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幽門螺旋桿菌之熱休克蛋白60誘導前發炎性細胞激素的分泌但卻抑制單核球細胞之活性

Heat Shock Protein 60 of *Helicobacter pylori* Induces Proinflammatory Cytokines Secretion but Diminishes Monocyte Activation

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幽門螺旋桿菌之熱休克蛋白 60 在之前的文獻中被發現它可以誘導胃上皮細胞及人類單核球細胞產生介白素-8 (IL-8)，以及巨噬細胞產生介白素-6。在此研究中，我們將幽門螺旋桿菌之熱休克蛋白 60 (rHpHSP60) 與人類單核細胞株 THP-1 共同培養，發現除了介白素-8 以及介白素-6 之外，腫瘤壞死因子-甲型以及介白素-1 乙型都會被 THP-1 產生。腫瘤壞死因子-甲型與介白素 1 乙型與炎症反應的開始有很大的關連。其他生物的熱休克蛋白雖然也會誘導前發炎細胞激素(pro-inflammatory cytokines)的產生，但 pro-inflammatory cytokines 產生的時間卻不盡相同。在此篇研究中發現，腫瘤壞死因子-甲型在受到 rHpHSP60 刺激後兩小時就開始顯著的產生而在 4 小時產量最高；而介白素-1 乙型，介白素-6，介白素-8 會隨著時間延長而小幅增加，直到 24 小時才大量的表現。腫瘤壞死因子-甲型在這些細胞激素中最早產，因此對單核球細胞的活化最具影響力。我們藉由偵測單核球細胞的吞噬能力以及細胞表面抗原之表現來觀察細胞的活化。結果顯示，細胞的吞噬活性明顯下降而且主要組織相容性複合體 II (MHCII) 的表現也顯著性的下降。而共同刺激分子 CD40、CD80、CD86 會顯著性的升高，而主要組織相容性複合體 I(MHCI)表現則無改變。我們更進一步用商業化之人類腫瘤壞死因子-甲型觀察其對單核球細胞活化的影響，結果顯示隨著商業化之人類腫瘤壞死因子-甲型的增加，單核球細胞的吞噬能力竟然減弱了，而共同刺激分子 CD40 的表現則如我們預期的增加了。在胃部慢性發炎的病人中發現轉化生長因子－β1 (TGF-β1)會大量表現；為了模擬胃部慢性發炎的環境，我們將商業化之人類腫瘤壞死因子-甲型與轉化生長因子－β1同時刺激單核球細胞，結果發現腫瘤壞死因子-甲型可協助轉化生長因子－β1造成的抑制作用，轉化生長因子－β1則更進一步抑制由腫瘤壞死因子-甲型引起的單核球 CD40 之表現，阻礙其成熟與分化。根據以上的結果顯示，幽門螺旋桿菌之熱休克蛋白 60 會刺激單核球細胞產生介白素-1 乙型，介白素-6，介白素-8 以及腫瘤壞死因子-甲型；其中，腫瘤壞死因子-甲型最早產生，但單核球細胞之活性卻仍受到抑制。在我們的研究中，腫瘤壞死因子-甲型並非扮演活化性的角色反而是抑制單核球細胞的活性。在慢性發炎反應中，腫瘤壞死因子-甲型結合轉化生長因子－β1 對於單核球細胞的活化造成更嚴重的影響。

中華民國九十七年七月

Heat Shock Protein 60 of *Helicobacter pylori* Induces Proinflammatory Cytokines Secretion but Diminishes Monocyte Activation

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ABSTRACT

Heat shock protein 60 of *Helicobacter pylori* has been found that it can induce interleukin-8 (IL-8) secretion in human monocytic cells and gastric epithelium cells. In this study, we further found that the IL-6, IL-8, TNF-α and IL-1β were induced in THP-1 cells after *H. pylori* HSP60 stimulation. The kinetic of cytokine expression showed that TNF-α was earliest secreted at 2 h, and reached a maximum at 4 h. This result consisted with the kinetic of TNF-α mRNA expression analyzed by quantitative real-time PCR. TNF-α may have a great effect on THP-1 cells activation. Dissimilarly, IL-1β, IL-6, and IL-8 were later produced by THP-1 cells. We further examined THP-1 cells activation by detecting its enodocytosis activity and surface marker expression. Surprisingly, the endocytosis ability of THP-1 cells was weakened after rHpHSP60 stimulation. However, the co-stimulatory molecules (CD40, CD80, and CD86) were up-regulated, whereas MHC class II which plays a central in presenting the foreign antigen to T helper cells was significantly down-regulated. MHC I expression was not influenced by rHpHSP60. Interestingly, the rhTNF-α mimicry experiments indicated that the endocytosis activity of THP-1 cells was diminished by rhTNF-α in a does-dependent manner. However, it can promote CD40 expression on THP-1 cells surface. To mimic the chronic inflammation area of *H. pylori*-infected patients, rhTNF-α and TGF-β1 were used to treat THP-1 cells. The inhibitory effect on endocytotic activity of THP-1 cells was observed by rhTNF-α and TGF-β1 synergy treatment. TNF-α seemed to synergize with TGF-β1 to decrease the engulf ability of cells. However TGF-β1 further inhibited TNF-α-mediated CD40 expression. This study suggested rHpHSP60 induced TNF-α, IL-1β, IL-6, and IL-8 secretion in THP-1 cells. Among these cytokines, TNF-α was earliest secreted. Even through the endocytosis ability of THP-1 cells was inhibited and the MHC class II was significantly decreased after rHpHSP60 stimulation. The role of TNF-α in our study was not an “effector” on THP-1 cells activation but diminished its activity. In the chronic inflammation, the inhibition effect of TNF-α combining with TGF-β1 on monocytes activation was more critical.

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List of abbreviation

APCs	Antigen presenting cells
Cag A	Cytotoxin-associated gene A
DC	Dendritic cells
HSP	Heat shock protein
IFN-γ	Interferon -γ
Ig	Immunoglobulin
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MDNCF	Monocyte-derived neutrophil chemotactic factor
MHC	Major histocompatibility complex
NCF	Neutrophil chemotactic factor
NAP-1	Neutrophil attractant/activating peptide-1
PAI	Pathogenicity island
PMN	Polymorphonuclear neutrophil leukocytes
RNIs	Reactive nitrogen intermediates
TCR	T cell receptor
Th	T helper cells
TNF-α	Tumor necrosis factor-α
TLR	Toll-like receptor
VacA	Vacuolating cytotoxin A

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Chapter 1 Introduction

1-1 *Helicobacter pylori*

Helicobacter pylori is a well-known gastric-parasitical pathogen. Since 1982, Dr. Marshell swallowed ten hundred million of *H. pylori* personally to prove that the persistence of *H. pylori* in stomach can result in some kind of gastric diseases, and lead a new epoch in human gastroenterology. *H. pylori* has been found in human in all parts of the world, with over half of the world's population infected with *H. pylori*. In developing countries, 70-90 % of the population caries *H. pylori*. In 20-30 % of cases, the end result of the infection can be life-threatening (1). Many publishes revealed that *H. pylori* infection was associated with acute or chronic gastritis, peptic ulcer, gatroduodenal ulcer, and gastric cancer development, which led to *H. pylori* becoming classified as a class Ⅰ carcinogen by the World Health Organization. The complete process of *H. pylori* invasion, infection, and proliferation is really complex. Host immune surveillance system plays an essential role in pathogen elimination at early or late stage. However, *H. pylori* seems not be effectively cleared by host immune system and persist in host stomach for a half life time. Recently, scientists work hard to investigate many subversives of *H. pylori* and found that *H. pylori* can utilize multiple factors to protect them living in host stomach where a horrible environment for most pathogens is.

1-1.1 The morphology of *Helicobacter pylori*

H. pylori organisms are spiral, microaerophilic, gram-negative bacteria that colonizes the gastric mucosa of humans. In gastric biopsy specimens, *H. pylori* organisms are 2.5 to 5.0 μm long and 0.5 to 1.0 μm wide; there are four to six unipolar sheathed flagella, which are essential for bacterial motility. Moreover, the surface of individual bacteria may be linked to gastric epithelial microvilli by thread-like extensions of the glycocalyx (2, 3). Interestingly, *H. pylori* is classified as a noninvasive bacterial organism because it typically does not traverse the epithelial barrier (4). Nevertheless, the bacterium is able to induce strong immune responses in such environment and results in some kind of gastric disease.

1-1.2 *H. pylori*-associated diseases

Colonization with *H. pylori* is not a disease in itself but a condition that affects the relative risk of developing various clinical disorders of the gastrointestinal tracts.

1-1.2.1 Acute gastritis:

Several reports showed that the acute phase of colonization with *H. pylori* may be associated with transient nonspecific dyspeptic symptoms, such as fullness, nausea, and vomiting, and with great inflammation of stomach mucosa. This phase is often associated with hypochlorhydria and it is unclear whether this initial colonization can be cleared spontaneously and prevents gastritis occurrence (5, 6).

1-1.2.2 Chronic gastritis:

Colonization with *H. pylori* always results in infiltration of the gastric mucosa with neutrophilic and mononuclear cells. *H. pylori* colonization relates to the chronic active gastritis, and other *H. pylori*-associated disorders result from this chronic inflammatory process. When colonization becomes persistent, a close correlation exists between the level of acid secretion and the distribution of gastritis. In subjects with intact acid secretion, *H. pylori* in particular colonizes the gastric antrum, where few acid-secretory-parietal cells are present. The pattern is associated with an antrum-predominant gastritis. Subjects with impaired acid secretion have a distribution of bacteria in antrum and corpus. The corpus bacteria in corpus are in closer contact with the mucosa, leading to a corpus-predominant pangastritis (7).

1-1.2.3 Peptic ulcer disease:

Duodenal ulcers (peptic ulcer) are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa. Duodenal ulcers usually occur in the duodenal bulb, which is the area most exposed to gastric acid. Both gastric and duodenal ulcer diseases are strongly related to *H. pylori.* It was showed that approximately 95 % of duodenal ulcers and 85 % of gastric ulcers occurred in the presence of *H. pylori* infection (8).

1-1.2.4 Atrophic gastritis, intestinal metaplasia, and gastric cancer:

Chronic *H. pylori*-induced inflammation can eventually lead to loss of normal mucosal architecture, with destruction of gastric glands and replacement by fibrosis and intestinal-type

epithelium. The risk atrophic gastritis depends on the distribution and pattern of chronic active inflammation (9). Patients with decreased acid output show a more rapid progression towards atrophy (10). It was reported that the risk of gastric cancer development via the sequence of atrophy and metaplasia, and the development of atrophy and cancer in the presence of *H. pylori* is related to host and bacterial factors, which influence the severity of the chronic inflammatory responses (6).

1-1.2.5 Gastric MALT lymphoma:

The gastric mucosa does not normally contain lymphoid tissue, after *H. pylori* infection, a lymphoid infiltrate appears, which constitute a chronic gastritis. In certain cases the lympoid tissue can be organized as lymphoid follicles. MALT lymphoma emerges from these lymphoid structures (11). The *in vitro* experiment showed that T lymphocytes sensitized for *H. pylori* produce cytokines which stimulate B lymphoid proliferation. It is a B cell lymphoma with a very unusual pathogenesis and evolution which slowly progresses and stays localized in the stomach for a long time (12).

1-1.3 The virulence factors resulting in *H. pylori* colonization and pathogenicity

1-1.3.1 The *cag* PAI and Cag A protein:

An intact *cag* PAI, which is associated with severe disease (13), encodes 31 proteins, which form a type IV secretion system capable of directly transferring bacterial proteins to the

cytoplasm of target cells, and it can stimulate human gastric epithelium cells to secrete IL-8, a mediator of serious gastric inflammation. (reviewed in Ref.(14)). Recntly, Nalini Ramarao *et al.* further investigated that type IV transporter of *H. pylori* is essential in preventing phagocytosis. The *H. pylori cag* PAI mediates the translocation of an effector protein, CagA, into gastric epithelial cells, and might also be directly involved in loosening of tight junctions (15). Furthermore, infection with *H. pylori* strains possessing CagA is associated with an increased risk of developing adenocarcinoma of the stomach (16).

1-1.3.2 VacA:

VacA is a secreted protein toxin, which causes vacuolar degeneration of epithelial cells in vitro and gastric epithelial erosion in vivo. In addition, VacA can loosen tight junctions in monolayers of polarized epithelial cells (17). A recent study showed that VacA alters the intracellular trafficking of proteins, increases the permeability of polarized epithelial cells, inhibits the process of antigen presentation, forms anion-selective channels in lipid bilayers, and interferes with cytoskeleton-dependent cell functions (18).

1-1.3.3 Urease:

The *H. pylori* urease can break down urea (CN_2H_4O) to form NH_3 and CO_2, which buffer the microenvironment and the cytosol of the bacteria (19). Furthermore, *H. pylori* urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokines production from immune cells and gastric epithelium cells (20) and the urease activity is also toxic to

human gastric epithelium cells (21).

1-1.3.4 NAP:

Helicobacter pylori neutrophil activating protein is also called HP-NAP. The main evidence supporting a role for HP-NAP in virulence is the ability to activate neutrophils to produce oxygen free radicals and adhere to cultured endothelial cells (22), however, oxygen free radicals production will result in gastric tissue damage in the future. NAP released by bacterial lysis directly interacts with neutrophils, monocytes, and mast cells, resulting in the activation of their inflammatory functions (23).

1-1.3.5 Arginase:

H. pylori produces an arginase that uses arginine to produce urea and L-ornithine. Furthermore, bacterial arginase allows *H. pylori* to evade the immune response by down-regulate eukaryotic NO production (24).

1-1.3.6 Cell wall and lipopolysaccharide:

Urease and HspB, a homolog of the GroEL protein of *Escherichia coli*, are abundant in outer membrane proteins (OMP) preparations. Urease and HspB are located strictly within the cytoplasm in early log phase cultures of H. pylori (25). However, in late-log-phase cultures, urease and HspB become associated with the bacterial surface in a novel manner. These cytoplasmic proteins are released by bacterial autolysis and become adsorbed to the surface of intact bacteria due to the unique characteristics of the outer membrane. The

lipopolysaccharide (LPS) of *H. pylori* has low biological activity, a property which may aid in the persistence of infection. *H. pylori* LPS disrupts the gastric mucus coat by interfering with the interaction between mucin and its mucosal receptor (26). However, the outstanding feature of the *H. pylori* LPS is its low proinflammatory activity.

1-1.3.7 Heat shock protein 60:

H. pylori HSP60 has been shown to play a role in the adherence and attachment of *H. pylori* to gastric epithelium and induce IL-8 secretion from human gastric epithelial cells (27, 28). In the immune cells, *H. pylori* HSP60 can induce IL-6 and IL-8 from macrophages and monocytes, respectively (29, 30). Chronic gastritis is initiated and maintained by cytokines that are secreted by gastric epithelial cells and macrophages. Interleukin 8 (IL-8) is one of the principal mediators of the inflammatory response. Moreover, Kobayashi *et al.* showed that development of lymphoid tissue in patients with MALT lymphoma was associated with HSP60 (31).

1-1.4 Interactions between *H. pylori* and immune cells

Neutrophils: Neutrophils are recruited when *H. pylori* initially colonizes the human stomach, and the gastric mucosal inflammatory response that occurs in the persistent *H. pylori* infection is characterized by infiltration of neutrophils. Several specific *H. pylori* factors are known to interact with neutrophils and modulate their function (32).

Mast cells: In vitro experiments indicate that whole *H. pylori* bacteria and various *H. pylori* components can activate mast cells. One *H. pylori* factor that can activate mast cells is VacA (33). VacA can induce mast cell chemotaxis and can stimulate mast cell expression of multiple proinflammatory cytokines, including IL-1, TNF-α, IL-6, IL-13, and IL-10 (34). VacA induces degranulation of the mast cell line but does not induce degranulation of murine bone marrow-derived mast cells. HP-NAP also can activate mast cells, resulting in IL-6 production. Activation of mast cells by *H. pylori* may contribute to the inflammatory response associated with *H. pylori* infection (35).

Macrophages: Contact between macrophages and intact *H. pylori* bacteria or *H. pylori* components results in macrophage activation and secretion of numerous cytokines and chemokines. Macrophage recognizes the intact *H. pylori* by mediating TLR2 or TLR4. Ingested *H. pylori* cells have at least some ability to resist intracellular killing. Another mechanism of *H. pylori* escaping from macrophage killing is by blocking the production of nitric oxide. This effect is mediated by *H. pylori* arginase, which competes with nitric oxide synthase for arginine. In addition to resisting killing by macrophages, in vitro experiments indicated that *H. pylori* can induce macrophage apoptosis (36). *H. pylori*-induced apoptosis of macrophages may result in impaired innate and adaptive immune responses.

Dendritic cells: In response to *H. pylori*, monocyte-derived human DCs express costimulatory molecules and major histocompatibility complex class II proteins (37), which

results in increased efficiency of antigen presentation. Similar to several other bacterial pathogens, *H. pylori* can bind to DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), a DC-specific lectin (38). Interactions between *H. pylori* antigens and DC-SIGN may contribute to suppress the inflammation.

B lymphocytes: *H. pylori* is reported to have several inhibitory effects on B lymphocytes. In one study, *H. pylori* VacA interfered with the prelysosomal processing of tetanus toxin in Epstein-Barr virus-transformed B cells, and the ability of these cells to stimulate human $CD4^+$ T cells were impaired in the presence of VacA. VacA inhibited the Ii-dependent pathway of antigen presentation mediated by newly synthesized MHC class II molecules but did not affect the pathway dependent on recycling MHC class II (39). Expression of CagA in B cells is reported to inhibit interleukin-3-dependent B-cell proliferation by inhibiting JAK-STAT signaling, which may result in inefficient antibody production and reduced cytokine expression (40).

T lymphocytes: One report indicated that *H. pylori* can have proapoptotic effects on T cells (41), but most of the observed effects occur in the absence of cell death. Coincubation of *H. pylori* with T cells results in diminished expression of IL-2 and IL-2 receptor (CD25), inhibition of activation-induced proliferation, and cell cycle arrest (42). The effects of *H. pylori* on T cells are mediated by several different bacterial factors, one of which is VacA. VacA interferes with the activity of nuclear factor of activated T cells (NFAT), a transcription

factor that regulates immune response genes, in Jurkat T cells, resulting in inhibition of IL-2 expression and G1/S cell cycle arrest (42). In addition to VacA and arginase, an uncharacterized low-molecular-weight protein of *H. pylori* has been reported to inhibit proliferation of T lymphocytes. This low-molecular-weight *H. pylori* factor is reported to block cell cycle progression at the G1 phase (43).

