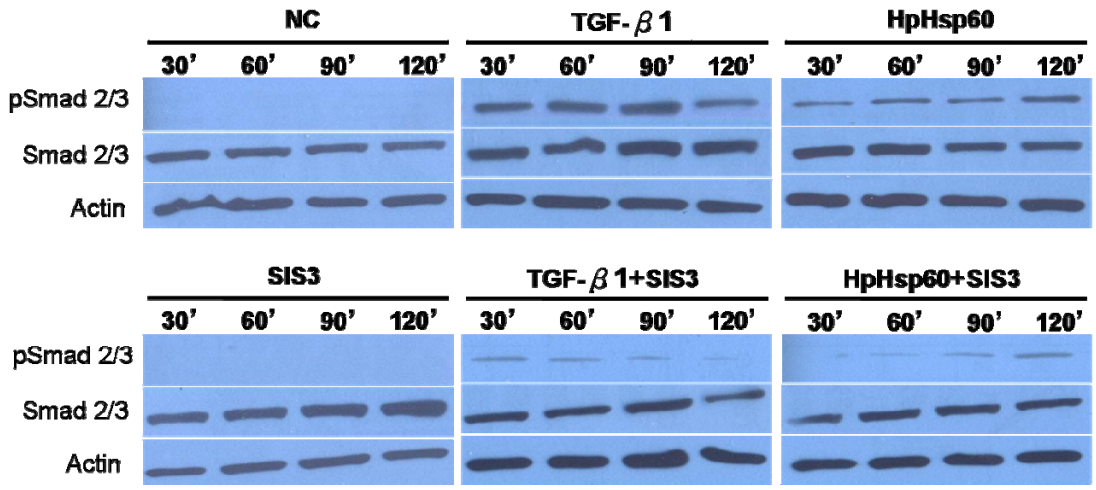
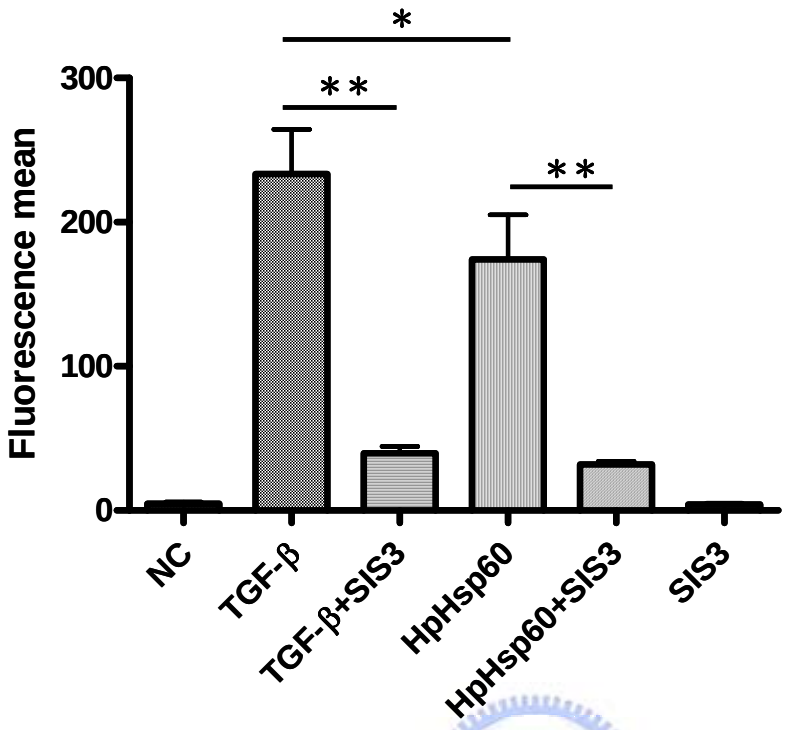


Fig. 8 The effects of HpHsp60 on SMAD 2/3 phosphorylation of THP-1 cells

THP-1 cells were treated either with TGF-β1 (1 ng/ml), HpHsp60 (10 μg/ml), TGF-β1 and SMAD2/3 specific inhibitor SIS3 (10 μM), HpHsp60 and SIS3, or SIS3 alone. Cell lysates were collected at 30', 60', 90' and 120 min to be performed the western blot.

a.


