

Antibodies against *Helicobacter pylori* heat shock protein 60 aggravate HSP60-mediated proinflammatory responses

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ABSTRACT

Anti-*Helicobacter pylori* heat shock protein 60 (HpHSP60) antibodies are usually found in *H. pylori*-infected patients and are known to be associated with the progression of gastric diseases. However, the effects of these antibodies on the functions of HpHSP60 have not been identified. This study aims to investigate the effects of the interaction between anti-HSP60 antibodies and HpHSP60 on inflammatory responses. Anti-HpHSP60 polyclonal sera and monoclonal antibodies (mAbs) were produced to evaluate their effects on HpHSP60-induced IL-8 and TNF- α activity. The results indicated that anti-HpHSP60 polyclonal sera collected from patients infected with *H. pylori* or from rabbit and mice immunized with HpHSP60 could significantly enhance HpHSP60-mediated IL-8 and TNF- α secretion from monocytic THP-1 cells. Similar effects were also found with anti-HpHSP60 mAbs. Further analysis revealed that this phenomenon was only carried out by anti-HpHSP60 antibody but not by other non-specific mAbs. Moreover, the non-specific mAbs decreased the synergism of HpHSP60 and anti-HpHSP60 mAbs in proinflammatory cytokine induction. Herein, we have examined the role of anti-HpHSP60 antibody in host immune responses for the first time. This study demonstrated that *H. pylori* HSP60/mAbs could modulate helicobacterial pathogenesis by increasing IL-8 and TNF- α production. The pathogen-specific antibodies may execute potential immune functions rather than recognize or neutralize microbes.

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1. Introduction

Helicobacter pylori (*H. pylori*) infection induces inflammatory responses that are closely associated with tumorigenesis through enhancing angiogenesis, remodeling, and suppression of antitumor immunity [1]. An array of proinflammatory cytokines such as IL-1 β , IL-8, and TNF- α [2] are stimulated by *H. pylori* to modulate host immune functions [3] or promote apoptosis of gastric epithelial cells [1], which have been suggested to play an important role in the pathogenesis of chronic gastritis or gastric cancer.

H. pylori heat shock protein 60 (HpHSP60) has been identified as one of the potential immunogens of the bacterium that induces IL-6, IL-8, TNF- α , and GRO production from monocytes or gastric epithelial cells [4,5]. Several examples have indicated that HpHSP60 plays a role in the induction of chronic mucosal inflammation and gastric atrophy [6–8]. In addition, HpHSP60-mediated inflammation could also facilitate angiogenesis through IL-8/GRO-CXCR2 engagement and Ca²⁺/PLC β 2 signaling activation [9]. The role of HpHSP60 in gastric carcinogenesis has been proved to promote inflammation, angiogenesis, and migration abilities of monocytes and gastric tumor cells [5].

Anti-HpHSP60 antibodies were consistently detected in *H. pylori*-infected patients, and the titers were associated with the progression of gastritis or gastric cancer [10–13]. However, few studies have attempted to disclose the contribution of these antibodies on the activities of HpHSP60. Herein, we sought to inspect the effects of the interplay between anti-HSP60 antibodies and

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HpHSP60 on inflammatory responses. The anti-HpHSP60 antibodies derived from different sources were evaluated for their influences on HpHSP60-mediated IL-8 or TNF- α induction. Surprisingly, anti-HpHSP60 antibodies derived from different sources did not inhibit the induction of HpHSP60-mediated proinflammatory cytokine but instead enhanced their secretion. Thus, the results suggested that anti-HpHSP60 antibodies in patient sera may intensify gastritis opposed to neutralize microbes.

2. Materials and methods

2.1. Cells, animals and reagents

THP-1 cells (BCRC, Hsinchu, Taiwan) were cultured in RPMI 1640 medium supplemented with 0.05 mM 2-mercaptoethanol (Sigma–Aldrich, MO, USA), 2 g/l of sodium bicarbonate, 50 μ g/ml of penicillin/streptomycin, and 10% heat-inactivated FBS. FO cells (BCRC) were cultured in DMEM medium supplemented with 1.5 g/l of sodium bicarbonate, 50 μ g/ml of penicillin/streptomycin, and 10% heat-inactivated FBS. Hybridomas were cultured with DMEM supplemented with 1.5 g/l of sodium bicarbonate, 50 μ g/ml of lipopolysaccharides (LPS), and 20% heat-inactivated FBS. PBMCs (peripheral blood mononuclear cells) were purified from fresh blood samples donated by healthy donors and cultured with RPMI 1640 medium supplemented with 50 μ g/ml of penicillin/streptomycin, and 10% heat-inactivated FBS. Spleen tyrosine kinase (SYK) inhibitor (BAY 61-3206) was purchased from Sigma–Aldrich®. SPF New Zealand rabbits and female BALB/c mice were maintained in the experimental animal facility of the College of Biological Science and Technology, National Chiao Tung University (NCTU), according to the guidelines approved by the Institutional Animal Care and Use Committee of NCTU. Anti-haptoglobin monoclonal antibodies (mAbs) were kindly provided by Dr. Simon Jen-Tan Mao of NCTU.