1-1.5 Immune activation and cell damage by *H. pylori* infection

The human gastric luminal pH is < 2, which prevents the proliferation of bacteria within the gastric lumen. *H. pylori* penetrates the gastric mucus layer after entering host stomach and thereby encounters a less acidic environment. *H. pylori* typically does not traverse the epithelial barrier, and it is classified as a noninvasive bacterial organism (4). Nevertheless, the bacterium is able to induce strong pro-inflammatory responses in these cells. Since *H. pylori* adherence, the production of a vacuolating cytotoxin and bacterial enzymes all contribute to epithelial damage. *H. pylori* infection, irrespective of their *cag* PAI phenotype leads to chronic gastric inflammation in the host. Recruitment and activation of immune cells in the underlying mucosa involves *H. pylori* chemotaxins, epithelial-derived chemokines such as IL-8 and pro-inflammatory cytokines liberated by mononuclear phagocytes (TNF-α, IL-1 and IL-6) as part of non-specific immunity. Moreover, gastric epithelial cells up-regulate expression of major histocompatibility complex (MHC) class II and costimulatioy molecules

on mococytes, macrophages, and dendritic cells in the gastric mucosa also play an important role in antigen presentation to activate adaptive immune cells activation (44). However, the infiltrated immune cells-induced inflammation response appears to be a primary cause of the damage to gastric surface epithelial layers and finally resulted in gastritis, peptic ulcer disease, and gastric cancer (40).

1-1.6 Immune subversion by *H. pylori*

Once arriving at the gastric epithelium, *H. pylori* must face the rapid onslaught of effector cells of the strong immune response. To overcome continually intense attack, *H. pylori* utilize some virulence factors to break host immune defense and successfully escape from killing by effector cells. In the innate immune stage, *H. pylori* first attack by nitrogen oxide (NO), which is an important component of innate immunity and an effective antimicrobial agent. To avoid killing by NO, *H. pylori* produces an arginase to regulate NO synthesis. Arginase can convert L-arginine to urea and L-ornithine, because L-ornithine is also used by iNOS to produce NO so that arginase can compete with iNOS for their substrate to decrease NO production. Even though *H. pylori* was unfortunately ingested by professional phagocytes, it is capable to resist phagocytic killing. Phagocytosis of *H. pylori* by macrophages becomes a large megasomes, which result from homotypic phagosome fusion and subsequent macrophage apoptosis might enable the escape of the bacteria. Interestingly,

the LPS of *H. pylori* is at least 1000-fold less active than *E. coli* LPS. The VacA protein of *H. pylori* is contributed to disrupt host adaptive immune response. Clear evidence has recently been obtained for VacA in suppression of T-cell response. Sundrud *et al.* showed that VacA inhibits human peripheral blood lymphocytes proliferation by TCR-CD28 co-stimulation by interfering with IL-2-dependent cell cycle progression. Gastric MALT lymphoma results from the uncontrolled polyclonal expansion of IgM memory B cells, T-cells inactivation might also contribute to the abnormal B-cell growth. Moreover, Cag A is also capable of preventing B-cell apoptosis by inhibiting p53 accumulation, which might involved in development of MALT lymphoma.

1-1.7 Chronic infection

Levels of numerous cytokines, including gamma interferon (IFN-γ), tumor necrosis factor (TNF), IL-1β, IL-6, IL-7, IL-8, IL-10, and IL-18, are increased in the stomachs of *H. pylori*-infected humans compared to uninfected humans (45). These cytokines have great effect on immune cells activity and attract these effector cells to the inflammation site. The concentration of various types of leucocytes was detected in gastric mucosal biopsies from human infected with *H. pylori.* Lymphocytes (both T cells and B cells), macrophages, neutrophils, mast cells, and dendritic cells (DCs) are usually present at the inflammation area and play an important role in antigen presentation (46). The relative abundance of

IFN-γ-producing T cells and the relative scarcity of IL-4-producing gastric T cells in the setting of *H. pylori* infection, it has been concluded that *H. pylori* infection leads to a Th1-polarized response (47). The chronic gastric mucosal inflammatory response to *H. pylori* probably reflects the combined effects of a cellular immune response and an ongoing stimulation of an innate immune response.

1-2 *Human innate immunity*

The human immune system defends against a spectrum of microbial pathogens, in terms of environmental prevalence, rang from common to rare. Invasion by common environmental microbes is prevented by constitutive innate immune defense in mucosal and epithelial tissues. Upon infection with highly virulent pathogens, auxiliary innate defenses are induced to combat the pathogens. Neutrophils, monocytes, macrophages, and dendritic cells are important cellular mediators of innate immune defense.

1-2.1 The characterizations of innate immune cells

Most cellular components of immune system derive from bone marrow. The typical developmental pathway begins with pluripotent bone marrow stem cells that give rise to progenitors that follow a variety of differentiation pathways to become mature cells with defined effector functions.

1-2.1.1 Monocytic cells:

Newly produced monocyte are released into blood where they circulate for 1-3 days before entering tissues to differentiate into mature resident macrophage (48).

1-2.1.1.1 Human monocyte subsets:

In humans, circulating monocytes are divided into two subsets on the basis of the expression of CD14, a component of the lipopolysaccharide (LPS) receptor complex, and CD16, the FcγRIII immunoglobulin receptor (49). These monocyte subsets express distinct chemokine, immunoglobulin, adhesion, and scavenger receptors (50). $CD14^{+}CD16^{-}$ ($CD14^{+}$) monocytes are large, ~ 18 μm in diameter, and represent ~ 80%–90% of circulating monocytes. In contrast, $CD14^{-}CD16^{+}$ ($CD16^{+}$) monocytes are smaller, ~14 μm in diameter, and constitute ~ 10% of circulating monocytes.

1-2.1.1.2 Monocytes differentiation:

Under inflammatory conditions, monocyte production in the bone marrow is increased and after released into the circulation monocyte are rapidly recruited to sites of injury and infection where they differentiate into inflammatory macrophage (51). Furthermore, monocytes can also give rise to dendritic cells (DCs) in vitro and in vivo, and microbial infection triggers in vivo monocyte differentiation into specialized DC populations.

1-2.1.1.3 Bacterial infection:

Circulating monocytes are increasingly implicated as essential players in defense against

a range of microbial pathogens. Monocytes kill bacteria by producing reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) and through the action of phagolysosomal enzymes (52, 53).

1-2.1.2 Macrophage:

Macrophage is a part of mononuclear phagocyte system and is professional antigen presenting cells for adaptive immunity. Mononuclear phagocytes migrate out from bone marrow, circulate briefly in the blood as monocytes, and then enter into the tissues and inflammatory foci where they differentiate into macrophages. Macrophages, which is a heterogeneous population of phagocytic cells found throughout the body that originate from the mononuclear phagocytic system (54).

1-2.1.3 Neutrophils granulocytes:

Neutrophils are abundant in blood, where they have a short half-life if they are not recruited to a site of inflammation by specific chemokines and cytokines. Once recruited to an inflammatory site, neutrophils migrate rapidly from blood to tissue, which is otherwise devoid of neutrophils (55). In response to inflammatory stimuli, neutrophils migrate from the circulating blood to infected tissues, where they efficiently bind, engulf, and inactivate bacteria. Phagocytosed bacteria are killed rapidly by proteolytic enzymes, antimicrobial proteins, and reactive oxygen species (56).

1-2.1.4 Dendritic cells:

Dendritic cells (DC) are one of the most potent antigen presenting cells (APCs) of the immune system and are thought to be crucial for the initiation of primary T cell-mediated immune responses (57). Resting DCs capture and process soluble or particulate antigens in late endosomal and lysosomal compartments that are rich in major histocompatibility complex (MHC) class II molecules (58). Mature DCs upregulate their expression of MHC class I and class II complexes that can be recognized by antigen-specific T cells. Once DC undergoes a process of maturation, they have a greatly diminished capacity for antigen uptake and processing but have gained the ability to present antigens effectively for priming T cells (59, 60).

1-2.2 Toll-like receptors (TLRs)-mediated immune activation

The role of Toll-like receptors is to activate phagocytes and tissue dendritic cells to response to pathogens by secreting cytokines and chemokines, and to express the co-stimulatory molecules essential to activate adaptive immunity. There are ten Toll-like receptors of humans are known. Each of them can recognize one or more particular molecule that is present in many pathogens. The well-known of these is Toll-like receptor 4, which is interacted with Gram-negative bacterium LPS (61) and the Toll-like receptor 5 recognized the flagellin of pathogens (62). When TLR-4 interacts with LPS bound to CD14, this sends a signal to activate the transcription factor NF-κB which result in several pro-inflammatory

cytokines secretion such as TNF-α (63). Moreover, TLR-1, TLR-2, and TLR-6, each of them is formed in dimer to recognize peptidoglycan, and lipoproteins. TLR-3 and TLR-9 recognizes double-stranded RNA and unmethylated CpG DNA, respectively. There are two different mechanisms by which activation of TLRs can contribute to host defense. Fist, activation of TLRs can directly mediate innate response by regulating phagocytosis and triggering antimicrobial activity (64). Second, activation of TLRs can trigger the release of cytokines and the differentiation of immature to mature dendritic cells, enabling the innate immune systems to instruct the adaptive immune response (65). Stephan *et al.* showed that DC-SIGN$^+$ cells have a macrophage-like phenotype, are phagocytic, and use DC-SIGN to facilitate the uptake of bacteria. In contrast, CD1b$^+$ cells have an immature dendritic phenotype, release pro-inflammatory cytokines and function as efficient antigen- presenting cells (66).

1-2.3 Phagocytosis of pathogens:

The most important phagocytic cell is monocyte/macrophage, which locates especially in connective tissue, in the submucosal layer of the gastrointestinal tract. The second major family of phagocytes such as the neutrophils or polymorphonuclear neutrophil leukocytes (PMNs) is short-lived cells that are abundant in blood, but they are not present in healthy tissue. Upon phagocytosis, macrophages and neutrophils can produce various toxic products

to kill the engulfed microorganism. The most important of these are nitric oxide (NO), the superoxide (O_2^-), and hydrogen peroxide (H_2O_2), which are directly toxic to bacteria. Dendritic cells, which is another type of phagocytic cell present in tissues, thus enabling these antigen-presenting cells to initiate adaptive immune response. After internalization of bacterial components, the expression of MHCII, CD80, and CD86 were up-regulation on the surface of these phagocytic cells, also called antigen presenting cells and subsequent activate the adaptive immune response. Both of these phagocytic cells have a key role in innate immunity because they can recognize, ingest, and destroy many pathogens without aid of adaptive immune responses.

1-2.4 Cytokines and chemokines production:

Cytokines are secreted proteins that many cell types produce; they are critical to both innate immunity and adaptive immune system responses. Cytokines play a crucial role in the immune system response to all kinds of disease. They interact with organs and cells, alone and in combination with each other. The diverse role that cytokines serve in the immune system makes them an ideal target for intervening or bolstering immune responses. Inflammation is mediated by a variety of cytokines. Inflammatory cytokines can be divided into two groups: those involved in acute inflammation and those responsible for chronic inflammation. Several cytokines play key roles in mediating acute inflammatory reactions,

namely IL-1β, TNF-α, IL-6, and chemokine, IL-8.

1-2.4.1 Interleukin-1β:

IL-1β accumulates as a 33 kDa pro-cytokine (proIL-1β) in the cytoplasm of monocytes and macrophages, and its activation depends on cleavage to the active, mature 17 kDa form (mIL-1β) by the enzyme caspase-1 (67). IL-1β is an important proinflammatory cytokine whose circulating levels are tightly regulated to prevent aberrant activation of pathways that can lead to chronic inflammation, septic shock, or death (68). In health person, these cells do not constitutively express IL-1β (69).

1-2.4.2 Interleukin-6:

Interleukin 6 (IL-6) is a pleiotropic cytokine that is produced by many different cell types and involved in a wide range of responses, such as immune response, and acute-phase reactions (70). IL-6 is produced by various types of lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, fibroblasts, and several tumor cells. The original classification of IL-6 as INF-β2, and a prominent regulator of T cell proliferation, differentiation, survival, and Ig secretion by B cells (71, 72). IL-6 can also regulate monocytes differentiation. Pascale *et al*. showed that once activated monocytes encounter stromal cells such as fibroblasts which will induce IL-6 productionthat, in return, increases functional M-CSFR on activated monocytes. As the activated monocytes spontaneously release M-CSF, the functional M-CSFR then transduces M-CSF signals, thereby initiating

macrophage differentiation (73). The role of IL-6 in the generation of human macrophages provides an explanation for the altered clearance of pathogens such as *Listeria* or *Mycobacterium* in mice (74). Hence, it has defined IL-6 as a factor for directing transition from innate to acquired immunity (75). However, nn anti-inflammatory function of IL-6 was also detected in 1989; it was showed that IL-6 can inhibit lipopolysacharide-indiced TNF-α production in monocytic cells (76).

1-2.4.3 Interleukin-8:

Interleukin 8 (IL-8), a proinflammatory chemokine, is produced by various types of cells upon stimulation with inflammatory stimuli and exerts a variety of functions on leukocytes, particularly neutrophils *in vitro*. It has been referred to as monocyte-derived neutrophil chemotactic factor (MDNCF), neutrophil attractant/activating peptide-1 (NAP-1), neutrophil chemotactic factor (NCF) (77, 78). The essential role of IL-8 in most inflammation reactions is recruiting and activating neutrophils, such as lysosomal enzymes, generation of superoxide/biolipis, and increase the expression of adhesion molecules on neutrophils (79). Whiles IL-8 has poorly influence on monocytes. According to recent study, it has shown us that IL-8 is certainly not involved in THP-1 cells activation (80).

1-2.4.4 Tumor necrosis factor-α:

Human Tumor necrosis factor (TNF) is translated as a 26-kDa protein that lacks a classic signal peptide. TNF-α is an important pro-inflammatory mediator produced predominantly by

activated monocytes and macrophages. TNF is not usually detectable in healthy individuals, but elevated serum and tissue levels are found in inflammatory and infectious conditions and serum levels correlate with the severity of infections (81, 82). Although cells of the monocyte/macrophage lineage are the main source of TNF-α in inflammatory disease, a wide range of cells can produce TNF-α, including mast cells, T and B lymphocytes, natural killer (NK) cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, fibroblasts and osteoclasts. One of the major biological roles of TNF-α is in the host defence to bacterial, viral and parasitic infections. Physiologically, TNF-α is important for the normal response to infection, but inappropriate or excessive production can be harmful. It has been found that the cytotoxic properties of TNF-α can both against tumor cells and against normal cells infected with intracellular pathogens and viruses, and performs many immunoregulatory functions (83, 84). TNF-α is also chemotactic to monocytes and neutrophils. Stimulation of these cells with TNF-α induces adherence of monocytes and neutrophils to endothelial cells, and enhancement the antigen presenting capacity of monoctes/macrophages (85).

1-2.4.4.1 TNF-α associated diseases

Rheumatoid arthritis: Rheumatoid arthritis is a chronic autoimmune inflammatory disorder affecting approximately 1% of the population, characterized by inflammation of synovial tissue, leading to progressive damage, erosion of adjacent cartilage and bone and chronic disability. The inflammation is associated with accumulation of inflammatory cells,

predominantly T cells and macrophages, but also B cells, plasma cells and dendritic cells. There is synovial hyperplasia and angiogenesis is a prominent feature (86).

Inflammatory bowel disease: TNF-α immunoreactivity is increased the lamina propria in intestinal specimens from patients with Crohn's disease and ulcerative colitis and mice overexpressing TNF-α develop a Crohn's disease-like inflammatory bowel disease (87, 88).

Psoriasis: Psoriasis is an inflammatory skin disorder, in which an inflammatory cell infiltrate is associated with hyperkeratotic lesions, giving rise to typical psoriatic plaques. TNF-α, TNFR1 and TNFR2 are upregulated in dermal blood vessels in involved skin from patients with psoriasis (89).

Disease of the central nervous system: In the central nervous system, TNF is produced primarily by microglia and astrocytes in response to a wide range of pathological processes, including infection, inflammatory disease, ischaemia and traumatic injury (90). TNF-α mediated protection against experimental autoimmune encephalomyelitis does not reqire TNFR1, although TNFR1 appears to be necessary for detrimental effects of TNF-α, which occur during the acute phase of the disease (91). Neutralization of TNF failed to benefit patients with relapsing–remitting multiple sclerosis, and significantly increased exacerbations (92).

Cardiovascular disease: TNF-α has also been implicated in the pathogenesis of a number of cardiovascular diseases, including atherosclerosis, myocardial infarction, heart failure,

myocarditis and cardiac allograft rejection, and vascular endothelial cell responses to TNF-α may underlie the vascular pathology in many of these conditions. Patients with chronic inflammatory conditions such as rheumatoid arthritis have an increased incidence of cardiovascular disease. Inflammatory mediators, including TNF-α, have been implicated in this increased cardiovascular risk, and there is some evidence that anti-TNF therapy ameliorates this risk in patients with rheumatoid arthritis (93).

Respiratory disease: TNF-α has been implicated in the pathophysiology of many inflammatory lung diseases, including chronic bronchitis, chronic obstructive pulmonary disease, acute respiratory distress syndrome and asthma (94). In asthma, TNF-α has been implicated in airway inflammation and remodelling, and may play a role in bronchial hyper-responsiveness. Leukocytes from bronchiolar lavage of asthma patients have increased release of TNF-α, and inhaled TNF-α increases airway responsiveness in normal subjects and is associated with a pulmonary neutrophil infiltration, assessed by induced sputum (95).

Renal disease: TNF-α has been implicated in the pathogenesis of many renal diseases, including ischaemic renal injury, renal transplant rejection and glomerulonephritis, which is often part of a systemic vasculitis. In diseases associated with renal inflammation, different forms of TNF-α blockade vary in their efficacy and adverse effects, and these differences may be attributed to different effects on signalling though TNF-α receptor subtypes (96).

1-2.4.5 Transforming growth factor-β1:

TGF-β stimulates cells at the resting state (monocytes), whereas activated cells (macrophage) are inhibited. Sharon *et al*. have shown that the function of TGF-β is depends on the concentration variation. It stimulates monocytic chemotaxis at 0.1 to 10 pg/ml, while higher concentrations, around 10 ng/ml, alter the production of cytokines such as IL-1, and TNF-α (97, 98), and inhibit killing of several invasive pathogens (99, 100). TGF-β is arguably the most potent endogenous immunosuppressive factor to be characterized (101). IFN-γ -induced expression of MHC class II molecules in macrophages is inhibited by TGF-β via the attenuation of CIITA (102).

1-2.4.5.1 TGF-β regulation of infectious diseases

Trypanosome: *Trypanosoma cruzi* is one parasite that makes direct use of the host's TGF-β signaling pathway. It uses TGF-β receptors I and II for successful entry into mammalian cells. Epithelial cells lacking TGF-β receptors are resistant to *T. cruzi* infection, and infectivity is restored following transfection of functional TGF-β receptors (103). TGF-β treatment of mouse and human macrophages blocks IFN-γ-mediated inhibition of parasite growth, and TGF-β-treated mice develop higher parasite loads and die faster than control mice. African trypanosome *T. brucei*, which does not invade host cells, might nevertheless possess a functional homolog of this TGF-β-activating moiety because this parasite has also been shown to release a factor that induces TGF-β mRNA expression (104).

Leishmania: *Leishmania* can survive and replicate in the phagolysosome of macrophages.

