



## *Hansenula polymorpha* expressed heat shock protein gp96 exerts potent T cell activation activity as an adjuvant

Yang Li<sup>a,1</sup>, Haolei Song<sup>a,1</sup>, Jin Li<sup>b</sup>, Yanzhong Wang<sup>a</sup>, Xiaoli Yan<sup>a</sup>, Bao Zhao<sup>a</sup>, Xiaojun Zhang<sup>a</sup>, Saifeng Wang<sup>a</sup>, Lizhao Chen<sup>a</sup>, Bingsheng Qiu<sup>a,\*</sup>, Songdong Meng<sup>a,\*</sup>

<sup>a</sup> CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences (CAS), No. 1 West Beichen Road, Beijing, China

<sup>b</sup> Beijing Tiantan Biological Products Co., Ltd, Beijing, China

### ARTICLE INFO

#### Article history:

Received 30 July 2010

Received in revised form

30 November 2010

Accepted 7 December 2010

Available online 15 December 2010

#### Keywords:

gp96

*Hansenula polymorpha*

Expression

Peptide binding

HBV

### ABSTRACT

Previous studies together with ours showed that heat shock protein gp96 as an adjuvant induces antigen specific T cell responses against cancer and infectious diseases. However, at present there is no efficient method to obtain high amount of full-length gp96 by in vitro expression. Here, we used the yeast *Hansenula polymorpha* as an efficient host for gp96 recombinant protein production. The transformant clones with highly expressed recombinant proteins were screened and selected by measuring the halo size which indicates enzymatic hydrolysis of starch in the medium. High-level production of gp96 (around 150 mg/mL) was achieved by using high-cell density fed-batch cultivations. We showed that peptide binding of the recombinant protein has similar specificity and intrinsic binding parameters as that of the native gp96. We next examined the self-assembly properties and high-order structures of the recombinant protein. Moreover, the *H. polymorpha* expressed recombinant gp96 can effectively induce HBV-specific CTL response in immunized mice while *Escherichia coli*-expressed gp96 cannot. Our results therefore may provide bases for structure and functional studies of gp96 and thereby potentially expedite the development of gp96-based vaccines for immunotherapy of cancer or infectious diseases.

© 2010 Elsevier B.V. All rights reserved.

### 1. Introduction

There is growing literature on immunoregulatory and adjuvant functions of heat shock protein gp96 in the development of preventive and therapeutic vaccines against cancer and infectious disease (Bolhassani and Rafati, 2008; Wood and Mulders, 2009). This extremely conserved molecule associates with antigenic peptides from tumor, virus and intracellular bacteria, presents these loaded antigens to both MHC class I and class II molecules and activates specific T cells (Srivastava, 2002; Doody et al., 2004; Robert et al., 2008). A more recent study provided a relay line model for antigenic peptide transfer from gp96 and calreticulin to MHC class I molecules in the endoplasmic reticulum (Kropp et al., 2010). Moreover, gp96 interacts with CD4<sup>+</sup> and CD8<sup>+</sup> T cells, increasing their proliferation and secretion of cytokines (Mirza et al., 2006). Recently gp96 was demonstrated to activate dendritic cells through interaction with toll-like receptors (TLRs) and initiate innate immune responses (Warger et al., 2006; Yang et al., 2007; McGettrick and O'Neill, 2010). Moreover, increased CD8<sup>+</sup> T cells and decreased Tregs in tumor tissues were detected in responder

mice after immunization with gp96, indicating that gp96 may overcome tumor induced immunosuppression (Schreiber et al., 2009). Owing to its remarkable immune-modulating activity and novelty of the mechanism of antigen presentation, autologous vaccines based on tumor-derived gp96 have been initiated in clinical trial for the treatment of cancer (Bolhassani and Rafati, 2008; Wood and Mulders, 2009).

It is well documented that gp96, when reconstituted in vitro with tumor associated antigens such as HER-2 (Pakravan et al., 2010), or antigens from virus, such as HPV or HIV (SenGupta et al., 2004; Li et al., 2005; Bolhassani et al., 2008; Gong et al., 2009), elicits potent CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses against tumor and virus. In agreement with these studies, our recent work showed that gp96 as an adjuvant has the ability to induce HBV specific CTL responses. We explored the mechanisms of gp96 mediated balance between CTL and regulatory T cells, which may potentially facilitate development of a more effective gp96-based therapeutic vaccine (Liu et al., 2009). However, at present there is no efficient method to obtain high amount of full-length gp96 by in vitro expression because of its very low expression levels in *Escherichia coli*, and furthermore, gp96 expressed in *E. coli* is easy to degrade and forms massive aggregates, and contaminating endotoxin is very difficult to be removed (Linderroth et al., 2001; Li et al., 2005; Warger et al., 2006; Bolhassani et al., 2008; Liu et al., 2009). In recent years, the methylotrophic yeast *Hansenula polymorpha* has gained increas-

\* Corresponding authors. Tel.: +86 10 64807350; fax: +86 10 64807381.

E-mail addresses: [qiubs@sun.im.ac.cn](mailto:qiubs@sun.im.ac.cn) (B. Qiu), [mengsd@im.ac.cn](mailto:mengsd@im.ac.cn) (S. Meng).

<sup>1</sup> These authors contributed equally to this work.

ing attention as a promising host for expression of heterologous proteins (Hollenberg and Gellissen, 1997; Gellissen, 2000; van Dijk et al., 2000). The aim of this study is to generate high yield of full-length gp96 in *H. polymorpha*. We examined the structure of the recombinant gp96 protein and its peptide binding capacity, and analyzed its adjuvanticity to elicit peptide specific CTL response.