2.2. Protein preparation of HpHSP60

HpHSP60 was prepared according the procedure described in our previous study [4]. Briefly, a DNA fragment containing the Hsp60 gene was amplified from *H. pylori* genomic DNA and cloned into the T7 promoter-driven pET 30a (+) expression vector (Novagen, Darmstadt, Germany). The protein was then expressed in *Escherichia coli* (BL21 strain) and purified using HisTrap affinity (Ni–NTA) chromatography (General Electric, NY, USA) and a Sephadex G-25 column (General Electric, NY, USA) to remove any salts associated with proteins. For Fc receptor binding assay, FITC-conjugated HpHSP60 was produced by co-incubating fresh prepared 1 mg/ml Fluorescein isothiocyanate (Sigma–Aldrich) and 10 mg HpHSP60 at room temperature for 45 min, uncoupled FITC was then removed with a Sephadex G-25 column.

2.3. Measurement of patient serum antibody against HpHSP60

Serum samples were obtained from National Taiwan University Hospital from the patients identified as having *H. pylori* infection. Serum antibodies against HpHSP60 were measured by enzyme-linked immunosorbent assay (ELISA) according to the procedure described in our previous study [4].

2.4. Preparation of polyclonal and monoclonal antibody against HpHSP60

2.4.1. Polyclonal antibodies

Purified HpHSP60 (250 μ g) in 1 ml of Freund's complete adjuvant was administered intradermally to each rabbit. Beginning 4 weeks after the initial immunization, 2 booster doses (250 μ g of HpHSP60 in 1 ml of incomplete Freund's adjuvant) were injected at 2- or 4-week intervals. Pre- and post-immunization blood was collected from the marginal ear vein. Blood was allowed to clot for 30 min and centrifuged at 5000g for 20 min to obtain the ser-

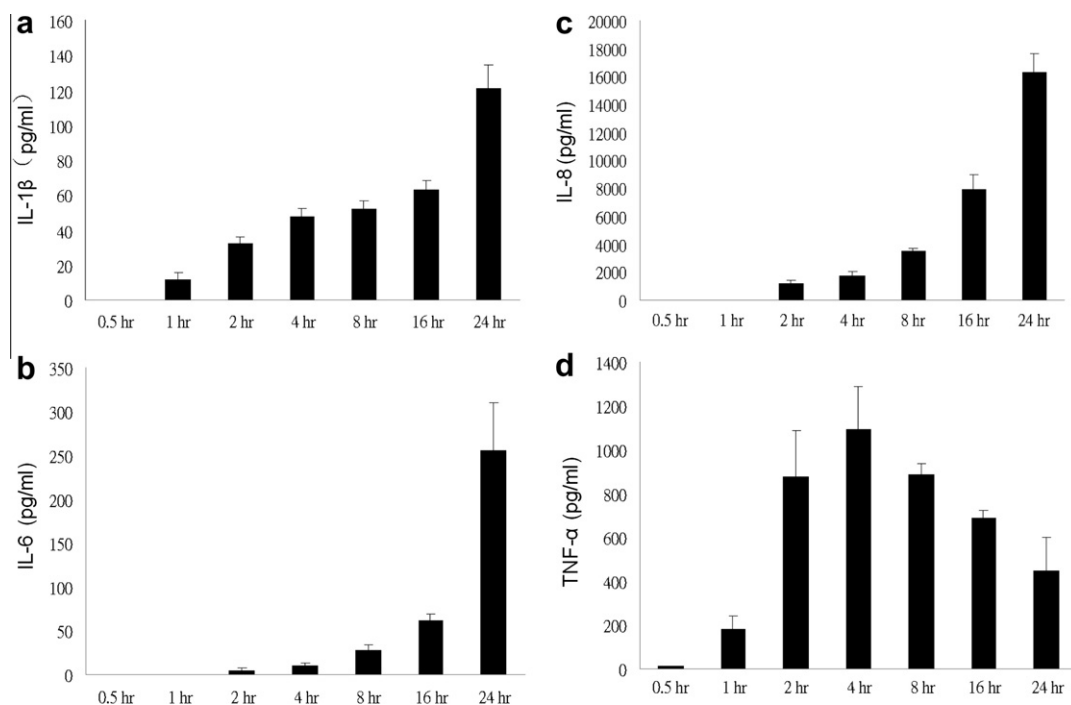


Fig. 1. Inflammatory cytokine expression stimulated by HpHSP60. THP-1 cells (1×10^5 cells/ml) were stimulated with HpHSP60 (10 μ g/ml). Spontaneous release of IL-1 β (a), IL-6 (b), IL-8 (c), and TNF- α (d) at given time points (0.5, 1, 2, 4, 8, 16, and 24 h) were assessed from the collected supernatants. ** $P < 0.01$.