Virulent strains of *Leishmania* have developed mechanisms to induce macrophages to produce high levels of active TGF-β, whereas non-pathogenic strains that produce low-grade infection induce relatively low levels of active TGF-β (105). TGF-β made by infected macrophages suppresses NO production and can influence T cell differentiation by inhibiting the production of TNF-α and IFN-γ (106).

Toxoplasma gondii: Toxoplasma is another obligate intracellular parasite that infects macrophages. As in the case of *Leishmania*, infection of mouse macrophages with *T. gondi* results in the release of TGF-β, which was associated with the down-regulation of TNF-α and its receptors (107).

Mycobacteria: Mycobacteria are obligate intracellular pathogens of macrophages that cause tuberculosis, leprosy, and opportunistic infections due to immunosuppression. Similar to most protozoan infections, mycobacteria induce macrphsge production of active TGF-β and suppress their antibacterial activity to aid their pathogenesis. Both purified protein derivative and lipoarabinomannan, a cell wall component of tuberculosis, induce TGF-β from human peripheral blood mononuclear cell (PBMC)-derived macrophages (108, 109).

Listeria: *Listeria monocytogenes* is a facultative intracellular bacterium and a strong inducer of Th1 response (110). Cytokines such as IFN-γ, TNF-α, and IL-6 play an important role in host resistance to *Listeria*, and TGF-β plays a protective role in *Listeria* infection. The mechanism by which TGF-β confers resistance to lethal doses of *L. monocytogenes* in mice is

not yet clear and requires more investigation (111).

1-3 *Heat shock proteins*

Heat shock proteins are constitutively present in eukaryotic and procaryotic cells. Expression of heat shock proteins (hsps) is markedly increased as part of the response to an array of stressors. These proteins participate in the refolding of denatured polypeptides that become damaged. Generally, during nonstress conditions, hsps participate in the folding of nascent polypeptides and the stabilization of receptors and signal transduction molecules (112). Extracellular HSPs are considered to belong to the heterogenous family of ‘‘alarmins’’ that are involved in tissue damage-associated inflammation (113). Furthermore, the stimulatory capacity of exogenous HSPs on antigen-presenting cells (APC) was detected. Combined with these reports, hsps can trigger immune activation at the certain environment.

1-3.1 Human heat shock proteins

The importance of the interaction of Hsps with the immune system is apparent from two important observations: first, the presence of anti-Hsp antibodies in serum and, second, the cytokine production induced in a number of cell types by exposure to Hsp60, or Hsp70 (114).

1-3.1.1 Human heat shock protein 60

HSP60 with a molecular mass of 60 kDa is mainly expressed in mitochondria; recently

the stimulatory capacity of extracellular HSP60 on the innate immune system has been recognized. Human macrophages respond to both bacterial and human HSP60 with the release of pro-inflammatory mediators such as TNF-α or IL-6 and of the Th1-promoting cytokines IL-12 and IL-15 suggesting that HSP60 might act as a "danger signal" for the innate immune system (115). There is evidence that CD14 and Toll-like receptors (TLR) 2 and 4 are involved in HSP60-mediated cell activation (116-118). Stefanie et al. revealed that HSP60 can promote dendritic cell maturation. Maturation of DC induced by human HSP60 was characterized by up-regulation of MHC class II and of the costimulatory molecules CD40, CD54, and CD86 and the pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-12 were also secreted. However, HSP60 is also an antigen for the adaptive immune system and T cell responses to HSP60 epitopes regulate inflammatory diseases like rheumatoid a arthritis, insulin-dependent diabetes mellitus, and artherosclerosis (119, 120). Taken together, maturation of DC is strongly induced by human HSP60 and paralleled by release of Th1-promoting cytokines. HSP60 might favor the development of Th1-dependent organ-specific autoimmune diseases when endogenous HSPs are released as observed in diabetes and arthritis.

1-3.1.2 Human heat shock protein 70

Hsp70 can be released from tumor cells in a complex with intracellular polypeptides and then recognized by components of the immune system, leading to antitumor immunity against the chaperoned tumor peptide antigens (121). Such Hsp70-peptide complexes interact with

APCs and induce tumor immunity by promoting Ag cross-presentation to T cells (122).

1-3.2 Bacterial heat shock proteins

HSPs are abundantly expressed in inflammatory lesions and produced by microorganisms during invasive infection and phagocytosis. Bacterial heat shock proteins have been reported to stimulate human monocytes to produce pro-inflammatory cytokines or to up-regulate the expression of adhesion molecules (123, 124).

1-3.2.1 *Escherichia coli* heat shock proteins: GroEL, DANK, and GroES

It was said that GroEL and DnaK were able to induce the release of TNF-α, IL-1α, IL-6 and sICAM-1 from keratinocytes. GroES showed significant activity only on the expression and release of IL-6. In the inflammatory reaction of the skin, keratinocytes play a determining role by synthesizing and secreting cytokines and adhesion molecules (125). In another study, DNAK and GroEL were able to induce the release of GM-CSF and IL-6 from HUVEC, in monocytes DNAk and GroEL were able to induce the release of soluble forms of E-selectin, ICAM-1, and VCAM-1, while GroES showed a significant activity only on E-selectin release (124). Release of cell surface adhesins may simply be a mechanism for breaking adhesive interactions between cells or may provide a means for clearing the cell surface of adhesins to control adhesivity. Taken together, HSPs may play an important role in the initiation of the inflammatory process that accompanies infections with microbial pathogens by regulating the

expression of cytokines involved in the activation of leukocytes and endothelial cells.

1-3.2.2 *Chlamydia* heat shock protein 60

Chlamydiae produce large amounts of heat shock protein 60 (HSP 60) during chronic, persistent infections, and *C. pneumoniae* localizes predominantly within plaque macrophages. *Chlamydia pneumoniae* infection has been associated with asthma and the aggravation of atherosclerosis. Kol *et al*. showed that *chlamydial* HSP 60 colocalizes with human HSP 60 within plaque macrophages and that HSP 60 from both species can induce macrophage production of TNF-α and matrix-degrading metalloproteinases, two mediators of atherosclerosis complications (126).

1-3.2.3 *Mycobacterial* heat shock protein 65

The 65 kDa heat-shock protein (Hsp65), a well-conserved and immunodominant antigen which elicits a cellular and humoral immune response may play a role in host defense against invading microorganisms and autoimmune disorders. Incubation with Hsp65 resulted in an enhanced release of TNF-alpha and IL-1 beta by human monocytes and monocyte-derived macrophages (MDM). The release of the proinflammatory cytokines TNF-α and IL-1β by human mononuclear phagocytes in response to Hsp65 indicates that this protein can contribute to both host defence and tissue damage in inflammatory lesions characterized by an abundant expression of Hsp65 (127).

1-3.2.4 *Helicobacter pylori* heat shock protein 60

H. pylori seems to bind to gastric epithelial cells and mucin via HSP60 (128) and induces IL-8 production from gastric epithelium or monocytes (28, 29). Interleukin-8 (IL-8) is a chemokine secreted by a variety of cell types, which serves as a potent inflammatory mediator recruiting and activating neutrophils. Several studies have demonstrated that *H. pylori* strains are capable of inducing IL-8 secretion from gastric carcinoma cells in vitro (129, 130). Not only IL-8 but also IL-6 was produced by macrophage via a toll-like receptor (TLR)-2, TLR-4 and myeloid differentiation factor 88-independent mechanism (30). *Helicobacter pylori* heat shock protein 60 and risk of coronary heart disease: a case control study with focus on markers of systemic inflammation and lipids (131). Tahenaka *et al*. showed that serum antibodies to *Helicobacter pylori* and its heat-shock protein 60 correlate with the response of gastric mucosa-associated lymphoid tissue lymphoma to eradication of *H. pylori* (132). It has been suggested that antibodies against heat shock proteins seem to be involved in the pathogenesis of coronary heart disease (CHD) and CagA positive H pylori infection may concur to the development of CHD; high levels of anti-Hsp60 antibodies may constitute a marker and/or a concomitant pathogenic factor of the disease (133).

Chapter 2 Materials and Methods

2-1 *Materials*

2-1.1 Reagent

The following reagents obtained were described as following: RPMI 1640, Fetal Bovine Serum (FBS), BSA, and Tryzol were from Invitrogen Inc. (Gaithersburg, MD, USA). Penicillin/ streptomycin/ amphotericin (PSA) were from Biological industries (Beithaemek, Israel). Restriction enzymes were from Promega Inc. (WI,USA). Kanamycin and Tris were from MDBio Inc. (Rockville, MD, USA). Ethidium bromide (EtBr), Isopropyl-beta-D-thiogalactopyranoside (IPTG), NaCl, yeast extract, agar, Tris-HCl, Triton X-100, TEMED and imidazole were from Amresco Inc. (Solon, OH, USA). Recombinant human TNF-α and TGF-β1 was from Peprotech Inc. (Rocky Hill, NJ). Sephadex G-25 Medium was from Amersham Bioscciences (Uppsala, Sweeden). Nitrocellulose (NC) paper was from PALL Inc. (Ann Arbor, MI, USA).

2.1.2 Antibody

PE-conjugated anti-CD86, Fluorescent isothiocyanate (FITC)-conjugated anti-CD40, anti-CD80, anti-HLA-ABC, and anti-HLA-DR antibodies were from BioLegend (Sandiego, CA, USA). HRP-conjugated rabbit anti 6X His antibody was from Novus (Littleton, CO, USA).

2-1.3 Kit

Human IL-1β, IL-6, IL-8, and TNF-α ELISA kit was obtained from R&D systems (Minneapolis, MN). Superscript III RT kit was from Invitrogen (Gaithersburg, MD, USA). RealQ-PCR master mix kit was from Ampliqon (Copenhagen, Denmark). Coomasie Plus™ Protein Assay Reagent kit and Enhanced chemiluminescence (ECL) system was from Pierce (Rockford, IL, USA), FITC-dextran was from SIGMA-ALDRICH (Steinheim, Germany).

2.1.4 Instrument

HisTrap™ HP column was from GE healthcare (Uppsala, Sweeden). ABI PRISM 7000 from was Applied Biosystems (USA). Flow cytometer was from BD (Bedford, MA, USA). Human heat shock protein 60 cDNA (complementary DNA) library were kindly provided from Dr. Chich-Sheng Lin (NCTU, Laboratory of Biomedical Engineering, Biological Science & Technology Lab).

2.1.5 Bacteria

Escherichia coli (BL21 and DH5α) was from Yeastern Biotech Co. *H. pylori* genome was from Department of Internal Medicine, College of Medicine, National Taiwan University.

2-1.6 Cell line

THP-1 cells, acute monocytic leukemia cell line was purchased from the Bioresource Collection and Research Centre (BCRC) (Hsinchu, Taiwan). Unlike other leukemic cell lines, THP-1 cells have no prominent chromosomal abnormalities (134).

2.2 Methods

2-2.1 Recombinant DNA techniques

The *H. pylori* strains were isolated from gastric biopsy specimens at National Taiwan University Hospital. The genome of *H. pylori* was prepared from the clinical isolates. The gene of Hsp60 was amplified from the genome of *H. pylori* by polymerase chain reaction (PCR) using the primers: 5'- ATC GAA TTC ATG GCA AAA GAA ATC AAA TTT TCA - 3' as forward primer and 5'-GAT CTC GAG TTA CAT CAT GCC GCC CAT G-3' as reverse primer. PCR condition was that 94 ℃ denaturation step followed by 35 cycles of 45 s at 95 ℃, 45 s at 50 ℃ and 2min at 72 ℃. After these cycles, incubate the PCR mixture at 72 ℃ 10min for complete elongation. PCR product was harvested, digested with EcoR1 and Xho I, and inserted into EcoR I and Xho I restriction fragment of the expression vector pET-30a with N-terminal His-tags. The recombinant plasmids were further identified by restriction enzyme and agarose gel. The resulting plasmid pET- Hsp60 was transformed into competent *E.coli* BL21 (DE3) cells growing on an agar plate with kanamycin for selection.

2-2.2 Transformation

Remove the appropriate number of competent cells tubes from the -80 ℃ freezer. DH5α is used for cloning and DNA amplification. BL21 is used for protein expression. After the cells have thawed, add 1ng DNA into the cells, mix by gently swirling the tip or by gently

tapping the tube. Incubate the competent cell on ice for 30 min. Heat shock the cell at 42 ℃ for 90 s. Place the cells on ice for 2 min and add 250 µl LB (10g tryptone, 10g NaCl, 5g Yeast extract) and incubate at 37 ℃ with shaking 225 rpm for 1 hr. Spread 100µl mixture onto each LB agar plate (10g tryptone, 10g NaCl, 5g Yeast extract ,20g agar) containing kanamycin (30 mg/ml) and incubate at 37 ℃ for 12~16 hr.

2-2.3 Expression and purification of *HpHsp60* gene in *Escherichia coli*

The colonies on the agar plate were picked, and shake in 100 ml LB with 30 µg/ml kanamycin at 37℃ overnight. Then, the 100 ml LB with bacteria was inoculated in 900 ml LB and the bacteria grew until the optical density at OD 600 nm reached 0.4-0.6. IPTG was added to a final concentration of 1 mM, and *E. coli* cells continually grew in 1L LB for 4 h. After induction, the LB containing *E. coli* cells were harvested by centrifugation at 5000 rpm for 15 min and the pellet was resuspended in 30 ml binding buffer (20mM Na_2HPO_4, 0.5M NaCl, 40mM imidazole, pH7.4). Then the homogenized samples were sonicated with short burst of 1 sec followed by intervals 1 sec and the sonication processing was maintained for 15 min. Centrifuge the samples at 12000 rpm for 30 at 4 ℃. Harvest the supernatant and passed the 0.45 µM filter to remove the particles.

2-2.4 Protein purification

In this experiment, we purify our proteins with HisTrap^{TM} HP column (GE healthcare). To prepare the column, wash the column with 5 column volumes of DDW and equilibrate the

column with 5 column volume of binding buffer at the flow rate about 1 ml/min. Apply the pretreated sample and wash with wash buffer (20mM Na_2HPO_4, 0.5M NaCl, 60mM imidazole, pH7.4) about 60 volume. Elute with elution buffer (20mM Na_2HPO_4, 0.5M NaCl, 200mM imidazole, pH7.4, filtered with 0.45μm filter) for 10 volumes. Detect several fractions containing proteins by coomasie blue reagent. Collect the fractions with high protein concentrations and use G25 column to remove the unnecessary salt from the solution and replace the buffer with PBS (Phosphate Buffered Saline, 140 mM NaCl, 2.7mM KCl, 10 mM Na_2HPO4, KH_2PO_4, pH 7.4). To prepare the G25 column, we need to swell the 7g Sephadex G-25 Medium with filtered PBS at room temperature for overnight. Fill the column with PBS and agitate the PBS containing G25 agarose, soon pour into the column along the edge of glass rod. After collecting the protein-containing fractions, we pour it into the G25 column and wash and elute with PBS. Detect which fractions contain proteins with coomasie blue reagent and collect the fractions. Poll the fractions together and filtered with 0.22 μm syringe filter. Quantitate the amount of protein concentration with coomasie blue reagent and dilute the solution to 1mg/ml. The quality of recombinant protein was checked by SDS-PAGE and Western blotting.

2-2.5 Cell cultures

The human monocyte THP-1 cell line was cultured according to the recommendations from ATCC. Briefly, non-adherent cells were grown in 75 T flasks in RPMI 1640 culture

medium. The culture medium RPMI 1640 was supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1% PSA (Biological Industris), 2.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate (from MP Biomedicals) and 0.05 mM 2-mercaptoethanol (Amersco). Cells were incubated in tissue culture incubator with 5 % CO_2 at 37 ℃ and split at a density of ~5x10^5 cells/ml. Cells were kept no more than 2 months in culture from the original stock.

2-2.6 Detection of cytokines production in the THP-1 cells using ELISA

THP-1 cells (5×10^5 cells per well) were dispensed into 24-well culture plates and then except for control treated with 10 μg/ml rHpHSP60. The supernatants with or without rHpHSP60 stimulation were collected at 24 h. To detect the kinetic of cytokine protein expression, the supernatants were collected at 5 min, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h and frozen at −80 ◦C. The concentration of TNF-α, IL-1β, IL-6, and IL-8 in the supernatants was measured by Enzyme-Linked ImmunoSorbent Assay (*ELISA*) kit (R&D systems).

2-2.7 FITC-dextran uptake of THP-1 cells

THP-1 cells were seeded in 24-well tissue culture plates at a density of 5×10^5/ml in a volume of 1 ml per well. Except for the control, 10 μg/ml of rHpHSP60 was added into the culture medium. To examine the effect of TNF-α or TNF-α combing with TGF-β1 on endocytotic activity of THP-1 cells, cells incubated with seven concentrations of TNF-α or 1 ng/ml TNF-α with three indicated concentration of TGF-β1. After 16 h treatment, cells with

culture medium were collected and centrifuged with 2000 rpm 5min. Then, the culture medium were removed and cells were incubated in 2% RPMI medium containing 1 mg/ml FITC–dextran in the tissue culture incubator with 5 % CO_2 at 37 ℃ or upon the ice (as the control) for 2 h. The uptake of FITC–dextran was analyzed using flow cytomestry.

2-2.8 Surface marker detection on THP-1 cells

Cells treated with 10 μg/ml rHpHSP60 or with TNF-α combing with TGF-β1 for 16 h or with three concentrations of TNF-α for 24 h. Then, cells with culture medium were collected and centrifuged with 2000 rpm 5min. The cell pallet was washed by wash buffer once time. Phosphate-buffered saline (PBS) with 1% bovine serum albumin and 0.05% sodium azide was used as wash buffer. Cells were incubated for 1 h on the ice at a volume of 50 λ with 1 λ of the following antibodies: PE-conjugated anti-CD86, Fluorescent isothiocyanate (FITC)-conjugated anti-CD40, anti-CD80, anti-HLA-ABC, or anti-HLA-DR antibodies. After three times washing, the surface marker expression of THP-1 cells was assayed by flow cytomestry.

2-2.9 RNA isolation and cDNA synthesis

At each time point, media was removed from the microcentrifuge tube after centrifugation. Total RNA was extracted from cells using Trizol reagent as described in the manufacturer's protocol. After centrifugation, pellet cells lysed in 1 ml Trizol reagent by repetitive pipetting. Incubated the homogenized samples for 5 minutes at room temperature to

permit the complete dissociation of nucleoprotein complexes. Add 200 λ of chloroform per 1 ml Trizol reagent. Cap sample tubes securely. Shake tubes fiercely by hand for 15 seconds and incubated them at room temperature for 3 minutes. Centrifuge the samples at 12000 rpm for 15 min at 4 °C. Following centrifugation, transfer the colorless upper aqueous phase to a fresh tube. Precipitate the RNA from the aqueous phase by mixing with 500 λ isopropyl alcohol. Incubated samples at room temperature for 10 minutes and centrifuged at 12000 rpm for 20 minutes at 4 ℃. After centrifugation, the gel-like pellet on the side and bottom of the tube. Remove the supernatant. Wash the RNA pellet once with 1 ml of 75 % ethanol. Mix the samples by vortexing and centrifuge at 7500 rpm for 10 minutes at 4 ℃. Following centrifugation, remove the supernatant and briefly air-dry the RNA pellet for 15 minutes. Dissolved RNA in RNase-free water and incubated for 10 minutes at 60 ℃. The concentration of RNA was measured by spectrophotometry (ideal $OD_{260/280} \cong$ 1.8-2.0) and the samples were stored at -80 ℃ until use. One microgram of total RNA was used to synthesis cDNA using random hexamer primers with the Superscript III Fisrst-Strand Synthesis kit (Invitrogen).

