Vaccine xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant

Yok Teng Chionh[a,b], Arthi Arulmuruganar[a], Elena Venditti[c], Garrett Z. Ng[a], Jia-Xi Han[a], Claire Entwisle[c], Ching-Seng Ang[d], Camilo A. Colaco[c], Shaun McNulty[c], Philip Sutton[a,b,*]

[a] *Mucosal Immunology, Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne, Parkville, VIC 3052, Australia*
[b] *Centre for Animal Biotechnology, School of Veterinary Science, University of Melbourne, Parkville, VIC 3010, Australia*
[c] *ImmunoBiology Ltd., Babraham Research Campus, Babraham, Cambridge, UK*
[d] *Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, VIC 3010, Australia*

ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form 24 January 2014
Accepted 12 February 2014
Available online xxx

Keywords:
Helicobacter pylori
Heat shock protein

ABSTRACT

Background: The development of a vaccine against the human gastric pathogen *Helicobacter pylori*, the main causative agent of gastric adenocarcinoma, has been hampered by a number of issues, including the lack of a mucosal adjuvant for use in humans. Heat shock proteins (Hsp), highly conserved molecules expressed by both bacteria and mammalian species, possess a range of functions, including acting as chaperones for cellular proteins and the ability to activate innate immune receptors. Hsp complex (HspC) vaccines, containing Hsp derived from pathogenic bacteria, are immunostimulatory without addition of an exogenous adjuvant and can induce immunity against their chaperoned proteins. In this study we explored in mice the potential utility of a *H. pylori* HspC vaccine.
Results: Vaccination with *H. pylori* HspC, by either the subcutaneous or respiratory mucosal route, induced a strong antibody response, elevated gastric cytokine levels and significant protection against subsequent live challenge with this pathogen. The level of protection induced by non-adjuvanted HspC vaccine was equivalent to that which resulted from vaccination with adjuvanted vaccines. While protection induced by immunisation with adjuvanted vaccines was associated with the development of a moderate to severe atrophic gastritis, that induced by *H. pylori* HspC only resulted in a mild inflammatory response, despite an increase in pro-inflammatory gastric cytokines. This reduced gastritis correlated with an increase in IL-10 and IL-13 levels in the gastric tissues of HspC vaccinated, *H. pylori* challenged mice.
Conclusions: H. pylori HspC vaccines have the potential to overcome some of the issues preventing the development of a human vaccine against this pathogen: HspC induced protective immunity against *H. pylori* without addition of an adjuvant and without the induction of a severe inflammatory response. However, complete protection was not obtained so further optimisation of this technology is needed if a human vaccine is to become a reality.

1. Introduction

The development of an effective vaccine against *Helicobacter pylori*, the main causative agent of gastric adenocarcinoma [1], gastric mucosal associated lymphoid tissue lymphoma [2] and peptic ulcer disease [3] has been hindered by several key obstacles. These include a poor understanding of the immune mechanisms required for protective immunity, the inability to reliably induce sterilising immunity that completely protects against this infection, and the lack of suitable adjuvants for use in humans [4,5]. An additional concern has been the development of a post-immunisation gastritis, whereby vaccinated mice subsequently challenged with *Helicobacter* commonly develop an exacerbated gastritis that is more severe than observed in unvaccinated, infected mice [6,7]. Hence new approaches are required to overcome these issues.

When released extracellularly, either from host cells or bacteria, heat shock proteins (Hsp) can act as danger-associated molecular pattern molecules (DAMP), potently activating the innate immune system [8]. This has resulted in their evaluation as potential vaccine components, both for the therapeutic treatment of cancers and as prophylactic inducers of protective immunity against pathogenic infections [9,10]. Hsp possess several key features that promote

* Corresponding author at: Mucosal Immunology, Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne, Parkville, VIC 3052, Australia. Tel.: +61 3 9936 6751; fax: +61 3 9348 1391.
E-mail address: phil.sutton@mcri.edu.au (P. Sutton).

http://dx.doi.org/10.1016/j.vaccine.2014.02.051
0264-410X/

their potential use in vaccines. Firstly, they are protein chaperones which, when purified from cells, carry bound to them a diverse protein cargo. Secondly, related to their role as a DAMP, they have intrinsic adjuvant activity due to their ability to activate toll-like receptors (TLR) [9,11,12]. Purification of Hsp from a bacterial pathogen therefore provides a potential vaccine containing the Hsp itself, as well as its complexed protein cargo, which is immunostimulatory without the requirement of adding an exogenous adjuvant.

The main Hsp possessed by *H. pylori* are the GroEL/S (58 kDa also called HspB/Hsp60 and 13 kDa also called HspA respectively) and the Dna K/J (also called Hsp70) chaperones [13,14], and their expression is controlled transcriptionally by the HspR and HrcA repressors [15]. Several studies have demonstrated the potential utility of *E. coli* expressed recombinant *H. pylori* GroEL and GroES as protective vaccine antigens when delivered in conjunction with a powerful exogenous adjuvant [16,17].