um. To produce mouse polyclonal antibodies, 5-week-old female SPF BALB/c mice were immunized subcutaneously with 100 µg of HpHSP60 at 2-week intervals. An identical dose with incomplete adjuvant was given intraperitoneally at the fourth week followed by an intramuscular injection without adjuvant at the fifth week. Serum was collected 1 week after the final booster.

2.4.2. Monoclonal antibodies

After immunization, anti-HpHSP60 titers that were determined to be over 1:6400 by ELISA were qualified to perform

hybridoma fusion. The monoclonal antibodies were produced according to published protocols [14,15]. Briefly, FO cells were fused with mice splenocytes using 50% polyethylene glycol (Sigma–Aldrich, MO, USA). Hybridoma culture supernatants were screened using ELISA and the positive hybridoma cells were cloned by limiting dilution. Subsequently, the mAbs were harvested and purified from the supernatants with an antibody purification kit according to the manufacturer's specifications (NAb™ Protein A/G Spin Kit, Thermo Scientific, USA), and the mAbs activity was characterized by Western blot.

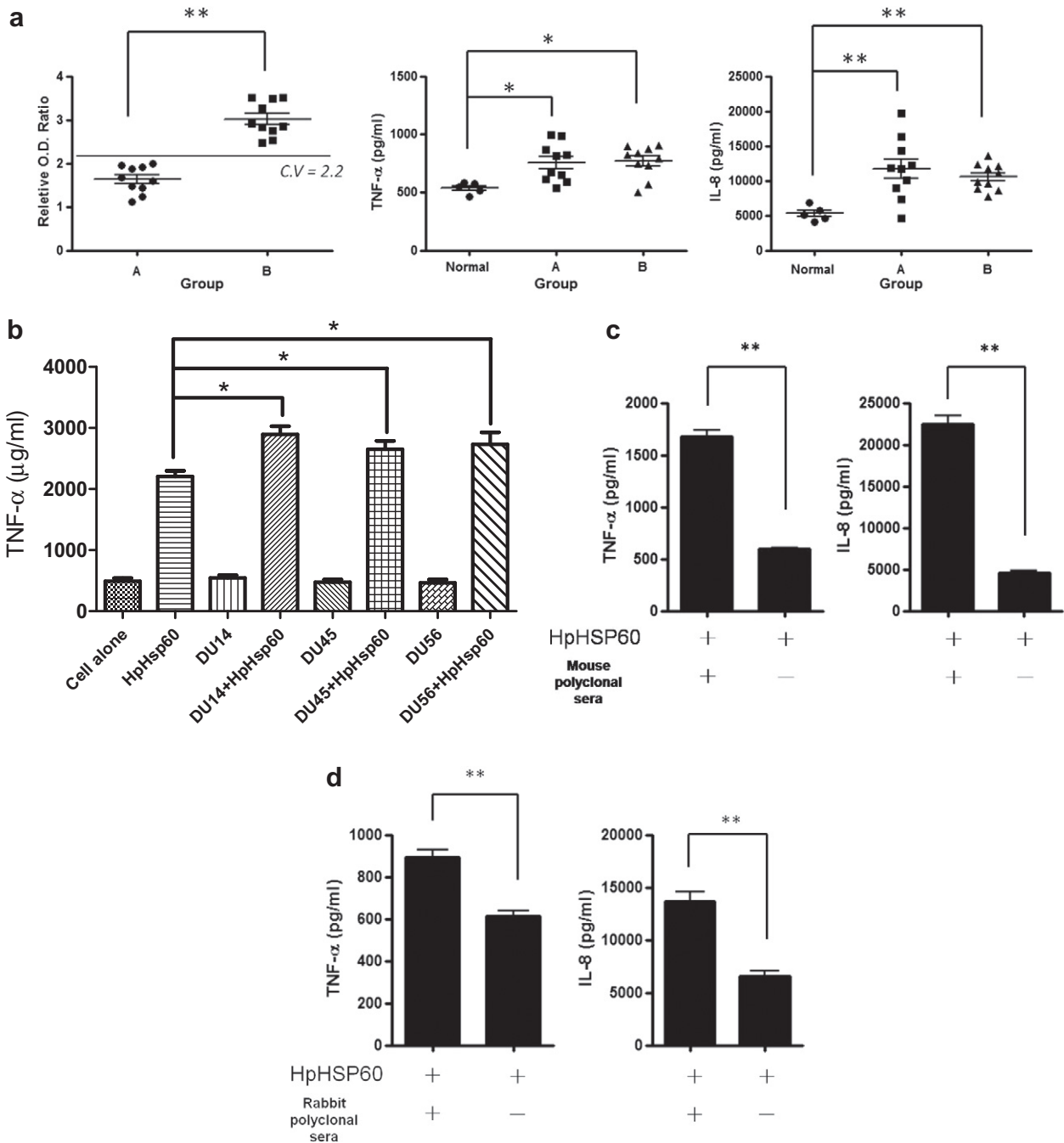


Fig. 2. The effects of anti-HpHSP60 polysera on HpHSP60-mediated TNF-α and IL-8 production. (a) Sera collected from *H. pylori*-infected patients were assessed for their anti-HpHSP60 titers (left panel). Co-administration of patient sera (1:250 dilution) with HpHSP60 (10 µg/ml) to THP-1 cells (1 × 10⁵ cells/ml) was used to assay TNF-α and IL-8 (middle and right panels) levels in the supernatants. (b) Sera of *H. pylori*-infected patients (DU14, DU45, DU56) were co-incubated with or without HpHSP60 (10 µg/ml) and then were added into human PBMCs (1 × 10⁶ cells/ml). For 24 h incubation, levels of TNF-α in the supernatants were measured (*P ≤ 0.05, N = 6). (c) Mouse or (d) rabbit polyclonal anti-HpHSP60 sera (1:1000 dilution) mixed with HpHSP60 were administered to THP-1 cells and the expression of TNF-α and IL-8 was assessed. *P < 0.05; **P < 0.01.

2.5. Fc receptor binding assay

About 2×10^5 THP-1 cells were pre-incubated with anti-Fc γ receptor mAb (0.5 μ g) for 30 min to block the Fc γ receptors. A mixture of FITC–HpHSP60 (1 μ g) and anti-HpHSP60 human polysera (1:100 dilution) was then stained with these cells and the mean fluorescence intensity (MFI) was assayed by flow cytometer.

2.6. ELISA for detecting cytokine expression