## 2. Materials and methods

### 2.1. Peptide synthesis

HBcAg<sub>87–95</sub> K<sup>d</sup>-restricted epitope SYVNTNMGL, and a control peptide HBcAg<sub>18–27</sub> FLPSDFFPSV used in this study was chemically synthesized by Jier Biological Company (Shanghai, China). The purity (>95%) and molecular weight of the peptide were determined by high-performance liquid chromatography and mass spectrometry.

### 2.2. Strains and media

The yeast strain NCYC495 (syn. CBS1976, ATCC14754) was used for most of genetic work, which was a gift from Dr Jan Kiel, University of Groningen, Netherlands. The yeast strains were grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose). The selection of transformants was performed on SD medium (0.67% YNB without amino acids, 2% glucose) at 30 °C. For high-cell density fed-batch cultivations, the medium and fermentation condition were performed previously described by Mayer (Mayer et al., 1999). *E. coli* DH5 $\alpha$  strain was used as a host for propagation of plasmids.

### 2.3. Construction, expression and purification of eukaryotic cell-expressed gp96

The plasmid pHGAPZ-B was constructed by replacing the *Pichia pastoris*-derived GAP promoter of the pGAPZ-B vector (Invitrogen, CA) with the *H. polymorpha*-derived GAP promoter (GenBank accession no. AY550078). Primers used in PCR amplification are listed in Table S-1. The loxP-GAP + AOxtt-loxP DNA fragment was amplified by PCR using pHGAPZ-B as the template. The PCR product was cloned into pHGAPZ-B via *Bgl*III and *Bam*HI to construct the plasmid pHGAPZ-BloxP. The *Amy* gene (GenBank accession no. EU200666.1) coding for acid  $\alpha$ -amylase was amplified from pWAG, and the PCR product was digested by *Not*I and *Xba*I and ligated with pHGAPZ-BloxP to generate the plasmid pHGAPZ-BloxP-Amy. Finally, the  $\alpha$ -amylase reporter system cassette was obtained using *Bgl*III and *Bam*HI to digest the plasmid pHGAPZ-BloxP-Amy. The PCR product containing the full coding region of gp96 (Li et al., 2005) was digested with *Eco*RI and *Xho*I and ligated into pHFMDZ-A plasmid (Song et al., 2003) to construct the plasmid pHFMDZ-gp96. The marker *LEU2* gene was amplified by PCR from pHIPX4 vector (Gietl et al., 1994). Finally, the expression plasmid pHFMDZ-GAmyL2-gp96 was constructed by inserting the *LEU2* gene and the  $\alpha$ -amylase reporter system cassette into the *Bam*HI site of plasmid pHFMDZ-gp96 in order.

*Bgl*III-linearized pHFMDZ-GAmyL2-gp96 plasmid was used to transform *H. polymorpha* by electroporation (Faber et al., 1994). When the transformants appeared, some clones with clear halos around them would be found on SD agar plate with 1% solution starch after fumigated by iodum owing to hydrolysis of starch in the medium. The clones with highly expressed HSP gp96 were screened and selected by determining the halo size of transformants, and the  $\alpha$ -amylase reporter system cassette was then removed from the selected clones using the Cre expression plasmid pHFMDRG-CRE via the Cre/loxP-mediated procedure as described previously (Qian et al., 2009). Small scale production of gp96 recombinant proteins was carried out in 5 L BIOTECH bioreactor interfaced with BIOTECH-FCS for data acquisition and control using the high-cell density

fed-batch cultivations. The fermentation condition and procedure control are as follows: after consumption of glycerol in medium, a glycerol-limited feeding phase would follow until the desired level of biomass (180 g/L, wet weight) is reached. Subsequently, methanol was added to a final concentration of 6 g/L to start HSP gp96 recombinant protein production. The cells were harvested and lyzed using a milling method with glass beads. The his-tagged recombinant protein was purified using Hitrap Q HP column and superdex-200 gel filtration (GE, NJ) on an AKTA FPLC system.

### 2.4. Dynamic light scattering (DLS) assay

The *H. polymorpha* expressed recombinant gp96 (rgp96) and gp96 purified from murine livers (mgp96) (Liu et al., 2009) were dissolved in 50 mM PBS (pH7.2) and centrifugated at 12,000  $\times$  g for 30 min at 4 °C. The radius and molecular weight of rgp96 and mgp96 were estimated using the DynaPro-Titan (Wyatt Technology Corp.) at room temperature, respectively.

### 2.5. Ethyleneglycol bis-succinimidylsuccinate (EGS) cross-linking assay

Protein samples were concentrated to 2 mg/mL in PBS (pH 7.2), and EGS (Sigma) was added to different final concentrations (0, 0.1, 0.3, or 0.5 mM). The reaction was stopped by the addition of 50 mM of glycine after 1 h of incubation on ice. Cross-linked products were analyzed by SDS-PAGE.