2-2.10 Quantitative Real-time PCR

The resulting cDNA was then subjected to quantitative real-time PCR and primers used were as Table 1. cDNAs were amplified using SYBR®-PCR mastermix (Applied Bio-systems) according to the recommendations of the manufacturer in a total volume of 25 μl in a ABI PRISM 7000 system (Applied Biosystems). The reactions were incubated at 50

℃ for 2 min to activate uracil *N*-glycosylase and then for 4 min at 95 ℃ to inactivate this enzyme and activate the Amplitaq Gold polymerase, followed by 40 cycles of 15 sec denaturation at 95 ℃, 25 sec annealing at 60 ℃, and 25 sec extension at 72 ℃. The expression of each gene was normalized to β-actin, a housekeeping gene. All samples were run in duplicate and non-template control.

Chapter 3 Results

3-1 Pro-inflammatory cytokines production

Several publishes revealed that *H. pylori* HSP60 would stimulate IL-8 or IL-6 secretion in human monocytic cells and mouse macrophage respectively (29, 30). The level of IL-1β and tumor necrosis factor-α will increase in the infected gastric tissues (135).

To examine whether the cytokines could be induced in THP-1 cells with rHpHSP60 treatment, THP-1 cells co-incubated with rHpHSP60 for 24 h, and the supernatants were collected. Pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, in the supernatant were analyzed by ELISA. The production of TNF-α, IL-1β, IL-6, and IL-8 in response to 10 μg/ml rHpHSP60 is significantly increased compared to the control. The concentration of IL-1β, IL-6, IL-8, and TNF-α in the culture medium was 101 ± 38 pg/ml, 277 ± 66 pg/ml, 16301 ± 1305 pg/ml, and 449 ± 153 pg/ml, respectively (Fig. 3a-3d). Among these cytokines, IL-8 which is a neutrophil attraction factor was greatly present in the culture medium. However, it has poorly effect on monocytes activation. According to the previous report (80), we know that the level of TNF-α induced by THP-1 cells in this system was able to induce monocytes activation. IL-6 is involved in activation of adaptive immunity dominantly. IL-1β, a product of phagocytic cells, has been reported that 20 ng/ml of IL-1 could cause maximal upregulation of monocyte phagocytosis (136). However, a little amount

of IL-1β was detected in the supernatant.

3-2 Release of TNF-α from THP-1 cells incubated with rHpHSP60 or heated rHpHSP60

We know that LPS is a general factor to stimulate TNF-α secretion from immune cells. Commercial recombinant protein from *E. coli* system would always contaminate LPS. In order to exclude the effect of rHpHSP60 contaminating with LPS to result in TNF-α secretion, we heated the protein at 95 ℃ for 1 h to cause proteolysis. As shown in Fig. 4, proteolysis induced by heating to 95 ℃ abolished TNF-α secretion. Thus, this evidence indicated that the observed effect of rHpHSP60 on TNF-α expression was not due to heat-stable LPS (Fig. 4).

3-3 Kinetic of cytokine protein expression

Friedland *et al.* showed that *mycobacterial* 65-kD heat shock protein induced a maximum expression of TNF-α at 4 h, IL-6 was obviously present at 8 h, and a great amount of IL-8 was detected at 24 h in THP-1 cells (136). However, GroEL, the 60 kDa HSP of *Escherichia coli*, time-dependent induced TNF-α, IL-6, IL-1α, and ICAM-1 expression (137). *Chlamydial* HSP 60 induces TNF-α production by mouse macrophages and it was secreted at a maximum at 6 h, and TNF-α expression kept in a stable level until 72 h (126).

In order to further confirm the cytokine expression kinetic, we treated THP-1 cells with

10 μg/ml rHpHSP60 and time-course collected the supernatants. The supernatants were collected at 5 min, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h. The amounts of cytokines were then analyzed by ELISA. IL-1β was slightly present at 2 h and kept low level until 24 h, whereas the amount of IL-6 and IL-8 were more than IL-1β in 24 h. The level of IL-8 was obviously appeared at 2 h. All of these three cytokines were time-dependent increase (Fig. 5a-5c). However, a dissimilar trend of TNF-α expression was observed. A little amount of TNF-α was measured at 1 h, and immediately increased at 2 h, then at 4 h reached a peak (Fig. 5d). This result showed that TNF-α was earliest secreted by THP-1 cells, and afterward other cytokines just appeared. According to this phenomenon, we speculated that TNF-α was the first cytokine THP-1 cells contacted after rHpHSP60 stimulation. Therefore, TNF-α might have a greater effect on THP-1 cells than other cytokines.

3-4 Kinetic of TNF-α mRNA expression

In the present results, we can see that TNF-α was earliest secreted. It was measured at 1h, and immediately reached maximal amounts between 2 and 4 h. In following experiment, we further investigated that the gene expression kinetic of TNF-α in THP-1 cells with rHpHSP60 treatment. As shown in Fig. 6, the level of TNF-α mRNA expression was slightly increased at 30 min, and reached a peak during 1 and 2 h after rHpHSP60 treatment, as compared to the control. This result revealed that there was a rapid, transient TNF-α mRNA accumulation

between 1 and 2 h after THP-1 cells by rHpHSP60 stimulation.

3-5 Detection of monocytic activation

Since THP-1 cells preferentially secreted TNF-α which was quite associated with monocytes activation. In the following experiments, we further examined THP-1 cells by detecting its endocytosis activity and mature surface marker expression after rHpHSP60 stimulation.

3-5.1 Endocytosis ability of THP-1 cells by rHpHSP60 treatment

THP-1 cells were stimulated with rHpHSP60 for 16 h and then incubated with FITC–dextran for 2 h in order to assess whether the engulf ability of monocytes influenced by rHpHSP60. As shown in Fig. 7, the fluorescence intensity was obviously decrease post-treatment rHpHSP60. The capacity of THP-1 cells to ingest the particles was significantly suppressed by rHpHSP60. The mean fluorescence intensity (MFI) value decreased from 47 ± 10 (untreated cells) to 30 ± 6 (rHpHSP60 treated cells), 43% inhibition was observed.

3-5.2 Surface marker expression on THP-1 cells

THP-1 cells were treated for 16 h with 10 μg/ml rHpHSP60, and the surface marker expression of CD40, CD80, CD86, MHC I and MHC II, being the monocytic activation markers, were evaluated by flow cytometric analysis. As shown in Fig. 8a, a considerable

level of CD40 was up-regulated in cells with rHpHSP60 treatment, and the mean fluorescence intensity (MFI) of treated cells was increased three folds than untreated cells. The expression of costimulatory molecules, such as CD80, CD86 was obviously increased by rHpHSP60 stimulation. The expression of MHC I seemed not influenced by rHpHSP60. Interestingly, the MHC II expression showed a significant down-regulation with the rHpHSP60 treatment. MHC II present antigens to $CD4^+$ T-helper cells and then control differentiation of B cells in antibody producing B-cell blasts (138). Histograms showed that a clear up-regulation was observed for CD40 and CD80 molecules whereas MHC II expression was obviously down regulated (Figure. 8b)

3-6 Effect of recombinant human TNF-α on CD40 expression and endocytotic activity of THP-1 cells

To examine if TNF-α can trigger THP-1 cells activation, we studied the effects of recombinant human TNF-α (rhTNF-α) on CD40 expression and endocytotic activity of THP-1 cells. Cells were respectively treated with 0.1, 0.5, and 1 ng/ml rhTNF-α for 24 h. As shown in Fig. 9, rhTNF-α significantly up-regulated CD40 expression under a dose of 0.1 ng/ml. The dose-dependent up-regulation of CD40 expression by rhTNF-α was observed. To observe the effect of rhTNF-α on endocytotic activity, we treated THP-1 cells with seven concentrations which were 0.1, 0.2, 0.4, 0.6, 0.8, 1, and 2 ng/ml respectively for 16 h. The

endocytosis level was expressed by relative median fluorescence intensity (RMF). To calculate RMF, we defined the endocytotic ability of cells without rhTNF-α treatment as 100 %. The RMF of cells treated with 0.1, 0.2, 0.4, 0.6, 0.8, 1, and 2 ng/ml rhTNF-α were 99.8 ± 6 %, 94.2 ± 5 %, 90.8 ± 2 %, 86.9 ± 4 %, 86.6 ± 9 %, 87.9 ± 7 %, and 79.4 ± 6 %, respectively. The data showed that rhTNF-α can dose-dependent decrease the endocytotic activity of THP-1cells (Fig. 10).

3-7 Effect of treatment with TGF-β1 and TNF-α on endocytotic activity and CD40 expression of THP-1 cells

TGF-β1 is a multifunctional cytokine that plays a central role in the pathogenesis of several chronic infectious diseases. A range of macrophage deactivating properties for TGF -β1 has been described previously (139). The TGF-β1-specific staining immunohistochemistry of patients infected with *H. pylori* from National Taiwan University Hospital also showed a high level of TGF-β1 expression at the infection area (Fig. 11). We further studied the effect of synergy of TGF-β1 and TNF-α on endocytotic activity and CD40 expression of THP-1 cells. First, to test the effect of TGF-β1 combined with TNF-α on endocytotic activity, cells incubated with 1ng/ml rhTNF-α alone, 1ng/ml rhTNF-α + 0.5 ng/ml TGF-β1, 1ng/ml rhTNF-α + 1 ng/ml TGF-β1, 1ng/ml rhTNF-α + 10 ng/ml TGF-β1, and 1 ng/ml TGF-β1 for 16 h. FITC-dextran assay was used to test its engulf ability and the

median fluorescence intensity was then analyzed by flow cytomestry. The data was expressed as relative median fluorescence intensity (RFI), and the cells without TNF-α and TGF-β1 identified as 100 %. The RFI of cells treated with 1ng/ml rhTNF-α alone was 88.2 ± 7 %. The RFI of cells incubated with 1ng/ml rhTNF-α and 0.5, 1, and 10 ng/ml TGF-β1 were 80.8 ± 8 %, 76.7 ± 15 %, and 69.6 ± 10 %, respectively. This result clearly showed that TGF-β1 dose-dependent synergized with TNF-α to suppress the endocytotic activity of THP-1 cells (Fig. 12).

Under the same condition, we also tested the CD40 expression on THP-1 cells. As shown in Fig. 13, the data showed as the relative mean fluorescence intensity, and cells without TNF-α and TGF-β1 identified as 100 %. The value of cells with 1ng/ml rhTNF-α treatment was 118.6 ± 15 %, and TGF-β1 can dose-dependent inhibit the TNF-α mediated CD40 expression (1ng/ml rhTNF-α + 0.5 ng/ml TGF-β1: 102 ± 26 %, 1ng/ml rhTNF-α + 1 ng/ml TGF-β1: 90.6 ± 24 %, 1ng/ml rhTNF-α + 10 ng/ml TGF-β1: 87.7 ± 28 %, and 1 ng/ml TGF-β1: 82.5 ± 15 %). This result revealed that TGF-β1 can influence TNF-α-mediated THP-1 cells maturation.

Chapter 4 Discussion

Heat-shock proteins (HSPs) also called stress proteins, are a group of proteins present in both prokaryotic and eukaryotic cells. They are induced when a cell undergoes distinc types of environmental stress. Extracellular HSPs are the most powerful ways of sending a ‘danger signal’ to the immune system in order to generate a response that can help the organism manage an infection or disease and also been reported that are closely associated with the innate or adaptive immune systems activation (140, 141). Bacterial HSPs have also been reported to have the capability to activate human monocytes and macrophages. The 60 kDa heat-shock protein (HSP60), an immunepotent antigen of *H. pylori*, induces IL-8 secretion from human gastric epithelial cells and monocytic cells (28, 29). Gobert *et al.* also showed that *H. pylori* HSP60 can induce IL-6 secretion from macrophages via Toll-like receptors (30). *H. pylori* infection leads to gastric inflammation, and the gastric mucosal levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) have been reported to be increased in *H. pylori*-infected subjects (142, 143). Recruitment and activation of immune cells in the underlying mucosa involves chemokines such as IL-8 and pro-inflammatory cytokines secreted by mononuclear phagocytes (TNF-α, IL-1 and IL-6) as part of non-specific immunity (135).

In this study, we detected that the expression of pro-inflammatory cytokines, including

IL-1β, IL-6, IL-8, and TNF-α in THP-1 cells with *H. pylori* HSP60 stimulation (Fig. 3). All of these cytokines were significantly increased compared to the cell without rHpHSP60 treatment. IL-1β is a soluble factor secreted by stimulated monocytes (Mo) was measured as 101 ± 38 pg/ml, however, Simms *et al.* revealed that 20 ng/ml of IL-1 can just cause maximal up-regulation of monocyte phagocytosisv (136). The production of IL-6 in the gastric mucosa is consistently induced by *H. Pylori* infection and correlate with the development of chronic gastritis (144, 145). The quantities of IL-6 released into culture medium were considerable less than that observed following treatment murine macrophages with rHpHSP60 (30). This phenomenon may be caused by different cell types or the level of LPS involved in the recombinant HpHSP60. A great amount of IL-8 was measured in the culture medium. IL-8 is a pro-inflammatory cytokine, the actions of which are reported to include neutrophil and T-lymphocyte chemotaxis, neutrophil activation and enhanced expression of neutrophil adhesion molecules (146), while IL-8 is certainly not involved in THP-1 cells activation (80). In contrast, Miyazawa *et al.* showed that TNF-α can directly activate THP-1 cells to up-regulate the expression of co-stimulatory molecules at concentrations ranging from 39.1 pg/ml to 2500 pg/ml (80). According to this result, we can speculate the concentration of TNF-α measured in the culture medium was able to induce THP-1 cells activation. To exclude LPS contamination from the observed effect, we heated the protein at 95 ℃ for 1 h and TNF-α secretion was abolished. The observed effect of rHpHSP60 on TNF-α expression

was not due to heat-stable LPS (Fig. 4).

Interestingly, the cytokine expression kinetic showed that IL-1β, IL-6, and IL-8 were time-dependent increase whereas TNF-α was secreted to a maximum at 4 h, and it was earlier present than other cytokines. When THP-1 cells stimulated with *mycobacterial* 65-kD heat shock protein, the secretion of TNF-α also reached a maximum at 4 h, but IL-6 was obviously present at 8 h, and the expression of IL-8 seems not time-dependent increase (123). Keratinocytes stimulated with GroEL, the 60 kDa HSP (HSP 60) of *E. coli*, would time-dependent secret TNF-α, IL-1α, IL-6, and ICAM-1 (137). These reports revealed that the HSP60 of various species even can induce the same cytokines production while the kinetic of cytokine expression was not exactly similar. In the result of kinetic of cytokine expression showed that TNF-α was the earliest secreted cytokine, it may have a great effect on THP-1 cells. TNF-α which is a pro-inflammatory cytokine associates with monocytes activation (Fig. 5). HSPs from pathogens might induce a localized accumulation of self HSP; in turn, would provide a stimulus for autoreactive T-cell proliferation, thereby triggering a cycle of events which may contribute to the pathological damage associated with autoimmune diseases (147, 148). Wei Chen *et al*. showed that human hsp60 is a danger signal to the innate immune system (115). Human hsp60 induces TNF-α secretion in monocyte-derived macrophage (149), whereas another study suggested that rhHSP60 does not induce TNF-α release from macrophages (150). We found that both rhHSP60 and rHpHSP60 have ability to induce

THP-1 cells to secret TNF-α (Fig. 1 and Fig 14). But the cell counts was not the same between these two experiments. Cells were used to detect human HSP60-induced TNF-α se cretion just at the concentration of 10^5/ml so that the level of TNF-α secreted from THP-1 cells was low (Fig. 14). Highly homologous between prokaryotic and eukaryotic cells hsp60 are strongly immunogenic and immune responses to microbial hsp60 are speculated to initiate chronic inflammatory diseases in which autoimmune responses to human hsp60 may be central to pathogenesis (151). In this regard, the immune system could be triggered by bacterial antigen, GroEL for example (bacterial hsp60), which share a high degree of homology with self hsp60 proteins, resulting in an aberrant immune response and chronicity of inflammation. Tabata *et al.* showed that affinity-serum purified antibodies to human hsp60 and *P. gingivalis* GroEL from selected patients reacted with *P. gingivalis* GroEL and human hsp60, respectively. They suggested that molecular mimicry between GroEL of the periodontopathic bacterium *P. gingivalis* and autologous human hsp60 may play some role in immune mechanisms in periodontitis (152). It has been revealed that *Chlamydial* HSP 60 frequently colocalizes with human HSP 60 in plaque macrophages in human atherosclerotic lesions. *Chlamydial* and human HSP 60 induce TNF-α and MMP production by macrophages. *Chlamydial* HSP 60 might mediate the induction of these effects by *C pneumoniae.* Induction of such macrophage functions provides potential mechanisms by which *chlamydial* infections may promote atherogenesis and precipitate acute ischemic events (126). Our colleagues also

detected whether the cross-reaction would occur when THP-1 cells stimulated by rHpHSP60. We further detected whether human heat shock protein 60 would be secreted from THP-1 cells after rHpHSP60 stimulation. The result showed that human hsp60 was not significantly induced from THP-1 cells after rHpHSP60 treatment (Fig. 15). The OD value of human hsp60 analyzing of 10 μg /ml rHpHSP60 + THP-1 cells and PBS + THP-1 cells was 0.091 ± 0.012 and 0.095 ± 0.005 respectively. In this sandwich ELISA system, the OD value of 1 μg recombinant human hsp60 was 0.574 ± 0.048 as the positive control. According to this result, we suggest that rHpHSP60 can not induce human HSP60 secretion from THP-1 cells. Next, we examined the limitation of human heat shock protein 60 could be detected by the sandwich ELISA system (Fig 16). The data showed that the OD value of 0, 1 pg, 10 pg, 100 pg, 1 ng, 10 ng, 100 ng, and 1 μg was 0.087 ± 0.003, 0.087 ± 0.021, 0.086 ± 0.001, 0.09 ± 0.019, 0.083 ± 0.002, 0.013 ± 0.021, 0.195 ± 0.03, and 1.237 ± 0.006 respectively. The concentration of human hsp60 has to reach 1 μg/ml or cannot be detected by the sandwich ELISA system. We concluded that 10 μg rHpHSP60 induced lower than 1 μg human hsp60 or not induction of human hsp60 expression. Thus, we can completely exclude rHpHSP60 cross reacting with human hsp60 to induce TNF-α secretion. The effect we observed was resulting from rHpHSP60.