*
**
**
Fluorescence mean
300
200
100
0
NC
TGF-β
TGF-β+SIS3
HpHsp60
HpHsp60+SIS3
SIS3

b.

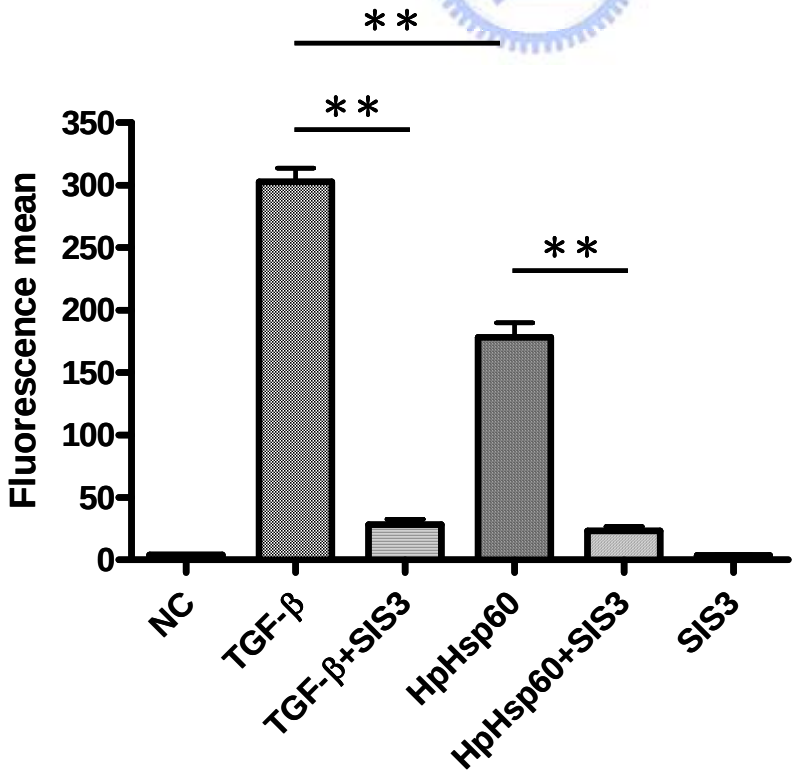
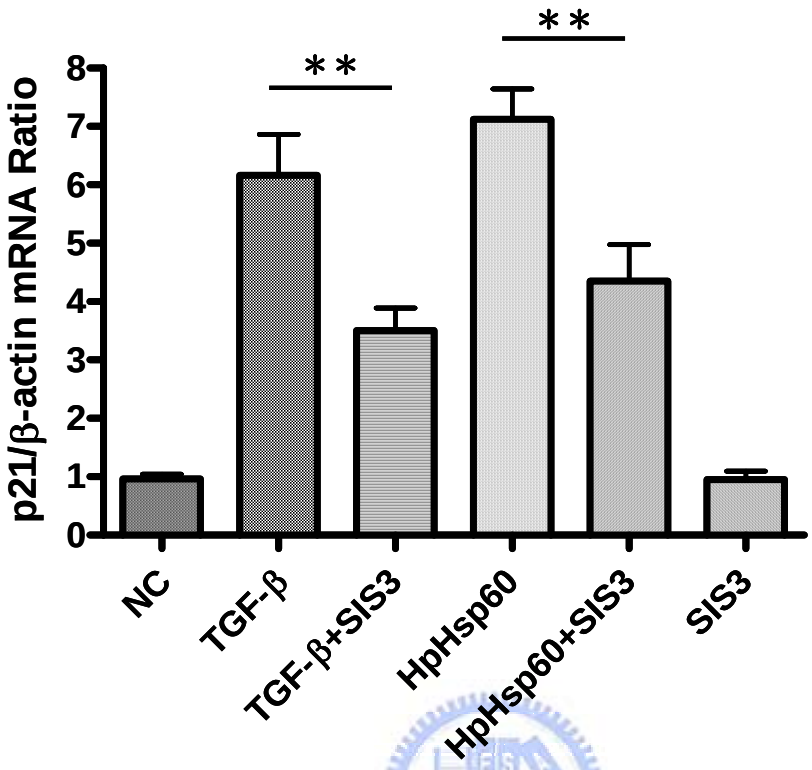