The supernatants collected from THP-1 cells with various treatments were measured for their levels of IL-1, IL-6, IL-8, and TNF- α by specific ELISA assays (R&D systems, MN, USA) according to the manufacturer's specifications.

2.7. Statistical analysis

All results were expressed as means and standard deviations and were analyzed with a 2-tailed Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. HpHSP60s rapidly induced high TNF- α and IL-8 production from THP-1 cells

HpHSP60 is viewed as a potential immunogen to induce proinflammatory cytokines including IL-1, IL-6, IL-8, and TNF- α . However, how rapidly this stimulatory effect is exerted remains undetermined. Herein, we treated monocytic THP-1 cells with HpHSP60 and collected the cell supernatants at various time points to evaluate the proinflammatory cytokine concentrations. Within only one hour after treatment, the expression of IL-1 β (Fig. 1a, 12.1 ± 3.4 pg/ml), IL-8 (Fig. 1c, 15.4 ± 3.6 pg/ml) and TNF- α (Fig. 1d, 184.0 ± 56.3 pg/ml) could be detected in the media; in addition, trace amounts of IL-6 (Fig. 1b, 5.3 ± 2.1 pg/ml) were also detected 2 h later. Once the cytokine release started, dramatic increases in TNF- α and IL-8 levels were sequentially found following HpHSP60 treatment. The peak secretion of TNF- α at the fourth hour (Fig. 1d, 1094.3 ± 195.4 pg/ml, $P < 0.01$) and IL-8 (Fig. 1c, 16301.2 ± 1305.7 pg/ml, $P < 0.01$) at the twenty-fourth hour revealed the strong immune-stimulatory effects of HpHSP60.

3.2. Polyclonal anti-HpHSP60 sera enhanced HpHSP60-mediated TNF- α and IL-8 production

To evaluate the effects of anti-sera on the activities of HpHSP60, patient sera were grouped into 2 clusters with low- (Group A) and high- (Group B) anti-HpHSP60 antibody quantities (Fig. 2a, left panel). Pre-incubating healthy individual or patient sera (1:250 dilution) with HpHSP60 (10 μ g/ml) for 30 min and then using it to treat THP-1 cells, which were also grouped into A and B clusters, could enhance TNF- α and IL-8 production compared to normal sera administration (Fig. 2a, middle and right panels). In addition, the patient sera could also increase the HpHSP60-induced TNF- α releases on human peripheral blood mononuclear cells from healthy individuals (Fig. 2b). Similar results were also found when applying either polyclonal mouse or rabbit anti-HpHSP60 sera. THP-1 cells subjected to anti-sera from different species had significantly increased TNF- α and IL-8 secretion compared to cells subjected to HpHSP60 treatment alone (Fig. 2c and d).