Here we present the first examination of a *H. pylori* Hsp complex vaccine. This study demonstrates that Hsp, derived from *H. pylori* along with chaperoned cargo, can induce protective immunity. Importantly this is achieved without the addition of an adjuvant, and without inducing a large inflammatory response at the site of infection. We further provide the first demonstration that Hsp complex (HspC) vaccines can be effective when delivered via a mucosal route.

2. Materials and methods

2.1. H. pylori culture

H. pylori strain SS1 [18] was cultivated in brain heart infusion broth (BHI; Oxoid) containing 0.02% Amphostat and 5% horse serum (Sigma, St Louis, MO, USA) and grown in microaerophilic conditions for 24 h at 37 °C.

2.2. Preparation of H. pylori heat shock protein complex vaccine

Heat shock protein complex vaccines were prepared essentially as described [19]. To prepare the HspC vaccine, *H. pylori* strain SS1 was lysed using a Triton X-100 solution (40 mM Tris, 1 mM $MgCl_2$, 20 mM NaCl, 0.5% Triton X-100, pH 8.0). The lysate was clarified by centrifugation at 13,000 rpm, filtered through a 0.2 μm filter, and 10 mg of the clarified lysate loaded (at 1.08 mg/mL) onto a 5 mL ion exchange column (Capto Q, GE Healthcare, Uppsala) at 0.5 mL/min. After washing the column with low salt buffer (40 mM Tris, 1 mM $MgCl_2$, 0.5% Triton X-100, pH 8.0), HspC were eluted using a high salt buffer (40 mM Tris–HCl pH 8.0 containing 300 mM NaCl and 0.5% Triton X-100). Fractions were analysed for protein concentration (by the Bradford assay) and Hsp60 content by Western blotting using the anti-Hsp60 antibody SPA-875 (Stressgen Bioreagents, Ann Arbor, MI, USA). The final vaccine, comprising pooled fractions containing most Hsp60, was sterile filtered (0.2 μm filter), diluted to 500 μg/mL in pH8.0 buffer containing 40 mM Tris, 1 mM $MgCl_2$, 300 mM NaCl and 0.1% Triton X-100, and stored at −80 °C.

2.3. Proteomic analysis of H. pylori heat shock protein complex vaccine

Protein content was visualised and 8 μg HspC compared with 8 μg *H. pylori* lysate on Coomassie stained SDS-PAGE gels as previously described [20]. Excised gel pieces were destained, reduced with 10 mM TCEP (Thermo Scientific Pierce, Waltham, MA, USA), alkylated with 55 mM iodoacetamide (Sigma) and digested overnight at 37 °C with sequencing grade modified trypsin (Thermo Scientific Pierce). The resultant peptides were then acidified with formic acid (Sigma) before analysis by liquid chromatography–tandem mass spectrometry (LC–MSMS).

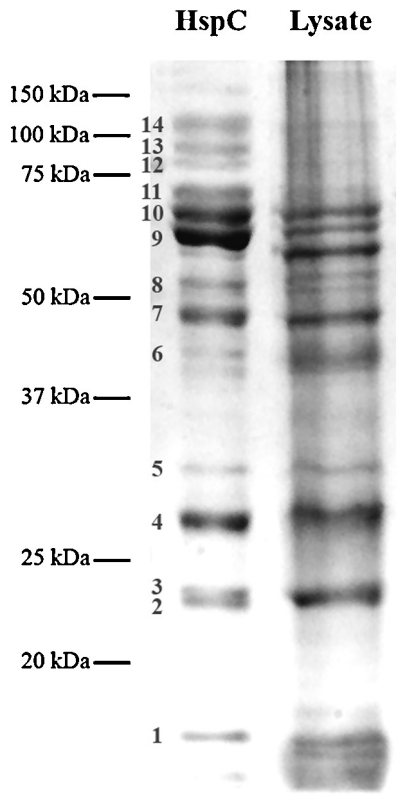

Fig. 1. *H. pylori* HspC: antigenic profile. The protein content of *H. pylori* HspC and lysate was compared by SDS-PAGE. HspC vaccine proteins identified by mass spectrometry: 1 = thioredoxin, 2 = protein grpE, 3 = alkyl hydroperoxide reductase, 4 = urease subunit B, 5 = fructose-bisphosphate aldolase, 6 = bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase, 7 = DNA polymerase III subunit beta, 8 = flagellin A, 9 = Hsp60, 10 = Hsp DnaK, 11 = Hsp90, 12 = fumarate reductase flavoprotein subunit, 13 = NADH dehydrogenase subunit G, and 14 = isoleucyl-tRNA synthetase.