### 2.6. Peptide binding assay

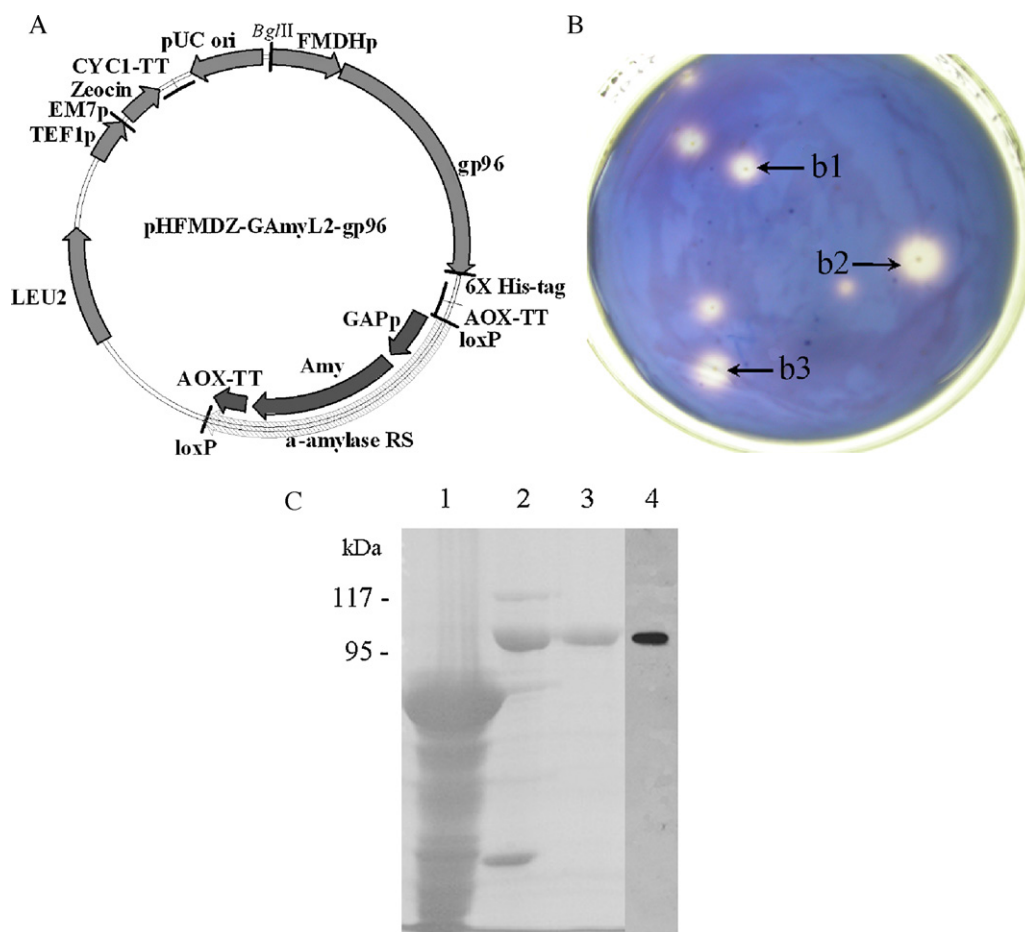
Binding of gp96 was quantified by ELISA assay. The solid-phase binding assay was performed as described previously (Gidalevitz et al., 2004) with some modifications. Briefly, 96-well streptavidin plates (Thermo Fisher Scientific, IL) were coated with the biotinylated peptide HBcAg<sub>87–95</sub>, and after blocking with 5% non-fat dry milk at 37 °C, serial concentrations of rgp96 or mgp96 protein ranging from 0.05 to 100  $\mu$ g/mL, along with or without the pan-HSP90 inhibitors radicicol (300  $\mu$ M), were added to each well in 100  $\mu$ L of binding buffer (20 mM HEPES (pH 7.2), 20 mM NaCl, 2 mM MgCl<sub>2</sub> and 100 mM KCl) to allow to bind for 1.5 h. Afterwards, the plates were incubated with the rat anti-gp96 antibody and the HRP-conjugated goat anti-rat antibody. The substrate TMB (3,3',5,5'-tetramethylbenzidine) was used for detection. The reaction was measured at 450 nm.

### 2.7. Immunization of mice and splenocytes isolation

Female BALB/c (H-2d) mice of 6–8 week old were immunized with 50  $\mu$ g of HBcAg<sub>87–95</sub> peptide bound to 20  $\mu$ g of rgp96 or mgp96, or with Freund's adjuvant (FA)/incomplete FA (IFA) as the positive control. gp96 or N355-peptide complexes were generated by incubating the mixtures of protein and peptide at 50 °C for 10 min, followed by 30 min at room temperature. All groups contained at least 5–8 mice. Each group was injected for three times (at 0, 1 and 3 weeks respectively). The mice were scarified after one week after the last immunization. Lymphocytes were isolated from the spleen as previously described (Liu et al., 2009).

### 2.8. ELISPOT assay

ELISPOT assay was performed according to the protocol supplied by the manufacturers (BD-pharmingen). Briefly, 100  $\mu$ L of culture medium containing 5  $\times$  10<sup>5</sup> splenocytes was incubated with 10  $\mu$ g/mL of HBcAg<sub>87–95</sub> peptide at 37 °C for 24 h. The peptide HBcAg<sub>18–27</sub> was used for background control. The number of spots was counted by an ELSPOT plate reader, and the result was expressed as the number of spot-forming cells (SFC) per 10<sup>6</sup> splenocytes.



**Fig. 1.** Screening of recombinant clones with highly expressed gp96 protein. (A) Physical map of the plasmid pHFMDZ-GAmyL2-gp96. (B) The plates with transformants were fumigated by 0.1% iodine at 80 °C for seconds. Colonies with different sizes of halos are indicated with arrows (b1, b2 and b3). (C) Recombinant His-gp96 expressed in *H. polymorpha* (lane 1) was purified by Ni<sup>+</sup> chelation chromatography (lane 2) and anion-exchange chromatography using Hitrap Q column (lane 3). The gp96 preparations were subjected to SDS-PAGE and stained with Coomassie Blue or immunoblotted with an anti-gp96 Ab (lane 4).

### 2.9. Pentamer assay

The indicated populations of T cells ( $10^6$  cells/sample) were stained with various fluorochrome-conjugated Ab against interested surface markers. The anti-CD8 $\alpha\beta$  mAb conjugated to FITC and anti-CD3 mAb conjugated to PE-Cy5.5 were purchased from eBioscience. PE-labeled H-2 Kd Pentamer complexes loaded with the peptide HBcAg<sub>87–95</sub> were purchased from ProImmune (Oxford, United Kingdom). Fourcolor flow cytometric analyses were performed using the FACSCalibur and CellQuest software (BD Biosciences).

### 2.10. Statistics

Student's *t* test or Mann–Whitney nonparametric *U* test were used for comparison between groups. Value of  $p < 0.05$  is considered as a significant difference.