**
**
**
Fluorescence mean
350
300
250
200
150
100
50
0
NC
TGF-β
TGF-β+SIS3
HpHsp60
HpHsp60+SIS3
SIS3

Fig. 9 The effects of HpHsp60 on SMAD transcriptional activity. HEK293 and AGS cells were transiently transfected with SMAD-dependent reporter plasmid (pSMAD 2/3/4-hrGFP) and then treatment either TGF-β1 (1 ng/ml), HpHsp60 (10 μg/ml), TGF-β1 and SMAD2/3 specific inhibitor SIS3 (10 μM), HpHsp60 and SIS3, or SIS3 alone. The cells were harvested and the fluorescence was analyzed with flow cytometer. Data are expressed as means ± SEM for four replicates from one of two representative experiments. (**, $P < 0.01$).

a.


p21/β-actin mRNA Ratio
0
1
2
3
4
5
6
7
8
**
**
NC
TGF-β
TGF-β+SIS3
HpHsp60
HpHsp60+SIS3
SIS3

b.

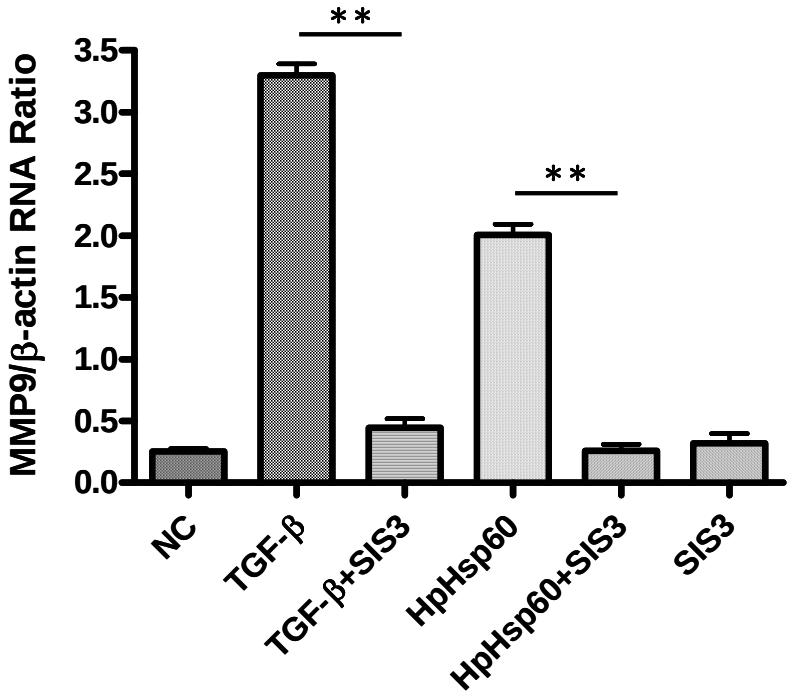
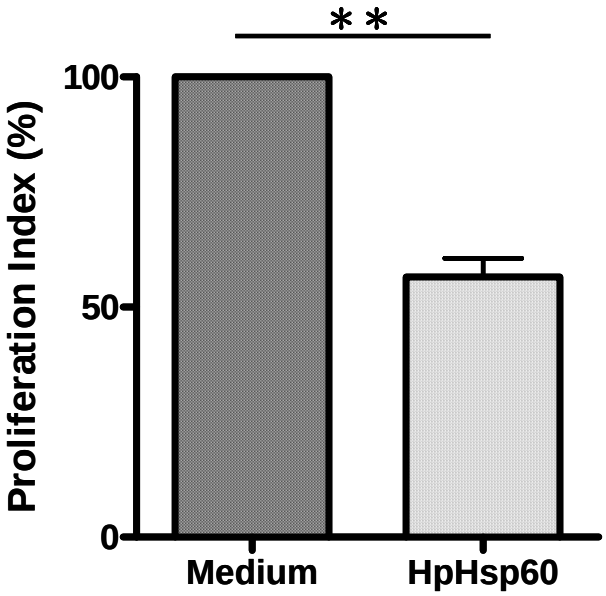

MMP9/β-actin RNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
**
**
NC
TGF-β
TGF-β+SIS3
HpHsp60
HpHsp60+SIS3
SIS3

Fig. 10 Relative gene expression levels in HpHsp60-treated THP-1 cells detected by quantitative RT-PCR.