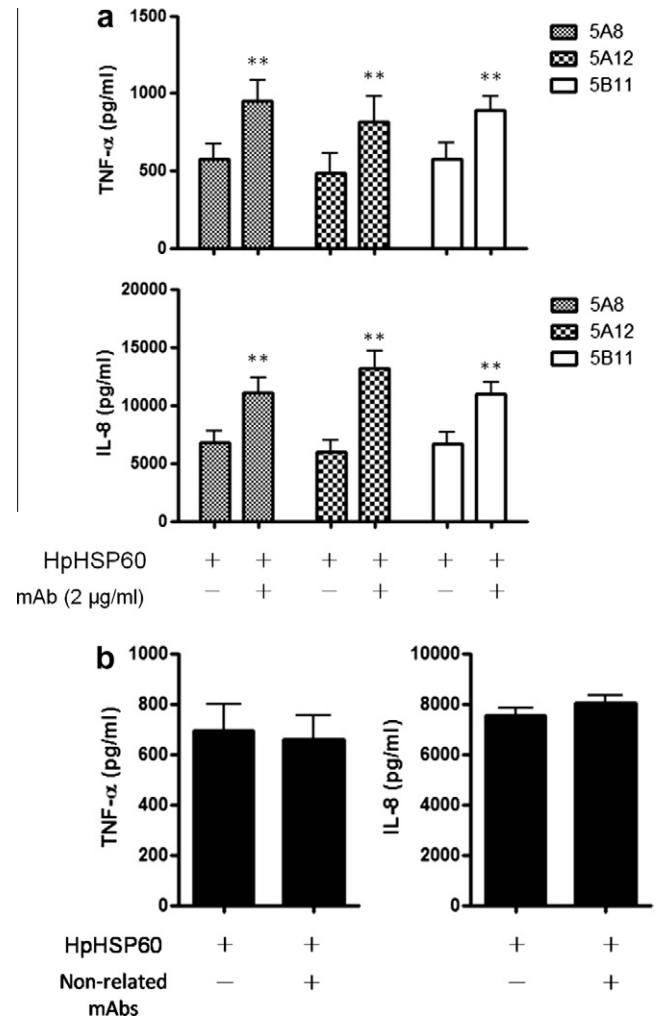


Fig. 3. The effects of anti-HpHSP60 mAbs on HpHSP60-mediated TNF- α and IL-8 production. HpHSP60 (10 μ g/ml) was incubated with various hybridoma clones (5A8, 5A12, or 5B11) (a) or anti-bovine haptoglobin mAbs (2 μ g/ml) (b) for 30 min and then used to treat THP-1 cells (1×10^5 cells/ml) for 24 h, after which TNF- α and IL-8 levels were measured in the supernatants. ** $P < 0.01$.

3.3. HpHSP60-specific mAbs strengthened TNF- α and IL-8 release stimulated by HpHSP60

It is interesting that anti-HpHSP60 sera could elevate HpHSP60-mediated proinflammatory responses. To further resolve this phenomenon, we produced mAbs specific to HpHSP60 to explore if the enhancing effect was derived from the antigen-specific antibodies and not other factors in sera. Three hybridoma clones (5A8, 5A12, and 5B11) were produced and their mAbs were used to treat THP-1 cells in the presence of HpHSP60. All results revealed that significantly higher levels of TNF- α and IL-8 were stimulated by mAbs with HpHSP60 than HpHSP60 alone (Fig. 3a, $P < 0.01$). In contrast, co-incubation of the non-related Abs (anti-bovine haptoglobin mAb) with HpHSP60 had no impact on TNF- α and IL-8 secretion from THP-1 cells (Fig. 3b). Taken together, the data indicated that HpHSP60-specific mAbs enhance the cytokine release induced by HpHSP60.

3.4. Non-specific mAbs interfered with the effects of HpHSP60-specific mAbs on HpHSP60-mediated TNF- α and IL-8 expression

Although non-specific mAbs failed to promote HpHSP60-mediated TNF- α and IL-8 expression by themselves, the ability of non-specific mAbs to influence the enhance effect of anti-