Monocytes express a variety of cell surface proteins that are thought to play an important role in antigen presentation and the cell contact-dependent interaction of monocytes with

other leukocytes. In this study, the expression of co-stimulatory molecules, such as CD40, CD80, and CD86, are increased of rHpHSP60 stimulation (Fig. 8). HLA-ABC (MHC I) was not significantly affected by rHpHSP60. Interestingly, the expression of HLA-DR (MHC II) was down-regulated by rHpHSP60 treatment (Fig. 8), as previously observed with heat-killed *Mycobacterium bovis* and *Escherichia coli* (153). CD40 binds to the CD40 ligand (CD40L) on T cells. In addition, CD40-CD40L interactions may also play an important role in the cell contact-dependent interaction that occurs between activated helper T cells and monocytes/macrophages during antigen presentation (154). In vitro, ligation of the monocyte CD40 surface protein with the T cell-derived CD40L increases the production of cytokines such as IL-1, TNF, and IL-6 through an ERK1/2-dependent pathway (154, 155). Nevertheless, the CD40-CD40L interactions would result in the up-regulation of matrix metalloproteinases which both facilitate the movement of the cells out of the vasculature into surrounding stroma and to sites of inflammation as well as accelerate the breakdown of ECM during chronic inflammatory diseases (156). The co-stimulatory ligands CD80 and CD86 play a crucial role in the initiation and maintenance of an immune response. The interaction between CD28 expressed on T cells and its counter-receptors CD80 (B7-1) and CD86 (B7-2) expressed on specialized APC provides the most important co-stimulatory signal (157, 158). In our results, the up-regulation of CD40, CD80, and CD86 was observed in THP-1 cells stimulated by rHpHSP60. The expression of MHC I seemed not influenced by rHpHSP60. However, the

down-regulation of HLA-DR (MHC II) was showed after rHpHSP60 treatment. Cytosolic proteins like viral proteins, or bacterial proteins with access to the host's cytosol, are degraded by the proteasome and bind to MHC class I molecules in the endoplasmic reticulum, leading to cytolytic $CD8^+$ T cell responses. Antigens sequestered from the cytosol in endocytic or phagocytic compartments encounter their partners through fusion with endocytic vesicles containing MHC class II molecules. MHC class II is believed to play a central role in initiating the immune response by presenting the foreign antigen to T helper cells. MHC II present antigens to $CD4^+$ T-helper cells and then control differentiation of B cells in antibody producing B-cell. Patients or mice failing to produce proper MHC II–peptide complexes will not produce efficient antibody responses to infection (138). According to these reports, the antigen presenting capacity of THP-1 cells seems diminished by rHpHSP60, and the adaptive immune response could not be exactly activated consequently.

Monocytes play a pivotal role as professional phagocytic other than antigen-presenting cells in the innate immunity. In our study, the engulf ability of THP-1 cells was manifestly inhibited after stimulation THP-1 cells with rHpHSP60 for 16 h (Fig. 7). The accurately mechanism was still unknown. But we can try to explain why the expression of MHC class II was down-regulated. Antigen presentation process that involves the uptake of complex forms of antigens. Then, antigens sequestered from the cytosol in endocytic or phagocytic compartments encounter their partners through fusion with endocytic vesicles containing

MHC class II molecules, leading to $CD4^+$ T cell responses (159, 160).

The findings above show that *H. pylori* HSP60 can stimulate THP-1 cells to produce pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α; hence it was quite correlated with the occurrence of *H. pylori*-associated gastric inflammation. However, the activity of THP-1 cells seems be weakened by *H. pylori* HSP60. Although the expression of co-stimulatory molecules (CD40, CD80, and CD86) was up-regulated, the MHC class II was contrarily down-regulated. Presumably the adaptive immune response would be influenced consequently. Furthermore, the engulf capacity of THP-1 cells was obviously abated by *H. pylori* HSP60. We tried to figure out whether these phenomenons were associated with TNF-α, the rhTNF-α was used in the following experiments. Treatment with rhTNF-α can augment CD40 expression in a dose-dependent manner, but down-regulated the endocytotic activity of THP-1 cells (Fig 9 and Fig 10). It seems that TNF-α can promote THP-1 cells maturation but suppress it's engulf ability under our systems. There were few studies directly using rhTNF-α to stimulate monocytes and examine the effect of rhTNF-α on endocytotic activity. According to previous reports, many publishes revealed that TNF-α was able to augment the monocytes antibacterial activity in vitro experiments with human monocytes (161, 162). Zerlauth *et al.* showed that TNF-α plays a crucial role in the activation of monocytes for growth restriction of intracellular microbes, including *Mycobacterium avium intracellulare* and *L. monocytogenes* (161). However, the studies which prove the TNF-α can enhance endocytotic

activity of THP-1 cells are really deficient. Roilides *et al.* revealed that incubation of human monocytes with TNF-α at 0.001 to 10 ng/ml for 2 days had no effect on the percent phagocytosis of conidia (163), however, Kathleen *et al.* showed that the effect of TNF-α on mature macrophages from mouse or human was to reduce the clearance of apoptotic cells rather than to enhance the uptake and the conclusion of the study was TNF-α stimulation of mature macrophages induces oxidant production through $cPLA_2$ activation and arachidonic acid release leading to increased active Rho and decreased efferocytic function (164). On the other hand, TNF-mediated skewing of monocyte differentiation toward DCs could be observed at a concentration of 1 ng/ml TNF and reached a peak at 10 ng/ml, however, the phagocytosis ability of various phagocytic cells was monocyte-derived macrophage ＞ monocytes ＞ immature dendritic cells ＞ polymorphonuclear neutrophilic leukocytes ＞ matute dendritic cells (165). We tried to explain the effect of TNF-α on THP-1 cells we observed was that if TNF-α earlier contact to THP-1 cells, it may skew cells to differentiate to dendritic cells phenotype and then cells turn to a professional antigen presenting cells and phagocytosis ability was weakened.

TGF-β1 is a multifunctional cytokine that plays a central role in the pathogenesis of several chronic infectious diseases. The ability of TGF-β1 to interfere with the activation and oxidative mechanisms of macrophages (139) has been implicated in promoting persistent infection by providing a survival advantage to pathogens. In this regard, the ability of

pathogens to elicit TGF-β1 expression has been hypothesized as a potential virulence trait (107, 166). The TGF-β1-specific staining of immunohistochemistry showed that gastric epithelium of people long-term infected with *H. pylori* expressed a high level of TGF-β1 (Fig. 11). As for the pro-inflammatory cytokines, the frequencies of TGF-β1-specific cells were also higher in the *H. pylori*-infected subjects than in the uninfected subjects (45). In this study, we further investigated the immune cells at the late stage of inflammation region where filled with pro-inflammatory cytokines and regulatory cytokines, such as TNF-α and TGF-β1 respectively. Monocytes require 1 to 10 ng/ml of TGF-β to inhibit peroxide secretion and microbial killing. TGF-β can impair human monocyte functions on release of H_2O_2 and 0_2^-, adherence and phagocytosis. Moreover, increased levels of TGF-β have been reported in the monocytes and granulomatous lesions of tuberculosis patients (167). Several pathogens have been found that they can utilize TGF-β1 to escape from host immune attack, such as *Trypanosoma cruzi* (168), *Mycobacterium avium* (99), and the *Mycobacterium tuberculosis* strain *(100)*. Our results showed that TNF-α alone (the concentration between 0.4 ng ~ 2 ng) could suppress endocytotic activity of THP-1 cells, and when synergized with TGF-β1 in a dose-dependent mannar, the inhibition effect was more obviously (Fig. 12). It seems like TNF-α synergized with TGF-β1 to diminish the endocytotic activity of THP-1 cells. Treatment of both HL60 and U937 cells with TNF-α induced a dose-dependent increase in expression of TGF-β receptors, suggesting that the synergy between TNF-α and TGF-β1 may

result from upregulation of TGF-β1 receptor expression by TNF-α (169). Thus, we reasonably supposed that TNF-α may favorably augment TGF-β1-mediated suppression effect on endocytosis ability of THP-1 cells. On the other hand, we also found that TGF-β1 would suppress TNF-α-mediated CD40 expression on THP-1 cells in a dose-dependent manner (Fig. 13). In terms of statistics, the value of this data was not significant between each condition but it can still give us a hint among this result.

In conclusion, *H. pylori* HSP60 can induce THP-1 cells to produce pro-inflammatory cytokines; including IL-1β, IL-6, IL-8 and TNF-α which are quite associated with gastric inflammation occurrence. Among these cytokines, TNF-α was earliest secreted by THP-1 cells and it may have a greater effect on cells than other cytokines. We can completely exclude from contaminating with LPS or cross-reacting with human hsp60 causing TNF-α expression. Although pro-inflammatory cytokines, especially TNF-α have been reported to activate immune activity, however, an inhibition effect of endocytosis activity was observed in our result. The costimulatory molecules (CD40, CD80, CD86) was up-regulated on THP-1 cells surface, whereas the expression of MHC class II was decrease. This effect would influence the $CD4^+$ T cell responses. Treatment THP-1 cells with recombinant human TNF-α surprisingly found that endocytosis activity of THP-1 cells was decrease in a dose dependent manner, whereas the CD40 expression was up-regulation on cell surface as our expected. This study suggested that rHpHSP60 induces proinflammatory cytokines secretion

but diminishes monocytes activation.

Table 1

mRNA targets	Oligonucleotides (5' → 3')		Product Sizes (bp)
Human β-actin	F926	TTGCCGACAGGATGCAGAA	80
	R1007	GCCGATCCACACGGAGTACT	
Human TNF-α	F275:	CCCAGGGACCTCTCTCTAATC	84
	R358:	ATGGGCTACAGGCTTGTCACT	

Table 1. Oligonucleotides for quantitative real-time PCR. F and R indicate forward and reverse primers, respectively; numbers indicate the sequence position.

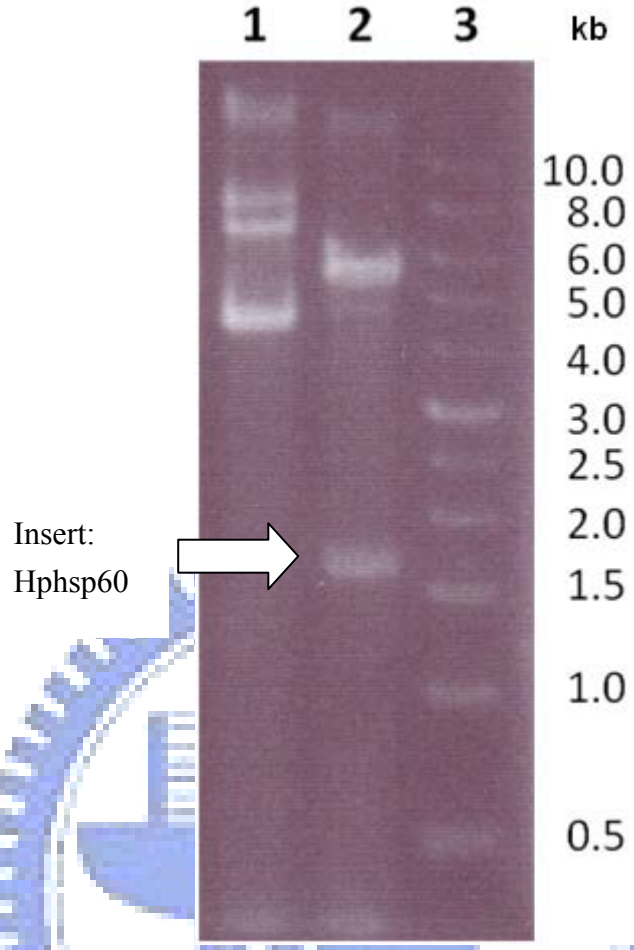
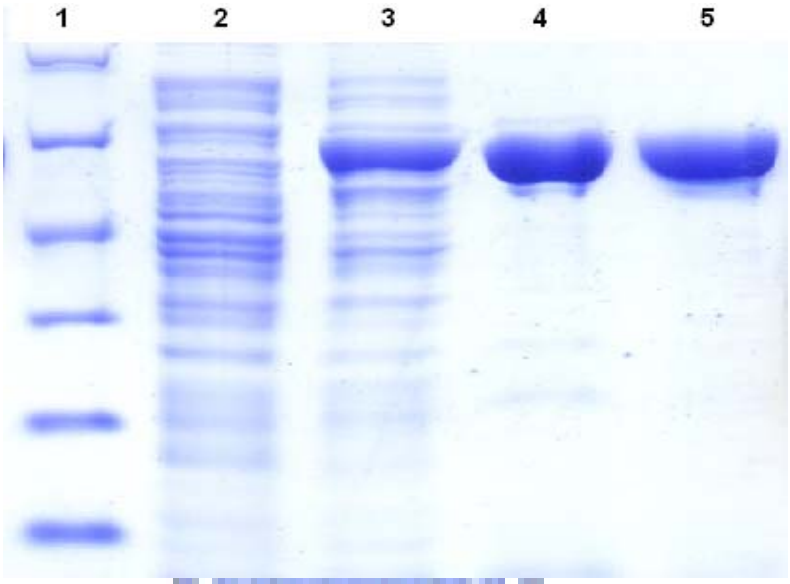


Figure 1. Identification of recombinant *H. pylori* hsp60 plasmid by restriction enzyme digestion. Lane 1: Recombinant plasmid without restriction enzyme, Lane 2: Recombinant plasmid digested with EcoR I and Xho I. Lane 3: Nucleotide marker.

(a)



(b)

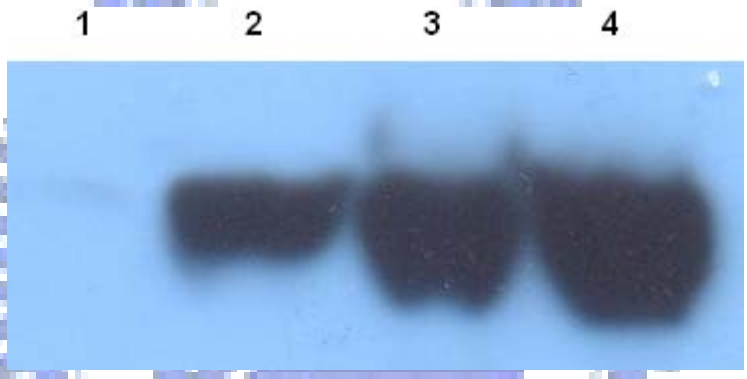


Figure 2. 1 SDS-PAGE and western blot analysis of the recombinant protein expressed in BL21. (a) SDS-PAGE analysis of the recombinant *H. pylori* protein expression. Lane 1: molecular weight marker (20,30,43,67, and 94 kD), Lane 2: Control strain BL21 (pET) before induction, Lane3: Control strain BL21(pET) after 4 h induction with IPTG, Lane 4: Purified recombinant protein Hsp60 by HisTrap™ HP column, Lane 5: Purified recombinant protein Hsp60 passing through Sephadex G-25 gel filtration column. (b) Western bolt analysis of the recombinant *H. pylori* protein expression. Lane 1: Control strain BL21(pET) before induction, Lane 2: Control strain BL21(pET) after 4 h induction with IPTG, Lane3: Purified recombinant protein Hsp60 by HisTrap™ HP column,Lane 4: Purified recombinant protein Hsp60 passing through Sephadex G-25 gel filtration column.

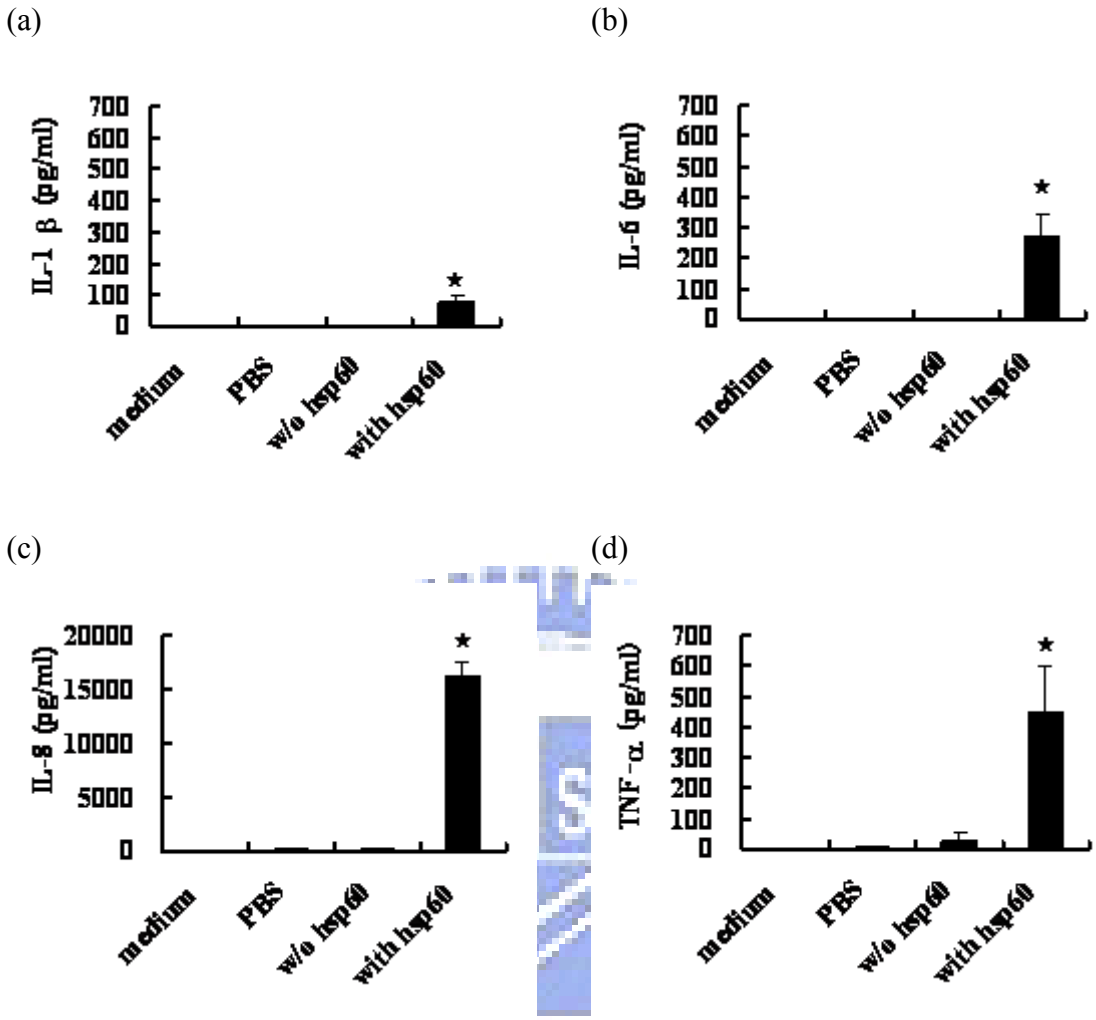


Figure 3. Production of pro-inflammatory cytokines in THP-1 cells. (a) IL-1β, (b) IL-6, (c) IL-8 and (d) TNF-α secretion in THP-1 cells in response to rHpHSP60. Recombinant heat shock protein 60 was added to medium containing 5×10^5 cells to a final concentration of 10 μg/ml incubated in 24-well plate for 24 h. Cytokines were measured by ELISA in 24 h culture medium. Results are representative at least three independent experiment. ★, $P < 0.001$ compared to without rHpHSP60 treatment. (n=3)