## 3. Results

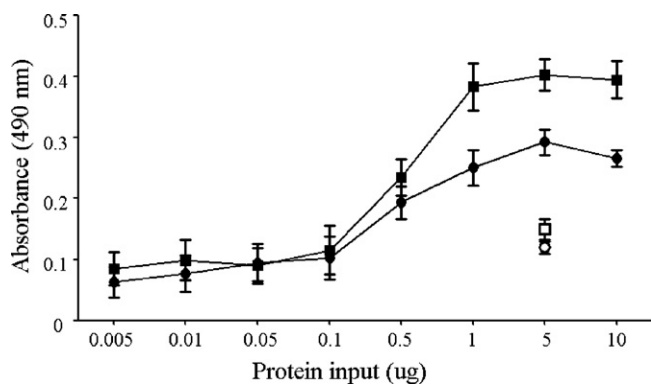
### 3.1. Screening of stable recombinant clones with highly expressed gp96 protein and high-cell density fed-batch cultivation

HSP gp96 expression plasmid *Bgl*II-linearized pHFMDZ-GAmyL2-gp96 was used to transform *H. polymorpha* by electroporation (Fig. 1A). The transformant clones with highly expressed recombinant proteins (rgp96) were screened and selected by measuring the halo size which indicates enzymatic

hydrolysis of starch in the medium (Fig. 1B). One recombinant clone with the highest expression of rgp96 protein was screened from about 500 candidate transformants. Subsequently, small scale fermentation production was carried out by using a simple high-cell density fed-batch cultivations protocol. The production level of rgp96 reached around 150 mg/L at 90–100 h.

### 3.2. Binding of HBV immunogenic 9-mer peptide HBcAg<sub>87–95</sub> to native mgp96 and recombinant rgp96

Our previous studies have shown that gp96 binds to the HBV-derived immunogenic peptide HBcAg<sub>87–95</sub> in HBV infected hepatocytes and gp96 or its N-terminal fragment has the ability to present the peptide to MHC class I molecules and augment peptide specific CTL responses in mice (Meng et al., 2001; Li et al., 2005). In this study, we first studied the interaction of the *H. polymorpha* expressed recombinant gp96 with HBcAg<sub>87–95</sub> peptide as the immunogenicity of 96 largely depends on its capacity of binding peptides. Toward this end, we analyzed the peptide binding capacity of both recombinant gp96, termed rgp96, and native mouse gp96, termed mgp96. Native mgp96 was purified as described (Meng et al., 2002), and Ni<sup>+</sup>-sepharose affinity chromatography and anion exchange chromatography (GE, USA) were used for purification of his-tagged rgp96. The preparations of rgp96 were analyzed by a Coomassie Blue-staining and immunoblotting with an anti-gp96 mAb (Fig. 1C).



**Fig. 2.** Dose binding of HBcAg<sub>87–95</sub> peptide to mgp96 (black circles) or rgp96 (black squares) in a 96-well plate assay. Empty symbols show inhibition of peptide binding by incubation with 300  $\mu$ M of radicicol. Results are presented as mean  $\pm$  SD from three independent experiments.

The biotinylated peptide HBcAg<sub>87–95</sub> was immobilized in the 96-well streptavidin plates. Fig. 2 shows that both rgp96 and mgp96 bound to HBcAg<sub>87–95</sub> in a dose-dependent fashion until a saturation level was reached at a concentration of 50  $\mu$ g/mL (100  $\mu$ L/well). Of note, the peptide binding activity was greatly abrogated by incubation of 300  $\mu$ M of the pan-HSP90 inhibitors radicicol, which is widely used as an inhibitor of gp96 chaperone function (Vogen et al., 2002; Gidalevitz et al., 2004). This indicates that the peptide binding of gp96 is specific as inhibition by radicicol is due to transmission of a conformational change along the protein. The recombinant rgp96 bound HBcAg<sub>87–95</sub> peptide with a binding curve similar to that of naturally purified mgp96, showing the ability of rgp96 to bind immunologically relevant peptides, such as the major T cell epitope of HBV, HBcAg<sub>87–95</sub>.

### 3.3. Biophysical analysis of mgp96 and rgp96

Previous studies showed that conformational properties of gp96 may play an important role in its immunological activities (Linderoth et al., 2001; Vogen et al., 2002; Gidalevitz et al., 2004; Thorne and McQuade, 2004; Fan et al., 2006). We next examined the self-assembly and aggregation properties of mgp96 and rgp96. The result of gel filtration showed that both mgp96 and rgp96 proteins formed large aggregates on the basis of their retention time and elution at or near the void volume of the column (Fig. 3A). Both mgp96 and rgp96 eluted within a relatively wide range of elution volume (10–15 mL). However, all peak fractions contained apparently homogeneous gp96 as judged by Coomassie Blue-stained gel (data not shown). The molecular masses of gp96 oligomers were therefore estimated by SDS-PAGE. Oligomers were cross-linked with EGS and separated on a 6% denaturing gel (Fig. 3B). Under treatment with increased concentration of EGS, the dominant band migrated from around 96 kDa (monomer) to more than 170 kDa (oligomers), indicating that both mgp96 and rgp96 exist mainly as soluble oligomers. Dynamic light scattering (DLS) was used to further characterize the oligomers (Fig. 3C). The mean molecular weight of mgp96 protein was around 1304 kDa, whereas for rgp96, it was 674 kDa, indicating that the rgp96 protein formed smaller oligomers. These values were comparable to the estimates from gel filtration analysis.

These data suggest that the multimeric aggregation properties of native mgp96 are shared by the yeast expressed recombinant rgp96. These results are also consistent with previous studies demonstrating that gp96 protein forms higher order self-associated complexes (Linderoth et al., 2001; Thorne and McQuade, 2004).