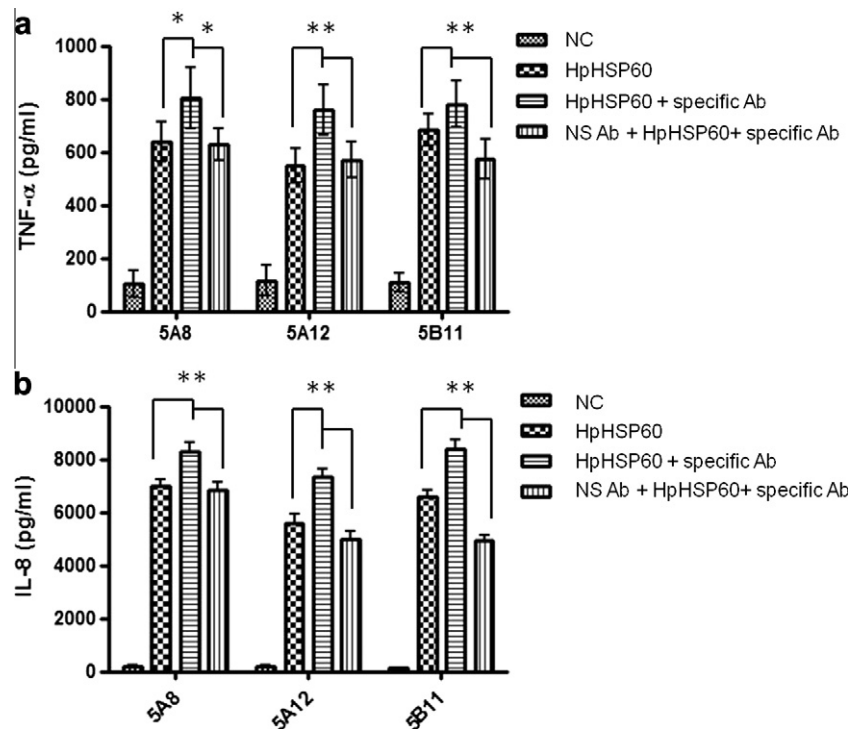


Fig. 4. The effects of non-specific mAbs on TNF- α and IL-8 release induced by HpHSP60 and anti-HpHSP60 mAbs. Hybridoma clones 5A8, 5A12, or 5B11 (2 μ g/ml) were cultured with HpHSP60 (10 μ g/ml) for 30 min then used to treat THP-1 cells (1×10^5 cells/ml) that were pre-treated with non-specific mAbs (NS Ab: anti-bovine haptoglobin mAbs) (50 μ g/ml) for 24 h to assess TNF- α (a) and IL-8 (b). * $P < 0.05$; ** $P < 0.01$.

HpHSP60 mAbs on the cytokine secretion induced by HpHSP60 was further examined. Pre-treating THP-1 cells with anti-bovine haptoglobin mAbs for 1 h followed by incubation with anti-HpHSP60 mAbs and HpHSP60 significantly reduced the production of TNF- α compared to that stimulated by treatments without non-specific mAbs (Fig. 4a). A similar result was also determined for IL-8 production (Fig. 4b). It appeared that non-related mAbs did not intensify HpHSP60-mediated proinflammatory responses but instead suppressed the synergism of HpHSP60 and anti-HpHSP60 mAbs on TNF- α and IL-8 induction.

3.5. The strengthening effects of HpHSP60 immune complex on inducing proinflammatory cytokines is mediated through Fc receptor signal pathway

Fc receptors is known to interact with Fc regions of antibodies [15] and Fc γ receptors were found to trigger inflammation in response to immunoglobulin-opsonized pathogens [16]. Therefore, the possible mechanism of increasing TNF- α and IL-8 production induced by HpHSP60 immune complex was investigated. Fig. 5a showed Fc γ receptor (Fc γ R) was obviously expressed on the THP-1 cell surface, and the murine or rabbit immunoglobulin could bind to the human Fc γ R (Fig. 5a). Blocking Fc γ R of THP-1 cells with specific mAbs was found to significantly reduce binding of HpHSP60/anti-HpHSP60 polysera (Fig. 5b). It indicated that HpHSP60 immune complex did interact with Fc γ receptor. Subsequently, since Fc γ R signal transduction is critically dependent on immunoreceptor tyrosine-based activation motifs (ITAMs) located in its cytoplasmic tail [17,18], and Spleen tyrosine kinase (SYK) binds these phosphorylated ITAM motifs and activates the events needed for downstream signaling, we next examine if interfering Fc γ R signaling might decrease the inflammatory cytokine induction. The data showed that treating THP-1 cells with SYK inhibitor suppressed TNF- α and IL-8 production stimulated by HpHSP60 immune complex (Fig. 5c) and suggested Fc γ R-SYK signal axis plays

important role in aggravating HSP60-mediated proinflammatory responses.