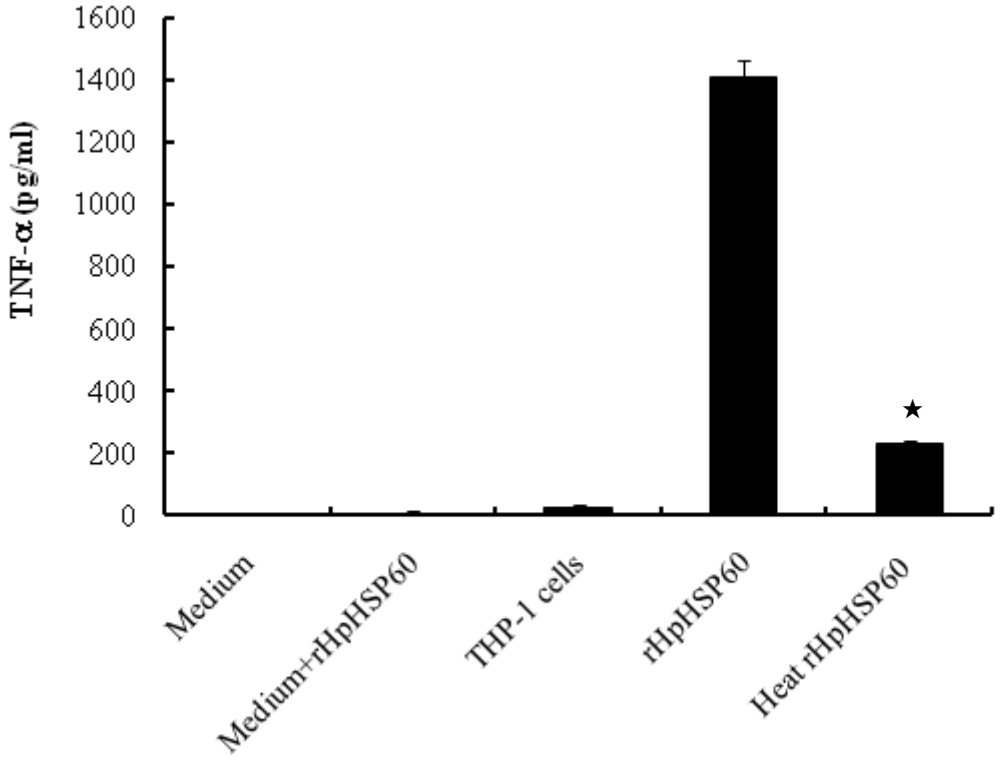


Figure 4. Release of TNF-α from THP-1 cells incubated with rHpHSP60 or heated rHpHSP60. THP-1 cells stimulated with rHpHSP60 or rHpHSP60 heating to 95 ℃ for 1 h. After 16 h, the production of TNF-α analyzed by ELISA. TNF-α expression due to rHpHSP60 but not to LPS is sensitive to heating 95 ℃ for 1 h, further indicating that the observed effect of rHpHSP60 is not a consequence of contamination by LPS. ★, $P < 0.01$

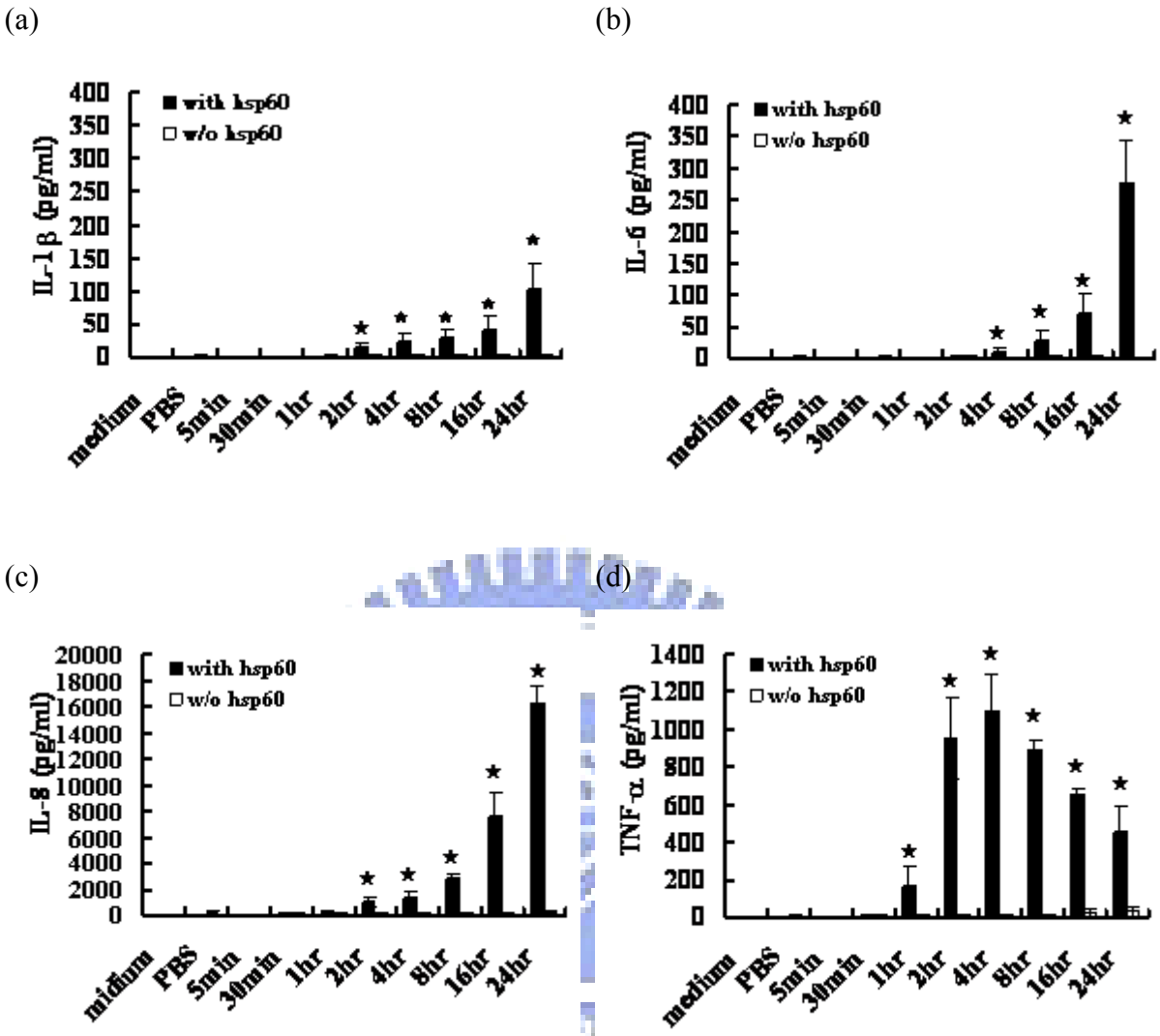


Figure 5. Kinetics of pro-inflammatory cytokine secretion in THP-1 cells. (a) IL-1β, (b) IL-6, (c) IL-8, and (d) TNF-α secretion by THP-1 cells stimulated with 10 μg/ml rHpHSP60. Cytokines were measured in culture supernatants at eight different time-points. Results are representative at least three independent experiment. ★, P <0.01 compared to without HpHSP60 treatment. (n=3)

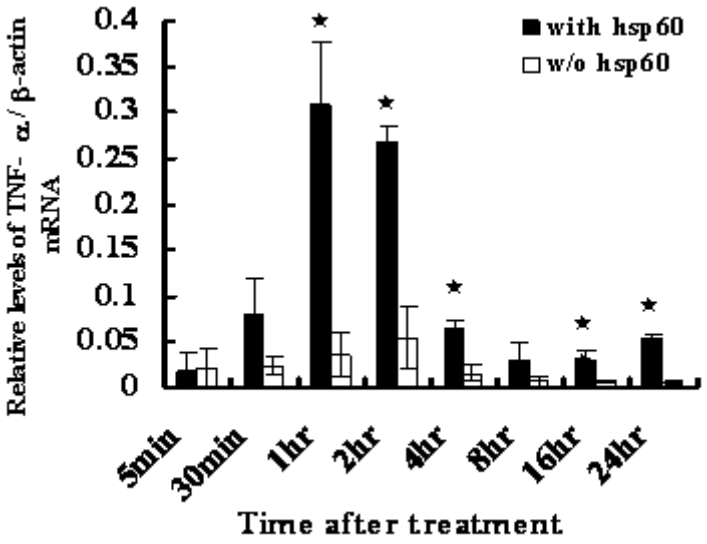
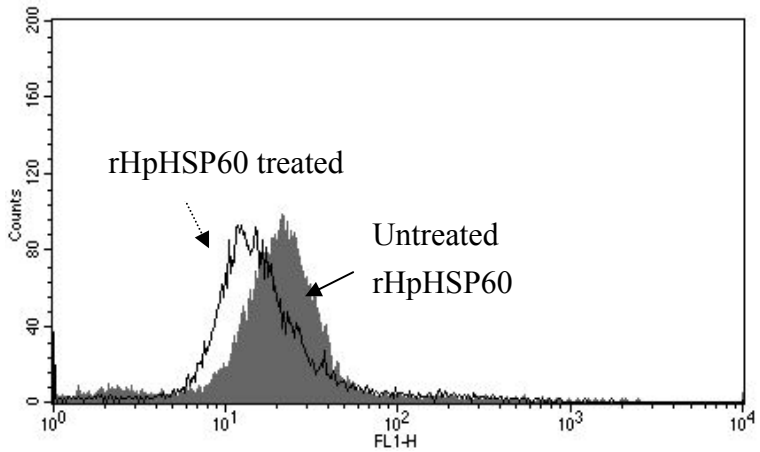


Figure 6. Kinetic of TNF-α mRNA gene expression in THP-1cells. THP-1 cells stimulated by 10 μg/ml rHpHSP60 and the cells were collected at 5 min, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h. TNF-α mRNA expression in THP-1 cells was analyzed by quantitative real-time PCR. Maximal TNF-α mRNA significantly expression between 1 and 2 h post-stimulation.★, $P < 0.01$ compared to without rHpHSP60 treatment. (n= 4)

(a)



(b)

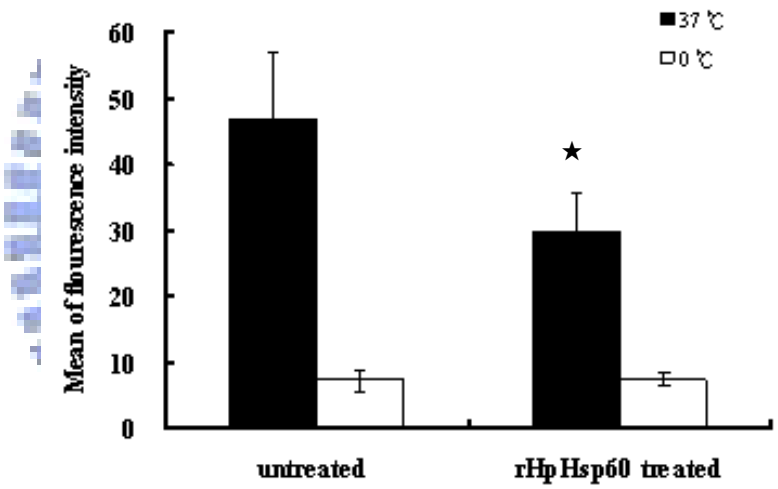
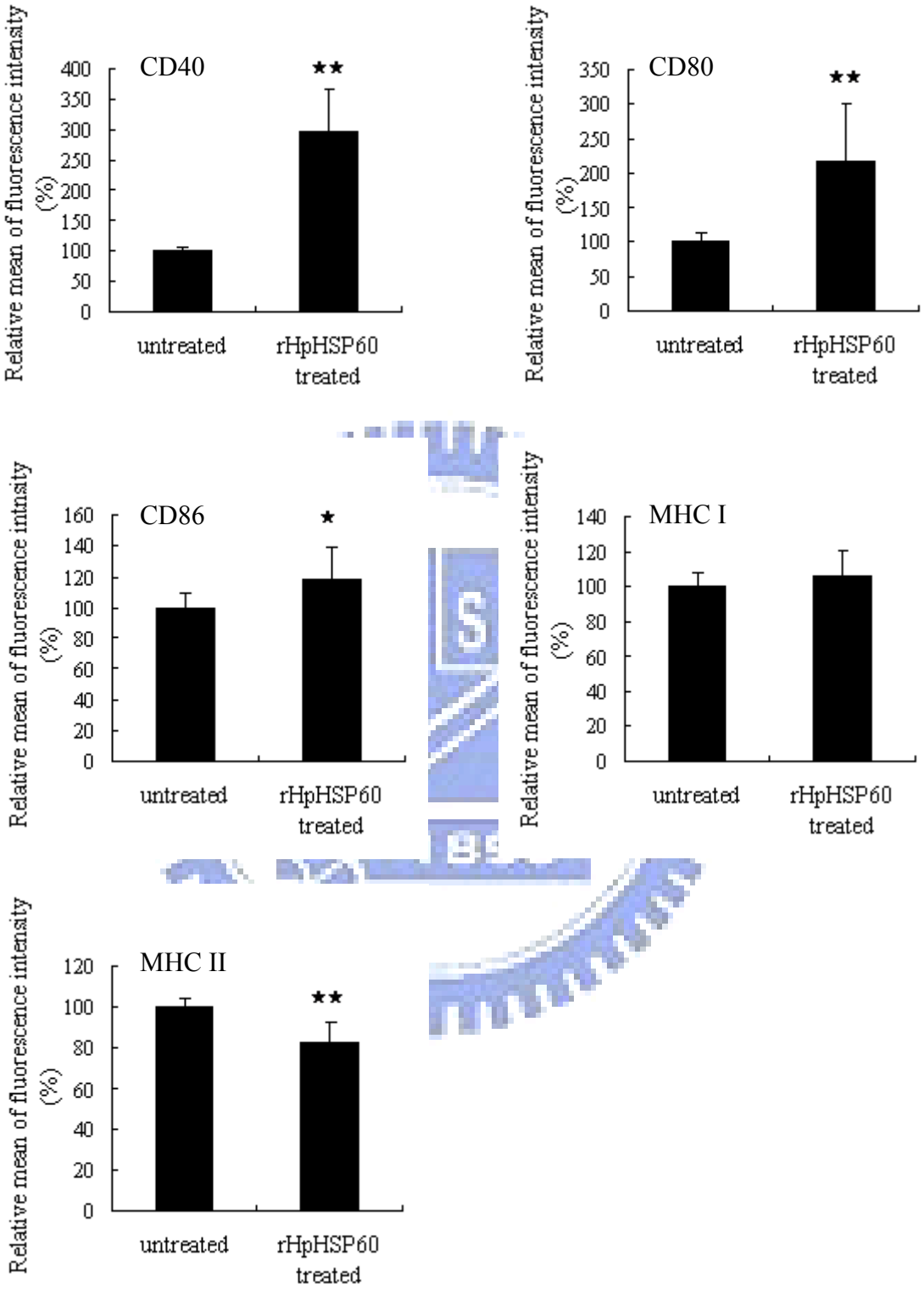


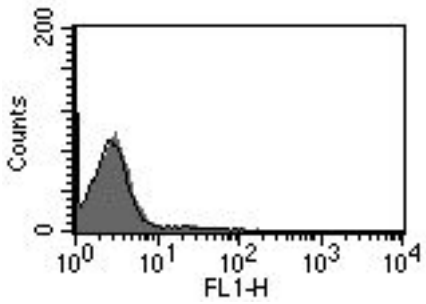
Figure 7. Endocytotic ability of THP-1 cells treated with rHpHSP60. THP-1 cells were cultured with or without rHpHSP60 for 16 h. and then incubated with FITC- dextran for 2 h at 37 ℃ or at 0 ℃ as a control for endocytosis. Cells were analyzed by flow cytometry. (a) The solid histogram indicated cells without rHpHSP60 treatment and the thick-lined histogram indicated cells with rHpHSP60 treatment. (b) The mean fluorescence intensity of cells is reported. Data are expressed as means ± standard deveations from three experiments. ★, $P<$ 0.05 compared to untreated cells. (n=3)

(a)

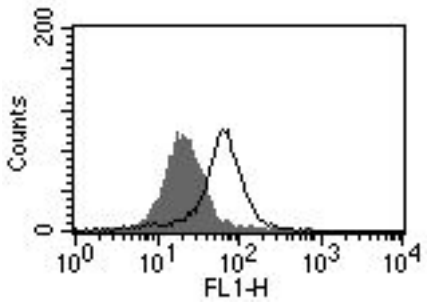

CD40
**
Relative mean of fluorescence intensity (%)
400
350
300
250
200
150
100
50
0
untreated
rHpHSP60 treated
CD80
**
Relative mean of fluorescence intensity (%)
350
300
250
200
150
100
50
0
untreated
rHpHSP60 treated
CD86
*
Relative mean of fluorescence intnsity (%)
160
140
120
100
80
60
40
20
0
untreated
rHpHSP60 treated
MHC I
Relative mean of fluorescence intensity (%)
140
120
100
80
60
40
20
0
untreated
rHpHSP60 treated
MHC II
**
Relative mean of fluorescence intensity (%)
120
100
80
60
40
20
0
untreated
rHpHSP60 treated

(b)

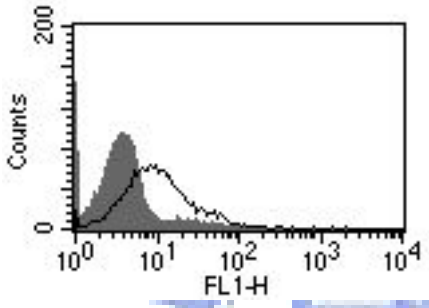
NC



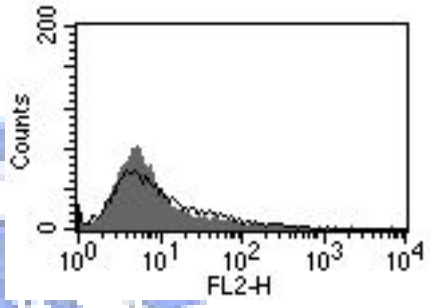
CD40



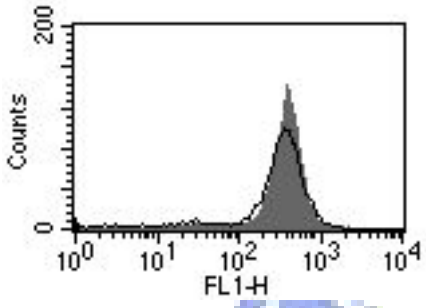
CD80



CD86



MHC I



MHC II

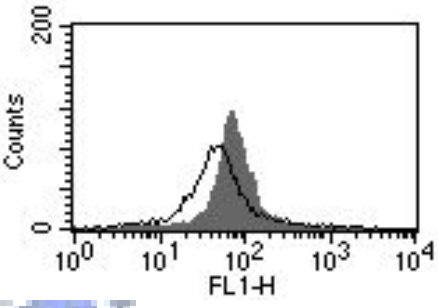


Figure 8. Surface marker expression on THP-1 cells treated with rHpHSP60. Flow cytometric analysis of THP-1 cells cultured with rHpHSP60 for 16 h. The expression of different surface markers on treated or untreated cells is reported. (a) Columns showed relative mean of fluorescent intensity of THP-1 cells treated or untreated rHpHSP60 (rHpHSP60 treated/untreated × 100%) (b) Histograms showed the expression of surface markers on THP-1 cells. Solid histograms indicated without rHpHSP60 treatment and open histograms indicated with rHpHSP60 treatment. Data are representative of five independent experiments. (n=5)