### 3.4. Enhanced T cell response in BALB/c mice induced by rgp96

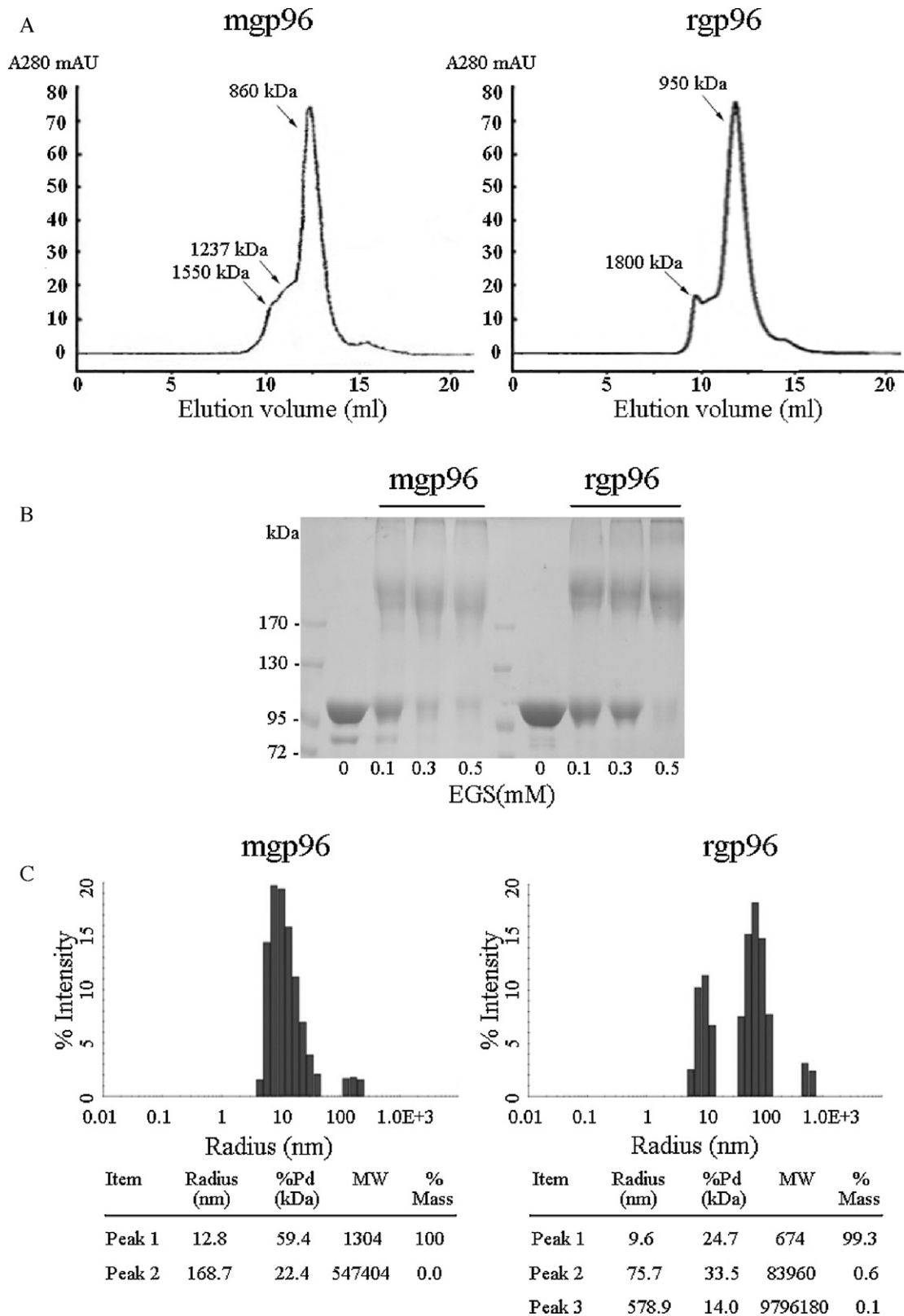
To examine the rgp96 function on T cell activity, we then tested if rgp96 could activate peptide-specific CTL response. Female BALB/c mice (6–8 wk old) were immunized s.c. with 20  $\mu$ g of rgp96 or mgp96-HBV peptide complexes. CTL expansion in response to HBcAg<sub>87–95</sub> peptide was detected one week after the last immunization. Splenocytes of immunized mice were detected by flow cytometric analysis for the presence of CD8<sup>+</sup> T cells and peptide-specific CTLs. As can be seen in Fig. 4, both mgp96 and rgp96 immunized mice exhibited significant increase of CD8<sup>+</sup> T cells and peptide specific CTLs (all  $p < 0.01$ ). Similar result was obtained in the ELISPOT assay. The results show that CTL responses can be greatly enhanced by rgp96, which is similar to mgp96. We also used *E. coli*-expressed gp96 as an adjuvant for immunization, and no significant enhancement of CTL activity was observed compared with control, suggesting that gp96 expressed in *E. coli* has low immune function maybe due to its fast degradation and massive aggregation.

Together, our data suggest that the yeast expressed recombinant gp96 has the ability to associate with antigenic peptides from HBV, and induce MHC class-I-restricted CTL responses, making rgp96 a powerful adjuvant for the generation of T-cell responses.

## 4. Discussion

One significant finding of the present work is the demonstration that peptide binding ability, self-assembly properties, antigen cross-presentation and T-cell activation activity were similar between *H. polymorpha* expressed recombinant rgp96 and naturally purified native mgp96, suggesting that rgp96 has great potential for development of effective gp96-based vaccines. The yeast *H. polymorpha* is an efficient host for gp96 recombinant protein production. High level production of rgp96 (around 150 mg/mL) was obtained by using high-cell density fed-batch cultivation. Peptide binding by rgp96 has the similar specificity and intrinsic binding parameters as that of mgp96. Moreover, our following experiments demonstrated that rgp96 can effectively augment HBV-specific CTL response in immunized mice. Since gp96 has been shown to be difficult to express efficiently as recombinant proteins, which thereby constitutes a bottleneck for functional and vaccine studies (Linderoth et al., 2001; Warger et al., 2006; Bolhassani et al., 2008; Liu et al., 2009), our current work therefore may provide bases for structure and functional studies of gp96 and expedite the development of gp96-based vaccines for immunotherapy of cancer or infectious diseases.