4. Discussion

Chronic infection of the gastric mucosa with *H. pylori* has long been recognized as a significant risk factor for gastric cancer [16]. As a prototypical model of inflammation-associated cancer, *H. pylori*-induced tumorigenesis is relevantly associated with the biological activity of the proinflammatory cytokines TNF- α and IL-8 within the circulation or the stomach [17]. TNF- α is a powerful proinflammatory cytokine produced from monocytes in response to *H. pylori* infection [17]. Similarly, IL-8 is known as a potent chemoattractant for neutrophils and lymphocytes, and it also has effects on cell proliferation, migration and tumor angiogenesis [18]. Although the function of both cytokines in gastric cancer development is well defined, host factors that modulate cytokine secretion remain largely unknown. The current study revealed that serum antibodies specific to HpHSP60 could intensity both TNF- α and IL-8 production from monocytic cells stimulated by HpHSP60. Though many surveys have been performed to analyze the correlation between anti-HpHSP60 titers and progression of gastric diseases [10,12,13], data shown here provide, for the first time, the role of anti-HpHSP60 antibody in the induction of HpHSP60-mediated proinflammatory cytokine release.

HpHSP60 has been demonstrated to be expressed on the surface of *H. pylori* to facilitate adhesion to host cells [19] and also to act as an active antigen to elicit strong immune responses [5,20,21]. Antibodies that specifically react with HpHSP60 were found in various studies, and the antibody titers were linked to the diagnosis of *H. pylori*-associated gastric diseases. Barton et al. reported that increasing IgA antibody titers to HpHSP60 was related to the incidence of gastric atrophy [12]; meanwhile, research from Ishii et al. and Tanaka et al. disclosed that higher titers of anti-HpHSP60

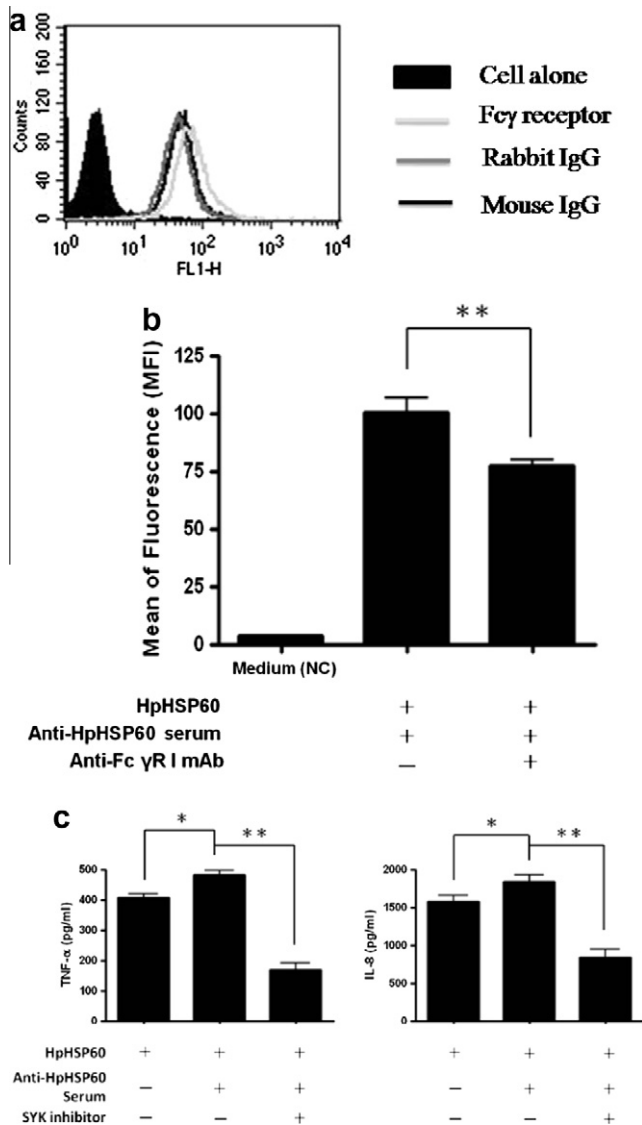


Fig. 5. Fc receptor-mediated signaling involves in HpHSP60/anti-HpHSP60 polysera-induced TNF- α and IL-8 production. (a) THP-1 cells (2×10^5) were stained with anti-human Fc γ R mAb, mouse IgG mAb or rabbit IgG mAb. (b) A mixture of FITC-conjugated HpHSP60 (1 μ g) and anti-HpHSP60 human polysera (1:100 dilution) was incubated with THP-1 cells (2×10^5) which were pre-incubated with anti-Fc γ receptor mAb (0.5 μ g) for 30 min to block the Fc receptors. The mean fluorescence intensity was analyzed by flow cytometer. (c) HpHSP60 (10 μ g/ml) and anti-HpHSP60 human polysera (1:100 dilution) were co-incubated for 30 min and then treated THP-1 cells (1×10^5 cells/ml) with SYK inhibitor (0.5 μ M) for 24 h to measure TNF- α (a) and IL-8 in the supernatants. * $P < 0.05$; ** $P < 0.01$.

antibodies were found in *H. pylori*-infected patients with gastric cancer or MALT lymphoma [10,13].