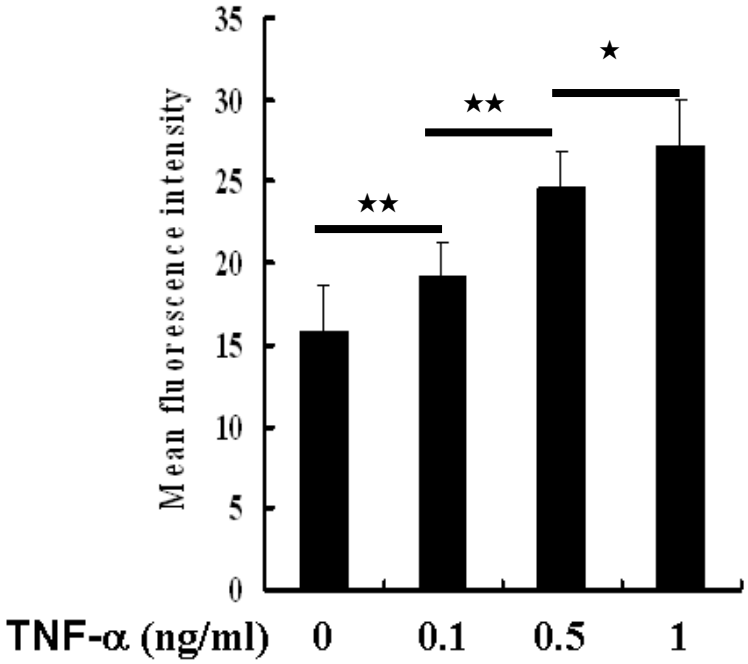


Figure 9. Effect of recombinant human TNF-α on CD40 expression. Cells were treated with rhTNF-α alone with three concentrations for 24 h and analyzed by flow cytomestry. Mean ± S.D. of CD40 expression is reported, four independent experiments is shown for each concentration of rhTNF-α tested. ★★, P <0.01; ★, P < 0.05 (n=4)

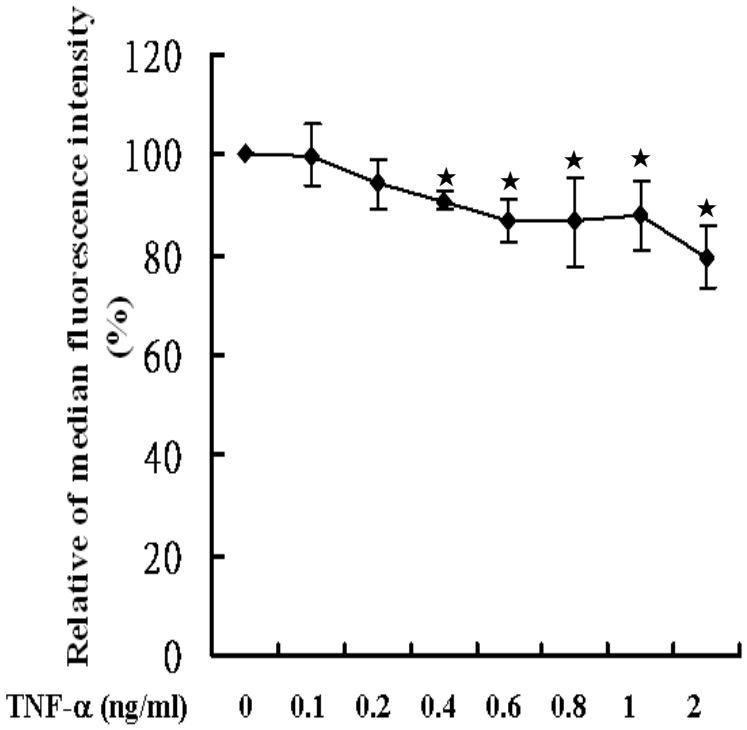
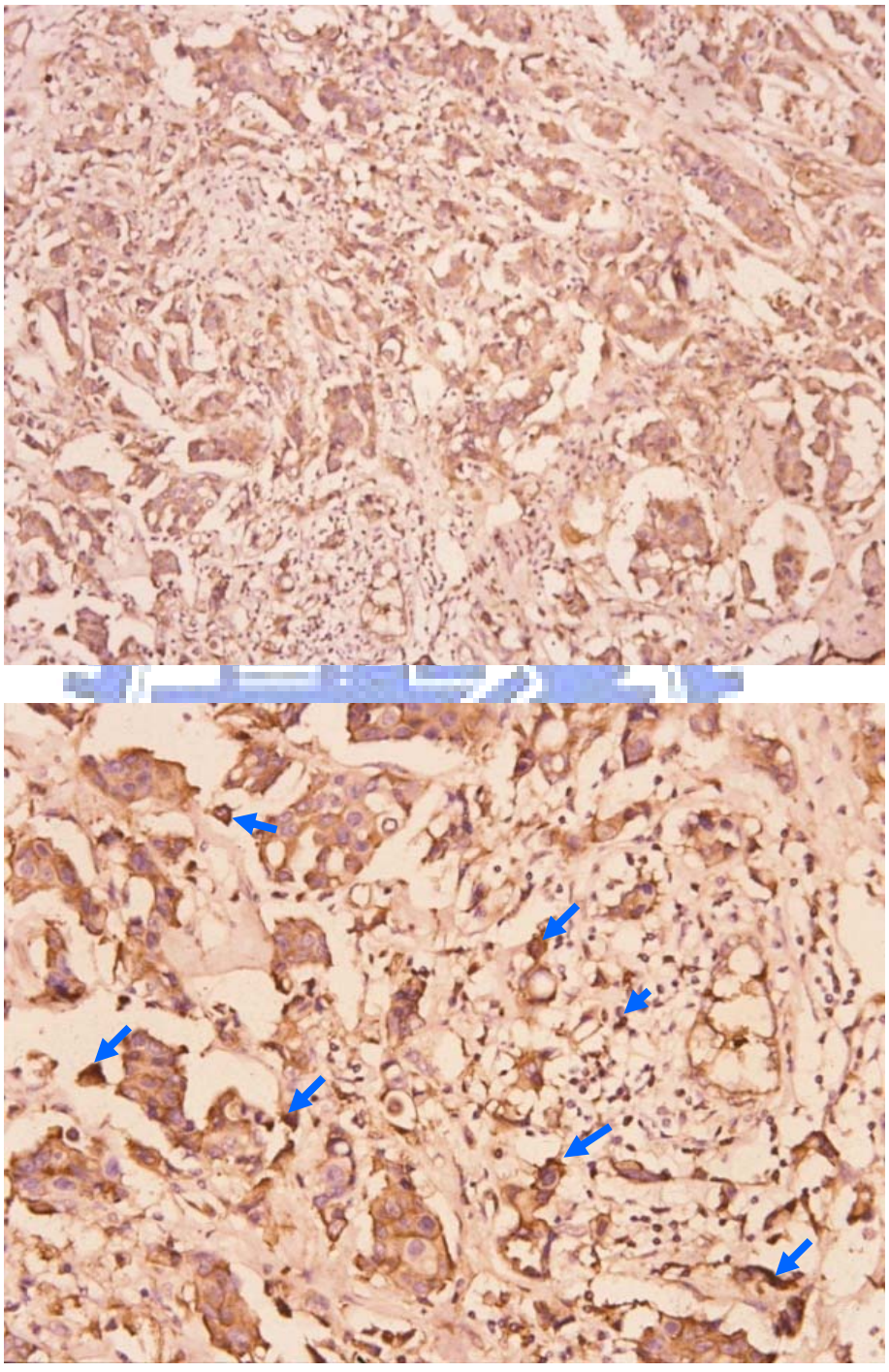


Figure 10. Effect of recombinant human TNF-α on endocytotic activity of THP-1 cells. Cells were treated with rhTNF-α alone with seven concentrations for 16 h and then incubated with FITC- dextran for 2 h at 37 ℃ or at 0 ℃ as a control for endocytosis. The median fluorescence intensity minus median fluorescence of control cells (0 ℃) was calculated. The data showed the relative median fluorescence intensity and three independent experiments are shown for each concentration of rhTNF-α tested. ★, $P < 0.05$ (n=3)

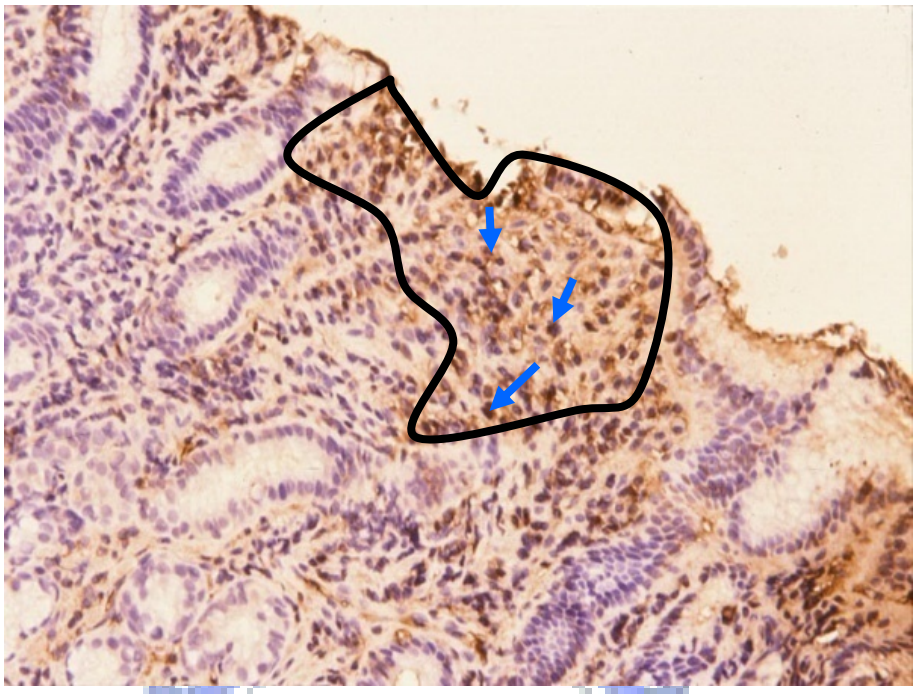
Figure 11.

(a)

(b)



(c)



(d)

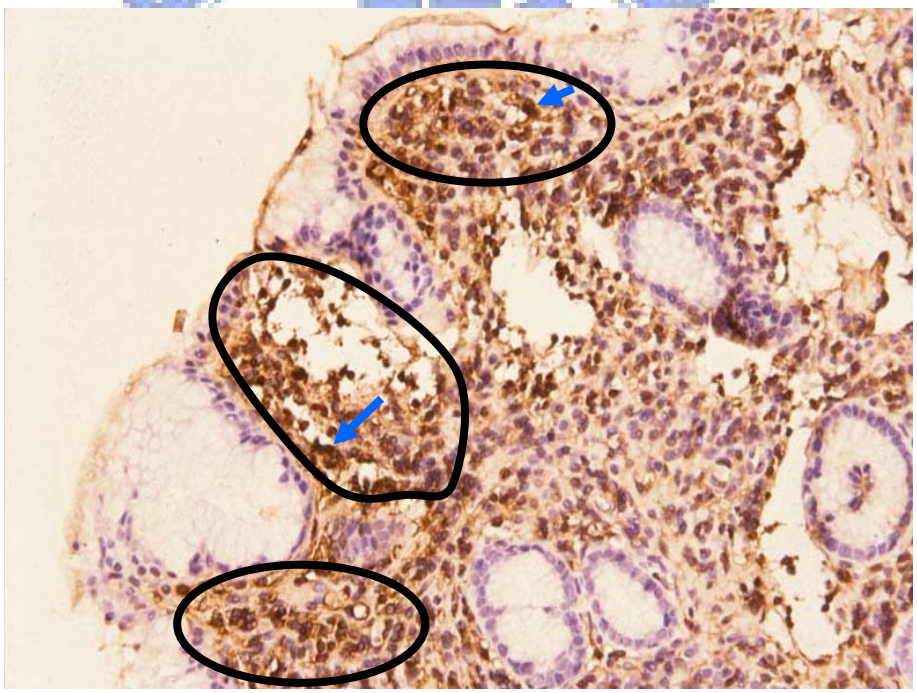


Figure 11. Immunohistochemistry of subjects infected by *Helicobacter pylori*. (a) and (b) are TGF-β1-specific staining in the patient with breast cancer used as positive control. (a) Original magnification, × 100. (b) Original magnification, × 200. (c), (d) TGF-β1-specific staining in gastric epithelium obtained from *H. pylori* -infected subjects. (c), (d) come from two individual patients. The brown color represents distribution of epithelial TGF-β1 reactivity (arrows) and the higher level of TGF-β1 was marked with a circle in black color.

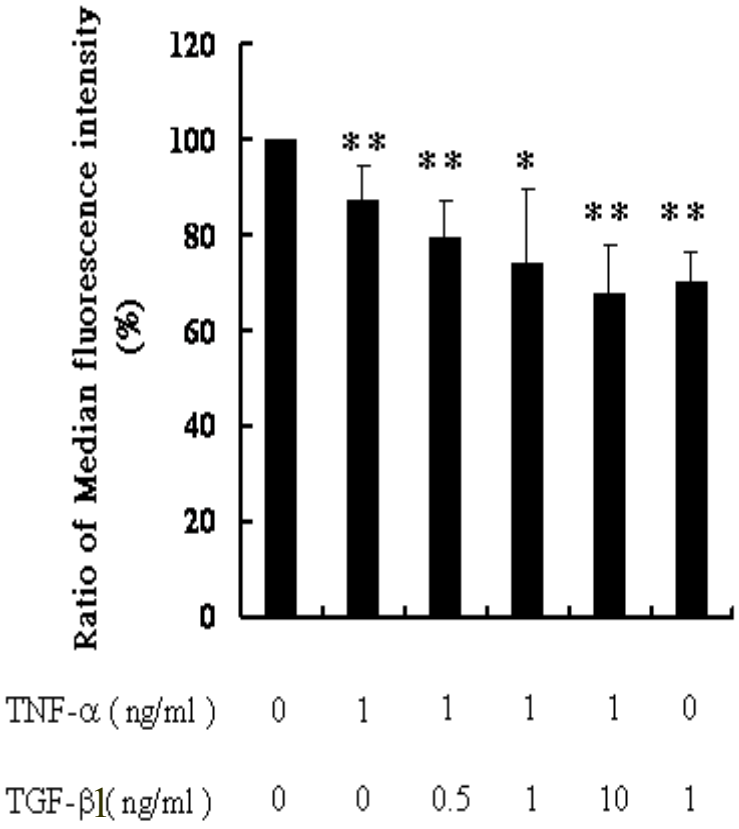


Figure 12. Effect of treatment with TGF-β1 and TNF-α on endocytotic activity of THP-1 cells. Cells incubation with indicated concentrations of TGF-β1 with or without 1 ng/ml TNF-α for 16 h. The median fluorescence intensity minus median fluorescence of control cells (0 ℃) was calculated. The data showed the relative median fluorescence intensity and three independent experiments are shown for each concentration of rhTNF-α tested. **, $P < 0.01$; *, $P < 0.05$ (n=3)

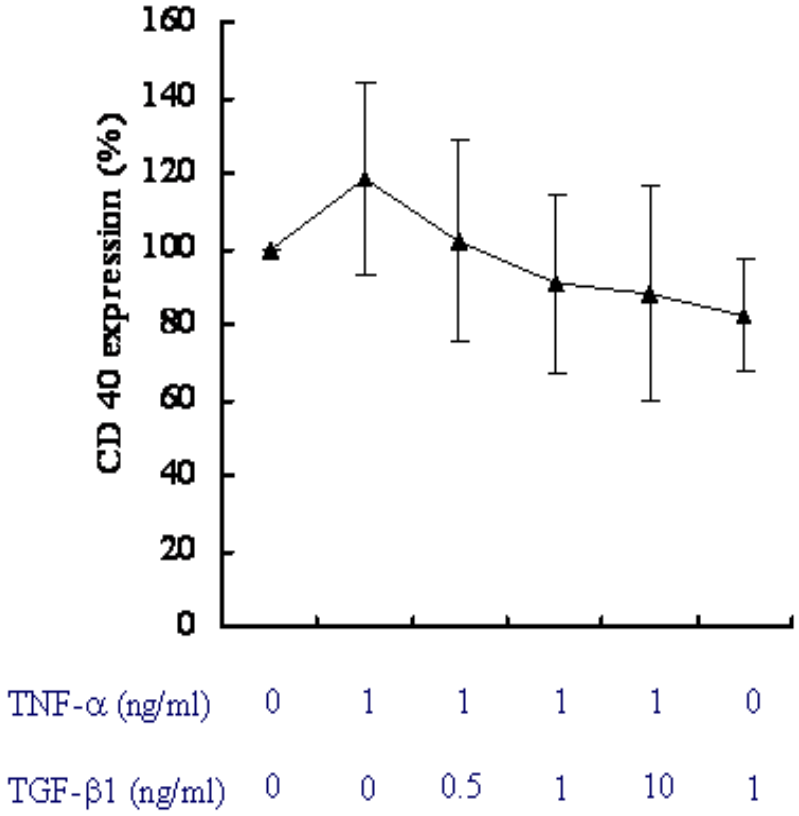


Figure 13. Effect of treatment with TGF-β1 and TNF-α on CD40 expression of THP-1 cells. Cells incubated with indicated concentrations of TGF-β1 with or without 1 ng/ml TNF-α for 16 h. The data showed the relative mean fluorescence intensity and three independent experiments are shown for each concentration of rhTNF-α tested. (n=3)

(a)