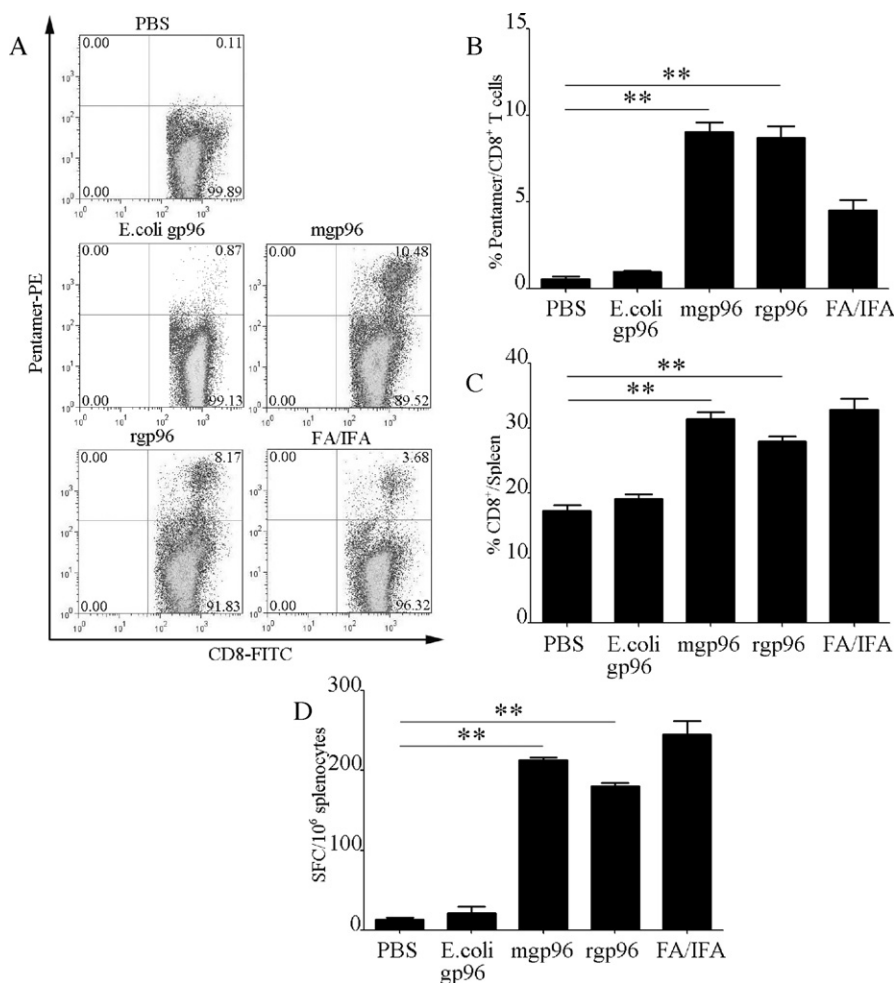
In this study, we have investigated the peptide binding capacity and higher order structure of rgp96. We compared peptide binding ability between rgp96 and mgp96 proteins. We found that the specificity and sensitivity to inhibitor were similar, and peptide binding affinity of rgp96 was even higher than that of mgp96 (Fig. 2). Peptide binding of both proteins could be inhibited by radicicol. Previous studies have shown that gp96 contains a peptide binding site in its N-terminal and peptide binding can be inhibited by radicicol due to a conformational change (Vogen et al., 2002; Gidalevitz et al., 2004). Based on these studies, together with our current results, we speculate that peptide binding in our assay is dependent on a structural aspect of rgp96 and is very likely to be related to the physiological function of gp96. For comparison, we also analyzed higher order structure of rgp96 and mgp96. Gel filtration, dynamic light scattering and cross-linking were used to investigate molecular self-association. The results showed that rgp96 and mgp96 form similar high order structures. Thus, the physicochemical properties of rgp96 are similar to those of mgp96, validating the potential of using rgp96 as an adjuvant.



**Fig. 3.** Characterization of mgp96 and rgp96. (A) Superdex-200 gel filtration analysis of mgp96 and rgp96. Estimated molecular weights are indicated in the chromatograms. (B) EGS-crosslinking assay. Protein samples were crosslinked with EGS at different concentrations (0–0.5 mM). The cross-linked products were analyzed by SDS-PAGE. (C) Dynamic light scattering analysis. Parameters obtained from dynamic light scattering measurements are presented. The results are similar in two independent experiments.

We observed self-assembly and aggregation properties of both mgp96 and rgp96. Dynamic light scattering analysis showed that the mean molecular weight for mgp96 and rgp96 was 1304 kDa and 647 kDa, and the mean radius was 12.8 nm and 9.6 nm, respec-

tively. These data are in agreement with a previous report showing that the predominant forms of gp96 are higher order oligomers (Linderoth et al., 2001). An intriguing question that arises from our observation is whether aggregation of gp96 affects its immuno-



**Fig. 4.** Enhanced T-cell response in mice immunized with mgp96 or rgp96-HBcAg<sub>87-95</sub> peptide complexes. *E. coli* expressed gp96 (*E. coli* gp96) was used for comparison. Flow cytometric analysis was performed to detect peptide-specific CTL (A and B) and CD8<sup>+</sup> T cell (C) populations from mouse spleens. HBcAg<sub>87-95</sub> specific CTLs were also detected by IFN- $\gamma$  ELISPOT assay (D). Data show mean  $\pm$  SD of five mice. \* $p < 0.05$ ; \*\* $p < 0.01$  by *t* test, compared with no gp96 immunization. Data are representative of two independent experiments.

genicity. The homooligomerization of gp96 has been linked to the enhancement of its peptide binding and chaperone activity (Thorne and McQuade, 2004). Conceivably, the unique ability of gp96 to act as a potent Th1 adjuvant may partly rely on its unique organizing aggregated structure (Ding et al., 2009). However, it is also possible that in gp96 oligomers the peptide-binding site may be concealed or less accessible, which will affect the efficiency of peptide binding and presentation to MHC molecules. This may explain why rgp96 with smaller aggregates has higher peptide binding ability than mgp96 with larger aggregates (Fig. 3). This should be addressed in more detail in future studies.