THP-1 cells were treated with either TGF-β1 (1 ng/ml), HpHsp60 (10 μg/ml), TGF-β1 and SMAD2/3 specific inhibitor SIS3 (10 μM), HpHsp60 and SIS3, or SIS3 alone for 6 hours and the cells were harvested for mRNA extraction and real-time PCR assay. The expression of the target gene was normalized with respect to β-actin served as an internal control gene. Relative fold expression was determined using the $2^{-\Delta\Delta Ct}$ method. (a) P21; (b) MMP9. Data are expressed as means ± SEM for three replicates from one of two representative experiments. (**, $P < 0.01$.)

a.


**
100
50
0
Proliferation Index (%)
Medium
HpHsp60

b.

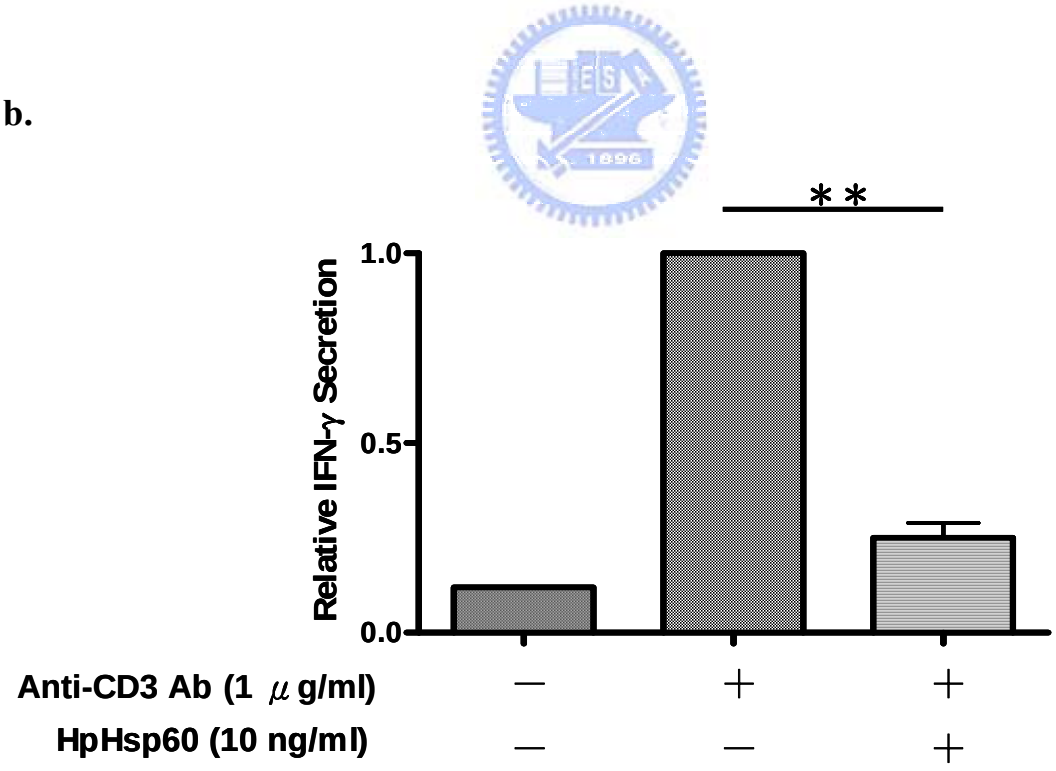
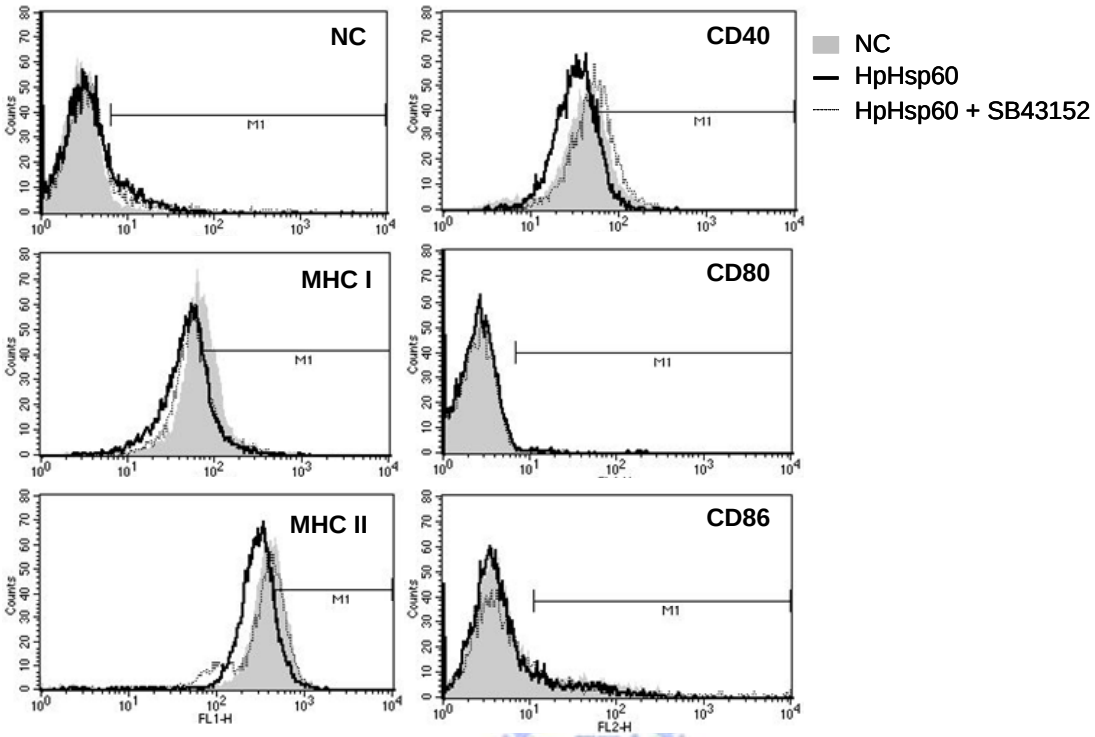
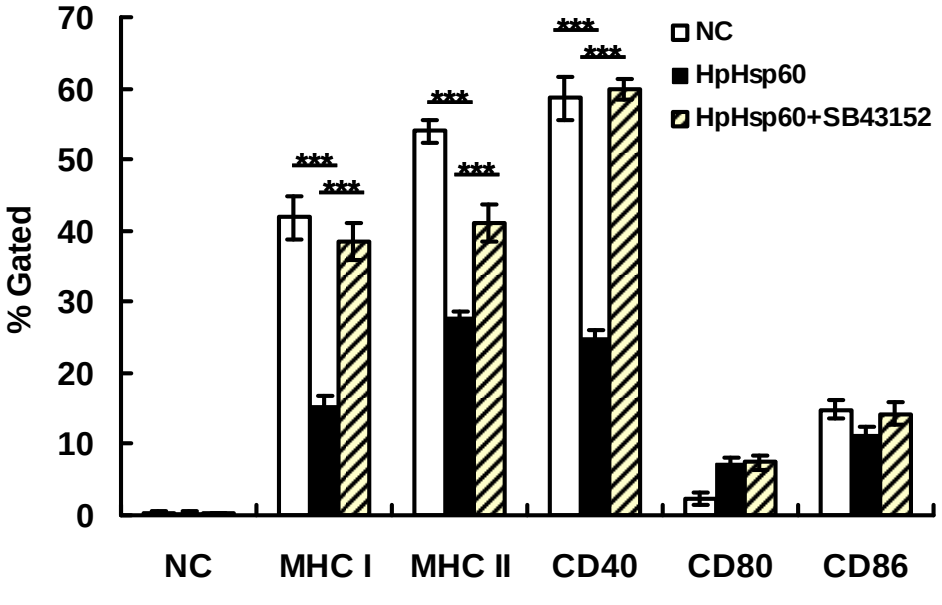