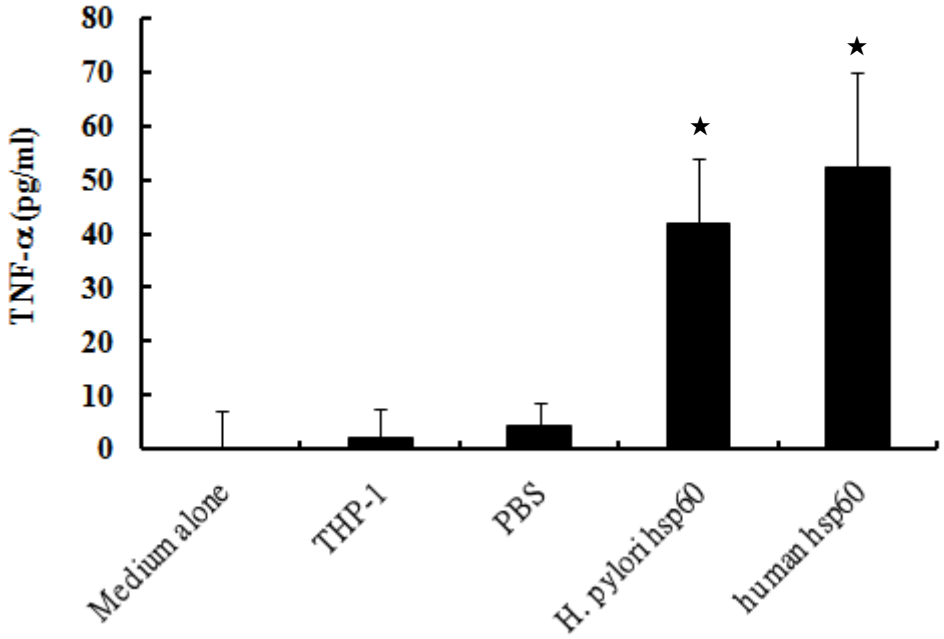


Figure 14. Production of TNF-α in THP-1 cells by *H. pylori* hsp60 or human hsp60 stimulation. 10^5/ml THP-1 cells were seeded in 24-well culture plate at a volume of 1 ml. Cell were then treated with 10 μg/ml *H. pylori* or human hsp60 respectively for 16 h. The concentration of TNF-α was analyzed by ELISA. ★, $P < 0.05$ (n=3)

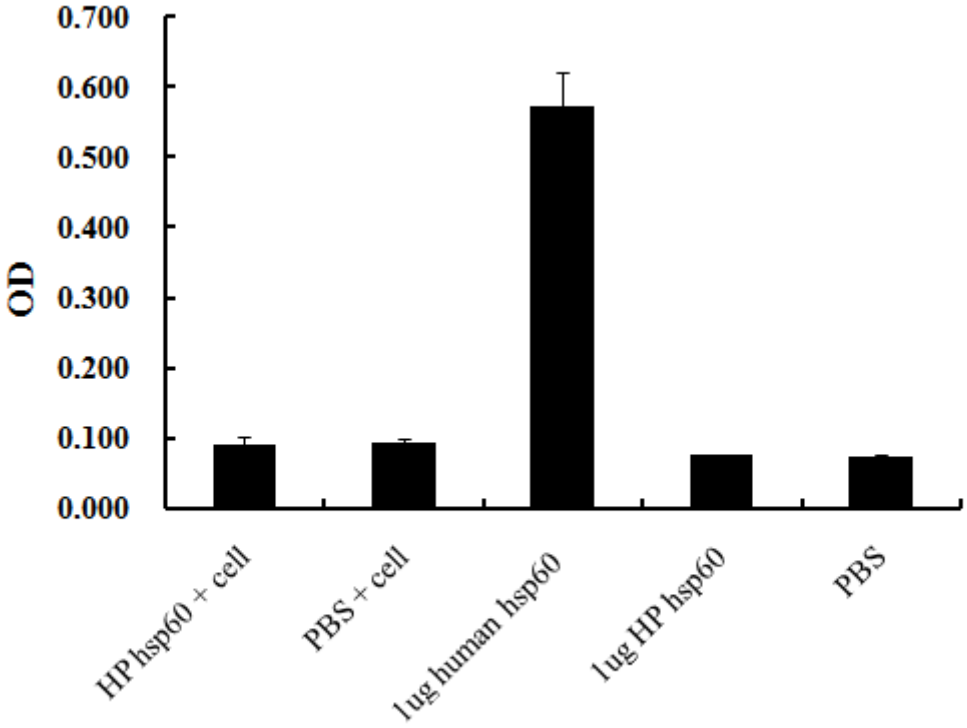


Figure 15. Detection of human heat shock protein 60 secreted from THP-1 cells after rHpHSP60 stimulation. 10^5/ml THP-1 cells were seeded in 24-well culture plate at a volume of 1 ml. Cells were treated with 10 μg/ml rHpHSP60 for 16 h and collected the supernatant. The amount of human HSP60 was detected by ELISA. 1 μg human HSP60 was the positive control of the ELISA system. 1μg rHpHSP60 and PBS were the negative control of the ELSA system to further confirm the antibody which detects the human HSP60 will not cross react to rHpHSP60 or nonspecific recognition respectively.

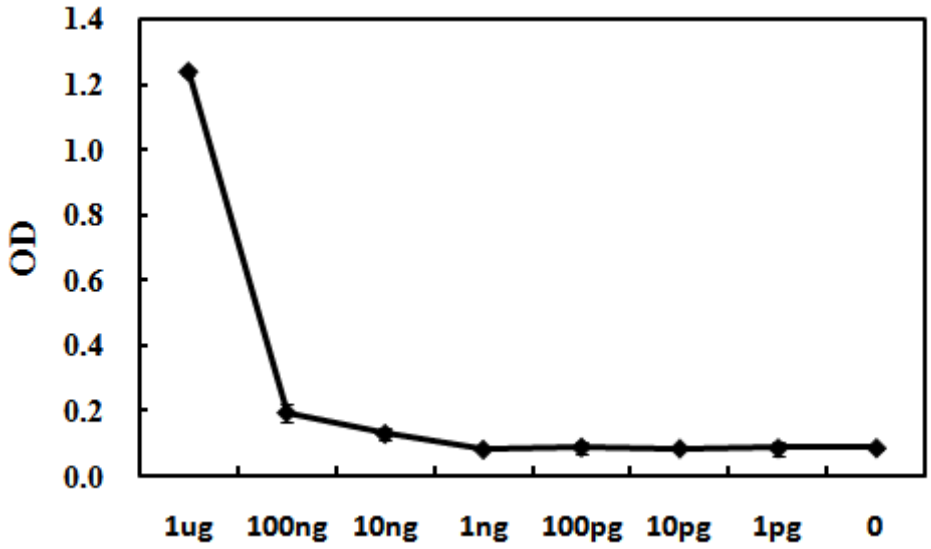
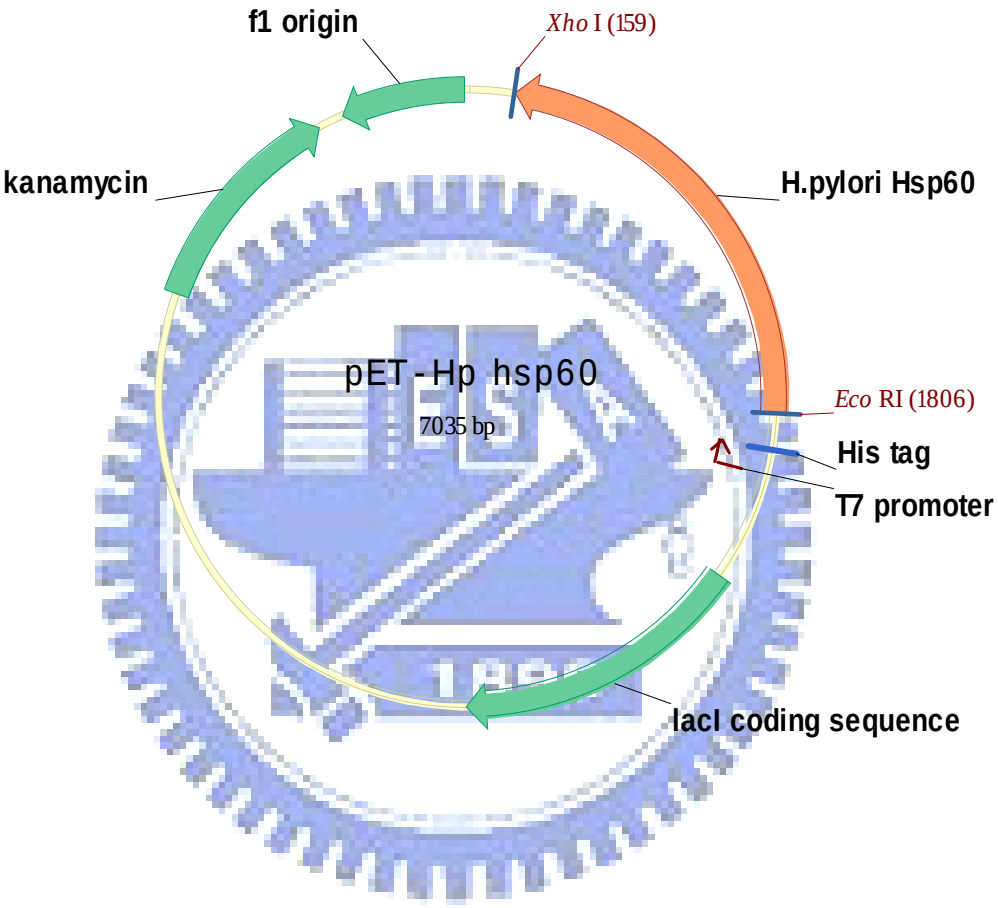


Figure 16. The limitation of human heat shock protein 60 detecting by the ELISA system. The rabbit anti-human HSP60 polyclonal antibody was used as the capture antibody at 37 ℃ incubator for 1 h. After washing three times, blocking buffer was added to 300 μl/well at 37 ℃ incubator for 1 h. After washing three times, the seven concentration of human HSP60 was added in the well and incubated at room temperature for 1 h. After washing three times, the mouse anti-human HSP60 monoclonal antibody was used as the detection antibody and incubated at room temperature for 1 h. Finally, the rabbit anti-mouse IgG conjugated HRP antibody was added after three times washing. Substrate solution was then added in the well and incubated at room temperature for 20 minutes. Add 50 μl of stop solution to each well and determined the optical density of each well by 450 nm wavelength.

Appendix

1-1 pET-Hp hsp60 map



1-2 *H. pylori* hsp60 DNA sequence

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1-3 *H. pylori* hsp60 protein sequence

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1-4 The whole DNA sequence of *H. pylori* hsp60

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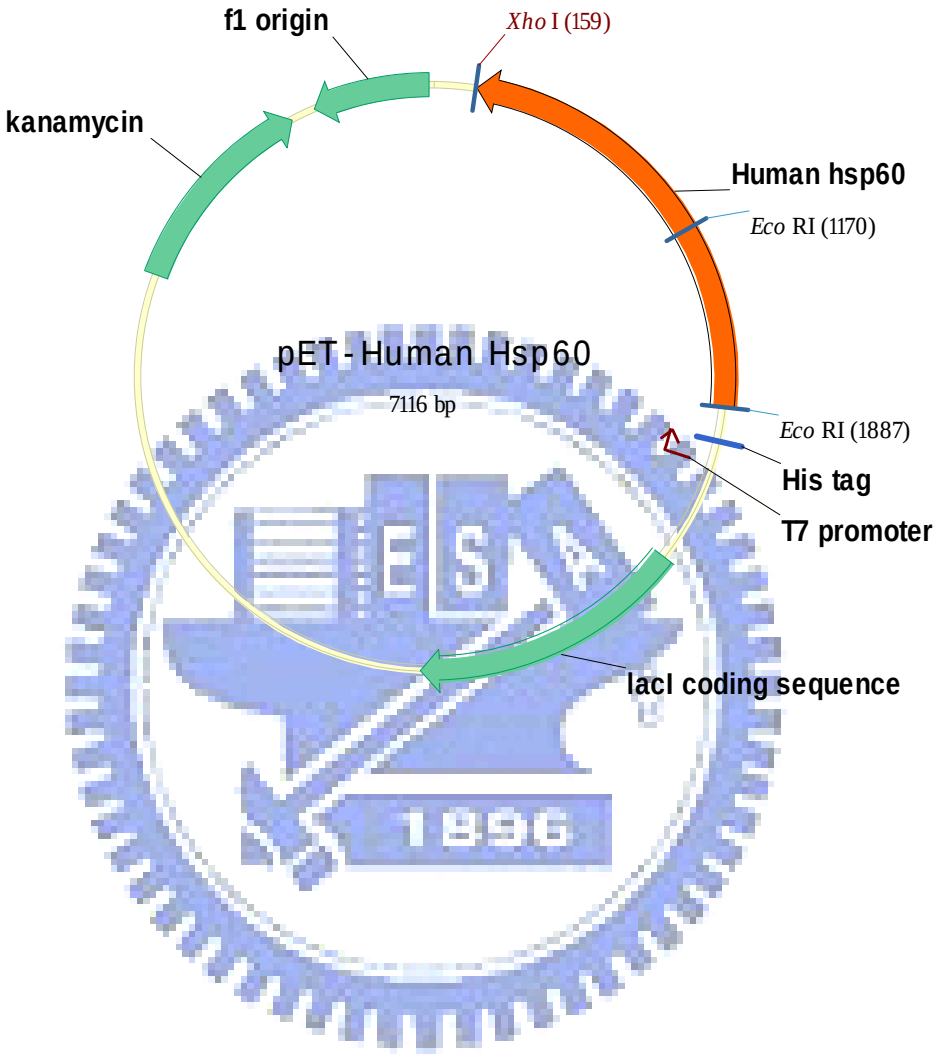
2-1 pET-Human hsp60 map



2-2 Human hsp60 DNA sequence

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2-3 Human protein sequence

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101 vanntneeag dgtttatvla rsiakegfek iskganpvei rrgvmlavda
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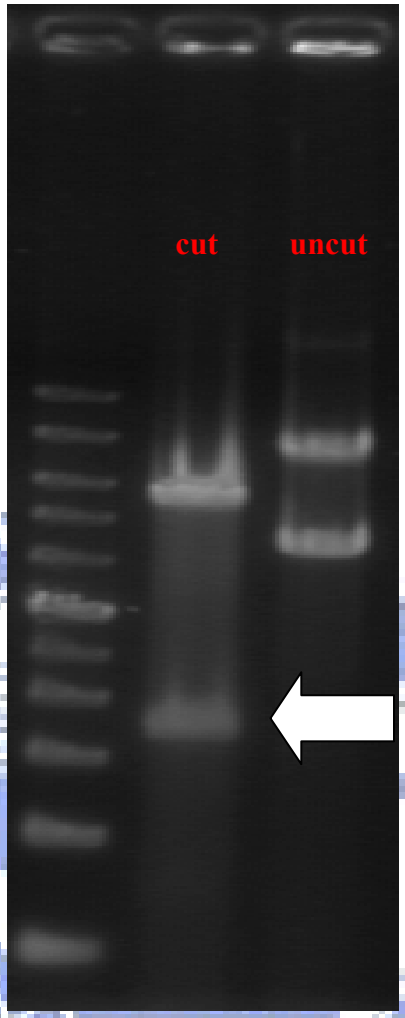
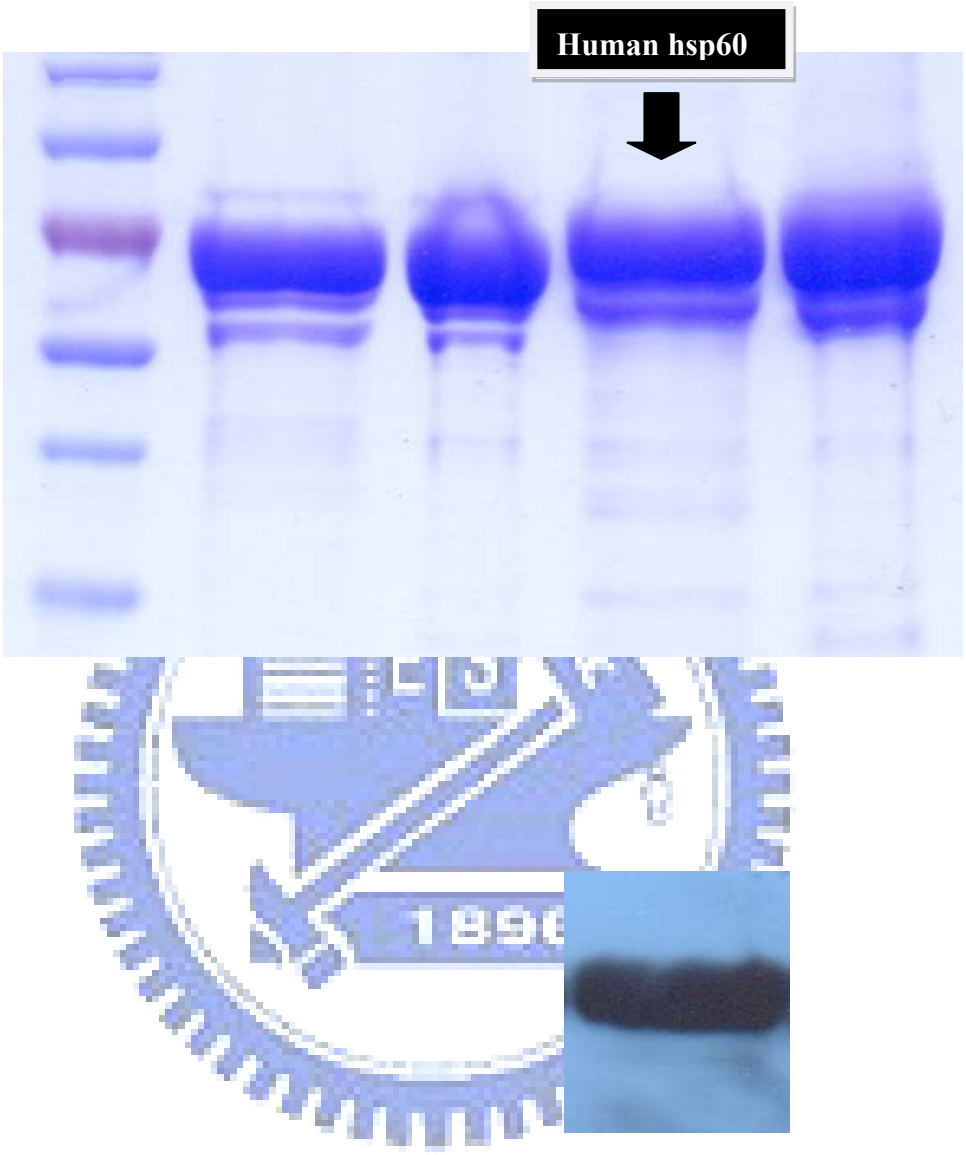
201 vkdgktlnde leiiegmkfd rgyispyfin tskgqkcefq dayvllsekk
251 issiqsivpa leianahrkp lviiaedvdg ealstlvlnr lkvglqvvav
301 kafgdnrpgk nqlkdmaiat ggavfgeegl tlnledvqph dlgkvgeviv
351 tkddamllkg kgdkaqiekr iqeiieqldv ttseyekekl nerlaklsdg
401 vavlkvggts dvevnekkdr vtdalnatra aveegivlgg gcallrcipa
451 ldsltpaned qkigieiikr tlkipamtia knagvegsli vekimqssse
501 vgydamagdf vnmvekgiid ptkvvrtall daagvasllt taevvvteip
551 keekdpgmga mggmgggmgg gmf
```

2-4 Identification of recombinant human hsp60 plasmid by restriction enzyme digestion



2-5 SDS-PAGE and western blot analysis of the recombinant human HSP60 protein expressed in BL21.



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