We found that, besides similar peptide binding and self-assembly properties between rgp96 and mgp96, their induction and activation of CTL responses were almost the same. As can be seen in Fig. 4, *H. polymorpha* expressed recombinant rgp96 has nearly equal ability as native mgp96 to enhance HBV-specific CTL activity by Pentamer and ELISPOT analyses. Compared with mgp96, rgp96 seems to have higher peptide binding ability and but form smaller oligomers. Despite the relatively higher peptide-binding activity compared with mgp96, rgp96 has similar immune activity as mgp96. This suggests that besides peptide binding ability, the self-assembly property may also affect the immunological activity of gp96. Based on molecular weight of mgp96 (1304 kDa) and rgp96 (674 kDa) determined by DLS, we speculate that mgp96 mainly forms a 14-mer complex of an Mr-96 kDa subunit, while rgp96 mainly forms a 7-mer complex. Conceivably,

the higher-order structure of gp96 may contribute its adjuvant ability to induce cellular immunoresponses. Moreover, we also detected antibody and CTL responses in ovalbumin (OVA)- and HBsAg vaccine-immunized mice using recombinant rgp96 as an adjuvant. The primary results showed that rgp96 could enhance both humoral and cellular responses to OVA and HBsAg (unpublished data), further validating the immunological potency of rgp96 as an adjuvant.

In conclusion, this study has significant implications in the application of *H. polymorpha* expressed recombinant rgp96 as an adjuvant for vaccine engineering. In the light of its specific peptide binding ability and induction of strong cellular immunoresponse, rgp96 appears to be a promising candidate for designing and engineering effective gp96-based vaccines aimed at eliciting T-cell responses for prophylactic and therapeutic applications.

#### Conflict of interest

The authors declare no financial or commercial conflict of interest.

#### Acknowledgements

We thank Lei Jin for his assistance in flow cytometric analysis. This work was supported by a grant from the National High

Technology Research and Development Program of China (No. 2006AA02A241), a grant from Major State Basic Research Development Program of China (No. 2007CB512802), a grant from Key Projects in the National Science & Technology Program of Eleventh Five-year Plan (2009ZX09503-007) and the CAS projects (KSCXZ-YW-R-1 and KSCXZ-YW-R-183). S.M. is a principal investigator of the Innovative Research Group of the National Natural Science Foundation of China (NSFC, Grant No. 81021003).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2010.12.006.