In the present study, we provided the first evidence that anti-HpHSP60 antibody could enhance TNF- α and IL-8 release induced by HpHSP60. The role of anti-HpHSP60 antibodies in inflammation is still not clear; however, similar findings were found in previous works. Mycobacterial protein PPD stimulates TNF- α secretion from monocytes, which is viewed as a major cause of tuberculosis-associated cachexia. Subsequently, a significant increase in TNF- α expression was observed when monocytes were co-cultured in the presence of TB patient plasma or anti-PPD antibodies [22]. Coinciding with this result, organs from *Histoplasma capsulatum*-infected animals treated with anti-HSP60 mAbs also had significantly increased levels of IL-2, IL-12, and TNF- α [23]. Our data, shown here, further confirm these discoveries and may imply a

direct link between *H. pylori* pathogenesis and elevated antibody titers.

HpHSP60 induces proinflammatory cytokines through activating TLR-2 and TLR-4 signaling pathways [20,21]. Augmentation of HpHSP60-mediated TNF- α and IL-8 release by anti-HpHSP60 antibodies could be attributed to the following mechanisms. First, Fc receptors on monocytes may partially account for the enhanced proinflammatory responses elicited by anti-HpHSP60 antibody. It is known that Fc receptors interact with Fc regions of antibodies [24] and Fc γ receptors were found to trigger inflammation in response to immunoglobulin-opsonized pathogens [25]. Immune complex-triggered inflammation was proven to be initiated by cell-bound Fc receptors and then amplified by cellular mediators and activated complement [26]. The increased TNF- α and IL-8 secretion in our case may therefore occur through engagement of HpHSP60 immune complexes with Fc receptors on monocytes. Additionally, this enhancement was not related to non-specific factors in plasma samples, since plasma samples from control donors as well as non-specific mAbs did not augment the cytokine secretion (Figs. 2a and 3b). Non-specific mAbs exerted no effects, indicating that the interaction among anti-HpHSP60 antibody/HpHSP60/TLRs was relevant to the proinflammatory cytokine upsurge. It could be proposed that HpHSP60 antigen/antibody complexes became immobilized for their binding to Fc receptors on monocytes. These ligations then made HpHSP60 more readily react with TLRs and therefore increased the efficiency of immune response stimulation. Pre-treatment of THP-1 cells with non-specific mAbs reduced the cytokine levels increased by HpHSP60 immune complexes, which further supported this hypothesis because these non-specific mAbs have already occupied the Fc receptors that are needed for engaging with anti-HpHSP60 antibody (Fig. 4).

Human HSP60 is considered as an important auto-antigen for certain autoimmune diseases such as autoimmune arthritis, type 1 diabetes mellitus, or atherosclerosis, etc. [27]. However, HpHSP60 could also trigger the production of host anti-HpHSP60 antibodies, and the anti-HpHSP60 antibodies have been demonstrated to cross-react with human HSP60 to induce cardiovascular injury thus lead to the occurrence of atherosclerosis or autoimmune arthritis [28,29]. Thus, the anti-HpHSP60 antibodies of patients with *H. pylori* infection may also increase the activity of the host endogenous HSP60 to induce more seriously inflammation, which will result in thus autoimmune diseases. Unlike HSP60, HSP70 has been shown that it could disrupt the expression of iNOS to lower the NO production, which can decrease the damage induced by *H. pylori* infection [30]. However, the expressions of several chaperons including HSP70, GRP, HSP60 were lowered by the infection of *H. pylori* [31–33]. Therefore, the cytoprotect effect of HSP70 in patients with *H. pylori* infection may not have the final conclusion. Furthermore, polymorphisms of HSP70 and TNF- α are related to inflammatory cytokines secretion in the infectious area of *H. pylori* [34]. Besides, host immuno-genetic background can influence the levels of anti-HpHSP60 antibody. Thus, we proposed that the genetic background of individual will control the expressions of these positive or negative factors for *H. pylori*-induced inflammation and result in different levels of inflammation and lead to the different damage of *H. pylori*-induced gastric diseases.

Collectively, it appears that mAbs against chronically infective pathogens tend to strengthen inflammatory responses toward their specific antigens. Resembling phenomena have been found in persistent *H. capsulatum* and *Mycobacterial tuberculosis* infections [22,23]. This study proved that *H. pylori* HSP60/mAbs could also modify *Helicobacter* pathogenesis through inducing TNF- α and IL-8 cytokine production. Further studies may provide important insights into the role of specific antibodies and proinflammatory cytokines in *H. pylori*-associated gastric diseases.

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