LC–MSMS was carried out on a LTQ Orbitrap Elite (Thermo Scientific) with a nanoelectrospray interface (nanoESI) coupled to an Ultimate 3000 RSLC nanosystem (Dionex) equipped with an Acclaim Pepmap nano-trap column (Dionex – C18, 100 Å, 75 μm × 2 cm) and analytical column (Dionex C18, 2 μm, 100 Å, 75 μm × 15 cm). 2 μL of peptide mix was loaded onto the trap column at 5 μL/min of 3% CH_3CN containing 0.1% (v/v) formic acid for 5 min before the enrichment column was switched in-line with the analytical column. Peptides were eluted with 0.1% formic acid and increasing concentrations of CH_3CN: 3–12% for 1 min, 12–35% for 20 min, 35–80% for 2 min, 80% for 2 min. The LTQ Orbitrap Elite mass spectrometer was operated in the data dependent mode with nanoESI spray voltage of +2.0 kV, capillary temperature of 250 °C and S-lens RF value of 60%. Data analysis was carried out using Proteome Discoverer (Thermo Scientific version 1.4) and the Mascot search engine (Matrix Science version 2.4). Search results were set to maximum of 1% false discovery rate and a minimum of 2 unique peptides required for positive identification. Relative protein concentrations were estimated using the exponentially modified protein abundance index [21].

2.4. Vaccination against H. pylori

Animal experimentation was performed under institutional guidelines and with approval from the University of Melbourne Animal Ethics Committee. Groups of age-matched, female C57BL/6 mice were dosed either subcutaneously or nasally as previously described [22,23]. Anti-*H. pylori* serum and intestinal antibody responses were determined by ELISA using plates coated with *H.*

Please cite this article in press as: Chionh YT, et al. Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.02.051