## References

- Bolhassani, A., Rafati, S., 2008. Heat-shock proteins as powerful weapons in vaccine development. *Expert Rev. Vaccines* 7, 1185–1199.
- Bolhassani, A., Zahedifard, F., Taghikhani, M., Rafati, S., 2008. Enhanced immunogenicity of HPV16E7 accompanied by Gp96 as an adjuvant in two vaccination strategies. *Vaccine* 26, 3362–3370.
- Ding, F.X., Wang, F., Lu, Y.M., Li, K., Wang, K.H., He, X.W., Sun, S.H., 2009. Multipeptide peptide-loaded virus-like particles as a vaccine against hepatitis B virus-related hepatocellular carcinoma. *Hepatology* 49, 1492–1502.
- Doody, A.D., Kovalchik, J.T., Mihalyo, M.A., Hagymasi, A.T., Drake, C.G., Adler, A.J., 2004. Glycoprotein 96 can chaperone both MHC class I- and class II-restricted epitopes for in vivo presentation, but selectively primes CD8<sup>+</sup> T cell effector function. *J. Immunol.* 172, 6087–6092.
- Faber, K.N., Haima, P., Harder, W., Veenhuis, M., Ab, G., 1994. Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*. *Curr. Genet.* 25, 305–310.
- Fan, H., Kashi, R.S., Middaugh, C.R., 2006. Conformational lability of two molecular chaperones Hsc70 and gp96: effects of pH and temperature. *Arch. Biochem. Biophys.* 447, 34–45.
- Gellissen, G., 2000. Heterologous protein production in methylotrophic yeasts. *Appl. Microbiol. Biotechnol.* 54, 741–750.
- Gidalevitz, T., Biswas, C., Ding, H., Schneidman-Duhovny, D., Wolfson, H.J., Stevens, F., Radford, S., Argon, Y., 2004. Identification of the N-terminal peptide binding site of glucose-regulated protein 94. *J. Biol. Chem.* 279, 16543–16552.
- Gietl, C., Faber, K.N., van der Klei, I.J., Veenhuis, M., 1994. Mutational analysis of the N-terminal topogenic signal of watermelon glyoxysomal malate dehydrogenase using the heterologous host *Hansenula polymorpha*. *Proc. Natl. Acad. Sci. U. S. A.* 91, 3151–3155.
- Gong, X., Gai, W., Xu, J., Zhou, W., Tien, P., 2009. Glycoprotein 96-mediated presentation of human immunodeficiency virus type 1 (HIV-1)-specific human leukocyte antigen class I-restricted peptide and humoral immune responses to HIV-1 p24. *Clin. Vaccine Immunol.* 16, 1595–1600.
- Hollenberg, C.P., Gellissen, G., 1997. Production of recombinant proteins by methylotrophic yeasts. *Curr. Opin. Biotechnol.* 8, 554–560.
- Kropp, L.E., Garg, M., Binder, R.J., 2010. Ovalbumin-derived precursor peptides are transferred sequentially from gp96 and calreticulin to MHC class I in the endoplasmic reticulum. *J. Immunol.* 184, 5619–5627.
- Li, H., Zhou, M., Han, J., Zhu, X., Dong, T., Gao, G.F., Tien, P., 2005. Generation of murine CTL by a hepatitis B virus-specific peptide and evaluation of the adjuvant effect of heat shock protein glycoprotein 96 and its terminal fragments. *J. Immunol.* 174, 195–204.
- Linderth, N.A., Simon, M.N., Rodionova, N.A., Cadene, M., Laws, W.R., Chait, B.T., Sastry, S., 2001. Biophysical analysis of the endoplasmic reticulum-resident chaperone/heat shock protein gp96/GRP94 and its complex with peptide antigen. *Biochemistry* 40, 1483–1495.
- Liu, Z., Li, X., Qiu, L., Zhang, X., Chen, L., Cao, S., Wang, F., Meng, S., 2009. Treg suppress CTL responses upon immunization with HSP gp96. *Eur. J. Immunol.* 39, 3110–3120.
- Mayer, A.F., Hellmuth, K., Schlieker, H., Lopez-Ulibarri, R., Oertel, S., Dahlems, U., Strasser, A.W., van Loon, A.P., 1999. An expression system matures: a highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. *Biotechnol. Bioeng.* 63, 373–381.
- McGettrick, A.F., O'Neill, L.A., 2010. Localisation and trafficking of Toll-like receptors: an important mode of regulation. *Curr. Opin. Immunol.* 22, 20–27.
- Meng, S.D., Gao, T., Gao, G.F., Tien, P., 2001. HBV-specific peptide associated with heat-shock protein gp96. *Lancet* 357, 528–529.
- Meng, S.D., Song, J., Rao, Z., Tien, P., Gao, G.F., 2002. Three-step purification of gp96 from human liver tumor tissues suitable for isolation of gp96-bound peptides. *J. Immunol. Methods* 264, 29–35.
- Mirza, S., Muthana, M., Fairburn, B., Slack, L.K., Hopkinson, K., Pockley, A.G., 2006. The stress protein gp96 is not an activator of resting rat bone marrow-derived dendritic cells, but is a costimulator and activator of CD3<sup>+</sup> T cells. *Cell Stress Chaperones* 11, 364–378.
- Pakravan, N., Soleimanjahi, H., Hassan, Z.M., 2010. GP96 C-terminal improves Her2/neu DNA vaccine. *J. Gene Med.* 12, 345–353.
- Qian, W., Song, H., Liu, Y., Zhang, C., Niu, Z., Wang, H., Qiu, B., 2009. Improved gene disruption method and Cre-loxP mutant system for multiple gene disruptions in *Hansenula polymorpha*. *J. Microbiol. Methods* 79, 253–259.
- Robert, J., Ramanayake, T., Maniero, G.D., Morales, H., Chida, A.S., 2008. Phylogenetic conservation of glycoprotein 96 ability to interact with CD91 and facilitate antigen cross-presentation. *J. Immunol.* 180, 3176–3182.
- Schreiber, T.H., Deyev, V.V., Rosenblatt, J.D., Podack, E.R., 2009. Tumor-induced suppression of CTL expansion and subjugation by gp96-Ig vaccination. *Cancer Res.* 69, 2026–2033.
- SenGupta, D., Norris, P.J., Suscovich, T.J., Hassan-Zahraee, M., Moffett, H.F., Trocha, A., Draenert, R., Goulder, P.J., Binder, R.J., Levey, D.L., Walker, B.D., Srivastava, P.K., Brander, C., 2004. Heat shock protein-mediated cross-presentation of exogenous HIV antigen on HLA class I and class II. *J. Immunol.* 173, 1987–1993.
- Song, H., Li, Y., Fang, W., Geng, Y., Wang, X., Wang, M., Qiu, B., 2003. Development of a set of expression vectors in *Hansenula polymorpha*. *Biotechnol. Lett.* 25, 1999–2006.
- Srivastava, P., 2002. Roles of heat-shock proteins in innate and adaptive immunity. *Nat. Rev. Immunol.* 2, 185–194.
- Thorne, M.E., McQuade, K.L., 2004. Heat-induced oligomerization of gp96 occurs via a site distinct from substrate binding and is regulated by ATP. *Biochem. Biophys. Res. Commun.* 323, 1163–1171.
- van Dijk, R., Faber, K.N., Kiel, J.A., Veenhuis, M., van der Klei, I., 2000. The methylotrophic yeast *Hansenula polymorpha*: a versatile cell factory. *Enzyme Microb. Technol.* 26, 793–800.
- Vogen, S., Gidalevitz, T., Biswas, C., Simen, B.B., Stein, E., Gulmen, F., Argon, Y., 2002. Radicol-sensitive peptide binding to the N-terminal portion of GRP94. *J. Biol. Chem.* 277, 40742–40750.
- Warger, T., Hilf, N., Rechtsteiner, G., Haselmayer, P., Carrick, D.M., Jonuleit, H., von Landenberg, P., Rammensee, H.G., Nicchitta, C.V., Radsak, M.P., Schild, H., 2006. Interaction of TLR2 and TLR4 ligands with the N-terminal domain of Gp96 amplifies innate and adaptive immune responses. *J. Biol. Chem.* 281, 22545–22553.
- Wood, C.G., Mulders, P., 2009. Vitespen: a preclinical and clinical review. *Future Oncol.* 5, 763–774.
- Yang, Y., Liu, B., Dai, J., Srivastava, P.K., Zammit, D.J., Lefrancois, L., Li, Z., 2007. Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. *Immunity* 26, 215–226.