**
1.0
0.5
0.0
Relative IFN-γ Secretion
Anti-CD3 Ab (1 μg/ml)
HpHsp60 (10 ng/ml)
−
+
+
−
−
+

Fig. 11 The effects of HpHsp60s on PBMCs proliferation and IFN-γ production

(a) PBMCs (10^4 cells/well) were seeded in the anti-CD3 mAb coated 96-well plate and treated with 10 μg/ml HpHsp60 for 4 days at 37°C, 5% CO2 incubator. After incubation, MTS reagent was added into each well for 4 h development to investigate cell proliferation. Data were calculated with following equation: Proliferation index (%) = OD 490 of sample/ mean of OD 490 from control (cells stimulated with anti-CD3 mAb). (b) PBMCs (2×10^5 cells/well) were seeded in the anti-CD3 mAb coated 24-well plate and treated with 10 μg/ml HpHsp60 for 24 hous at 37°C, 5% CO2 incubator. The supernatants were harvested for assaying IFN-γ and the data were processed with following equation: Relative IFN-γ secretion = IFN-γ concentration (conc.) of sample/ mean of IFN-γ conc. from control (cells stimulated with anti-CD3 mAb). Data are the results of three independent experiments and expressed as means ± SEM. (**, $P < 0.01$.)

a.


NC
CD40
MHC I
CD80
MHC II
CD86
M1
Counts
FL1-H
FL2-H
NC
HpHsp60
HpHsp60 + SB43152


% Gated
NC
MHC I
MHC II
CD40
CD80
CD86
***
NC
HpHsp60
HpHsp60+SB43152

b.

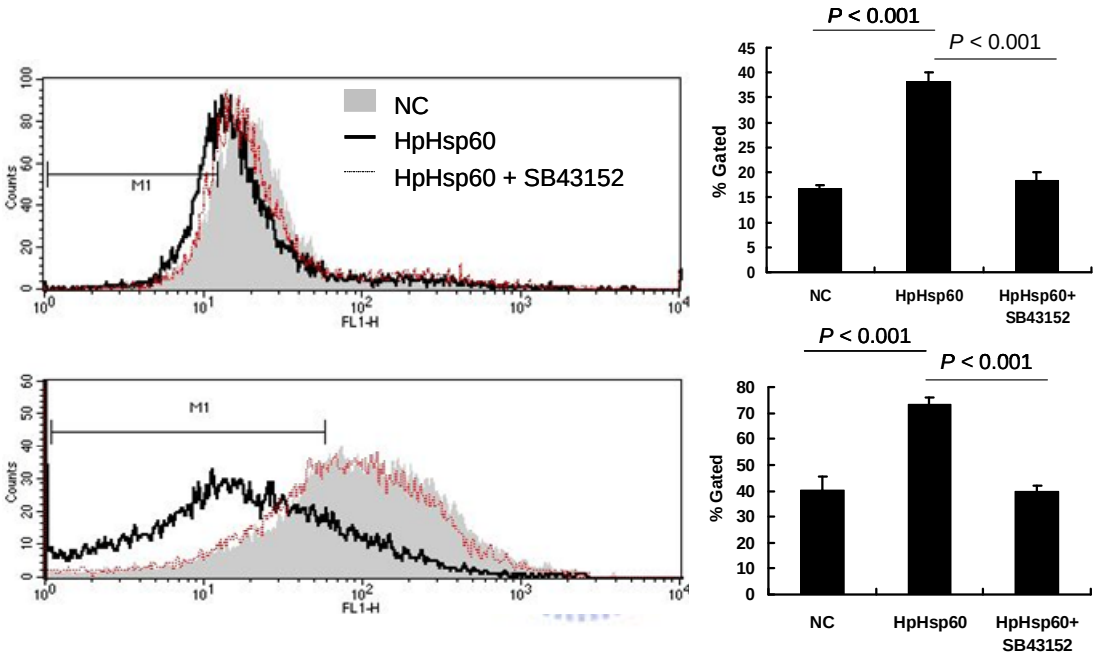

NC
HpHsp60
HpHsp60 + SB43152
M1
Counts
FL1-H
P < 0.001
P < 0.001
% Gated
NC
HpHsp60
HpHsp60+ SB43152
P < 0.001
P < 0.001
% Gated
NC
HpHsp60
HpHsp60+ SB43152

Fig. 12 Antigen uptake and expression of THP-1 cell surface molecules in the presence of HpHsp60.

(a) THP-1 cells were previously treated with SB431542 (10 μM) for 30 minutes, followed by the addition of HpHsp60 (10 μg/ml) into THP-1 cells for the remaining 16-h incubation. Expression of surface markers of THP-1 cells was examined by FACS. (b) THP-1 cells were incubated with FITC-dextran or FITC-labeled *E.coli* at 4℃ or 37℃ for 2 hours to measure endocytosis and phagocytosis. Fluorescence showed dextran or *E.coli* internalization and no staining was observed after incubation at 4℃ (not depicted). Data are expressed as means ± SEM for four replicates from one of three representative experiments. (***, $P < 0.001$)

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