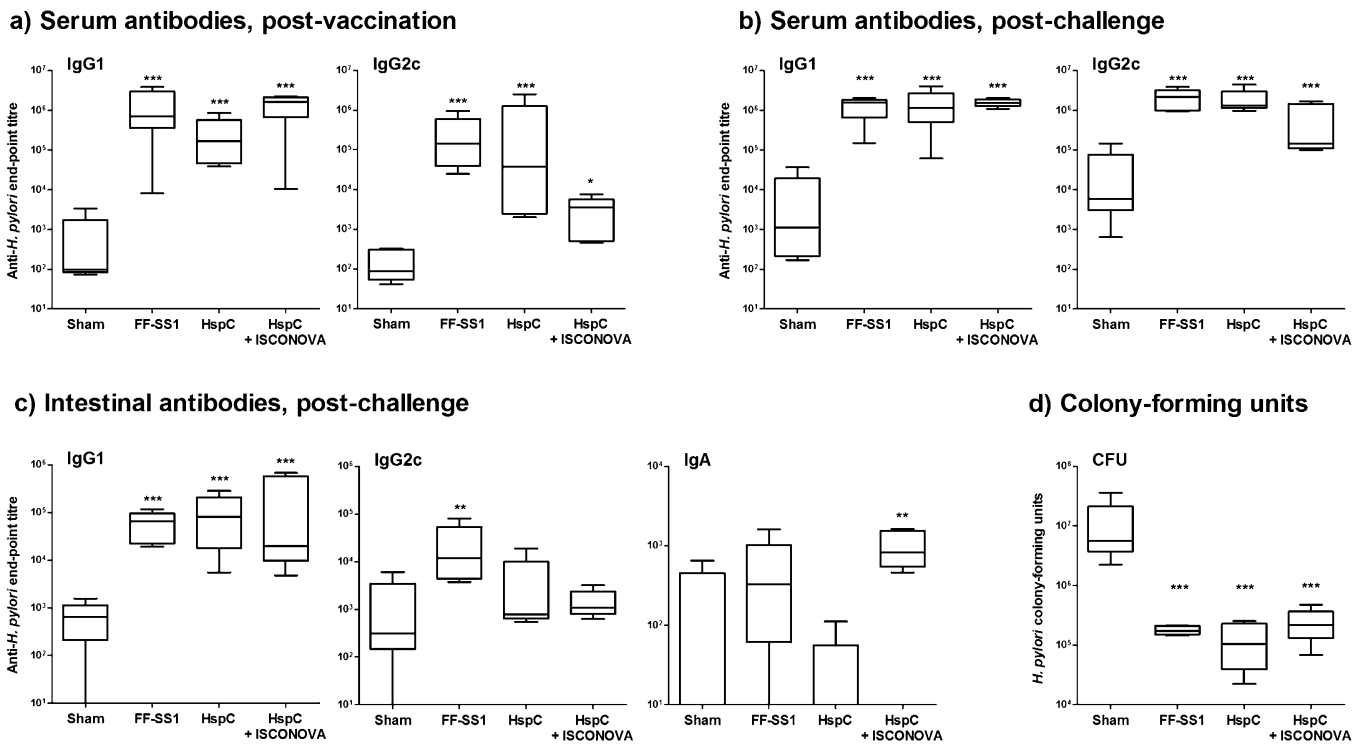

Fig. 2. Antibody response and protective immunity induced by subcutaneous delivery of *H. pylori* HspC. C57BL/6 mice ($n = 5$) were vaccinated by two subcutaneous injections of 25 μg *H. pylori* HspC in PBS either with or without addition of 12 μg ISCONOVA adjuvant. Negative controls were sham treated with PBS while positive controls were vaccinated with formalin-fixed *H. pylori* strain SS1 (FF-SS1) [22]. Vaccinations were spaced by 3 weeks, and sera collected 1 week post-vaccination (a). Four weeks after the second vaccination, all mice were challenged with *H. pylori* SS1. Four weeks later, post-challenge sera (b) and intestinal scrapings (c) were collected for antibody analyses and stomachs for quantification of *H. pylori* colonisation levels by colony-forming assay (d). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ cf sham control (ANOVA).

pylori lysate as described previously [24]. *H. pylori* infection levels within mouse gastric tissues were quantified by a colony-forming assay as described previously [25], with colony forming units calculated per stomach [26]. Gastric inflammation was determined by grading of cellular infiltration, mucus metaplasia and gastric atrophy on blinded Haematoxylin and Eosin stained stomach sections, as described previously [25].

2.5. Evaluation of gastric cytokine levels

Half stomachs were homogenised using a Polytron® PT2100 (Kinematica, Luzern, Switzerland) in 2 mL BHI. Cell debris was removed by centrifugation and protein concentrations in the supernatant quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA) prior to ELISA.

Cytokine concentrations were determined by coating 96-well Maxisorp plates (Nunc, Roskilde, Denmark) with purified anti-mouse MIP2 (100 ng/well; R&D systems, Minneapolis, MN, USA), IL-13 (50 ng/well; eBioscience, San Diego, CA, USA), IL-10 (100 ng/well; BD Biosciences, San Jose, CA, USA), IFNγ (100 ng/well; BD Biosciences), IL-17F (40 ng/well; R&D systems), or IL-17A (50 ng/well; eBioscience) overnight in bicarbonate coating buffer, pH 9.6. Plates were blocked with 1% BSA (Sigma) in PBS (blocker) for one hour prior to addition of samples in duplicate for three hours at room temperature or 4 °C overnight. Captured cytokines were then labelled with biotinylated anti-mouse MIP2 (3.7 ng/well; R&D systems), IL-13 (25 ng/well; eBioscience), IL-10 (50 ng/well; BD Biosciences), IFNγ (50 ng/well; BD Biosciences), IL-17F (10 ng/well; R&D systems) or IL-17A (25 ng/well; eBioscience) in blocker for one hour prior to the addition of 50 μL horseradish peroxidase conjugated streptavidin (Pierce) 1/5000 in blocker for 30 min. Colour was developed with 100 μL of TMB solution prepared as 0.1% of 10 mg/mL TMB (Sigma) in DMSO and 0.006% hydrogen peroxide in phosphate-citrate buffer, pH 5.0, and the reaction stopped with an equal volume of 2 M sulphuric acid prior to reading absorbance at 450 nm. Sample concentration was determined against a standard curve of recombinant IL-10, IFNγ (BD Biosciences), IL-13, IL-17A (eBioscience), IL-17F or MIP2 (R&D systems).

2.6. Statistics

For statistical analyses, all data were log-transformed then compared by analysis of variance (ANOVA), with Dunnett's post hoc analysis, except for histopathology which was analysed by Mann–Whitney non-parametric analysis, using SPSS software, version 21.0.

3. Results

3.1. H. pylori HspC is immunogenic and induces protective immunity when delivered via the subcutaneous route

One-dimensional gel and LC–MSMS analysis of the *H. pylori* HspC revealed that this complex contained abundant amounts of four different heat shock proteins (Hsp60, DnaK, Hsp90 and grpE) as well as a range of other antigens. Fourteen highly abundant proteins that were identified by LC–MSMS have been marked according to their apparent molecular weights in Fig. 1.

We first examined whether *H. pylori* HspC was effective when delivered parenterally by injection. Subcutaneous delivery of *H. pylori* HspC, without addition of an exogenous adjuvant, induced an antigen-specific serum antibody response that was significantly greater than unvaccinated controls both following vaccination alone (Fig. 2a) and vaccination then subsequent challenge with live *H. pylori* (Fig. 2b). The HspC antibody response, which comprised both IgG1 and IgG2c subclasses, was comparable or greater than

Please cite this article in press as: Chionh YT, et al. Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.02.051

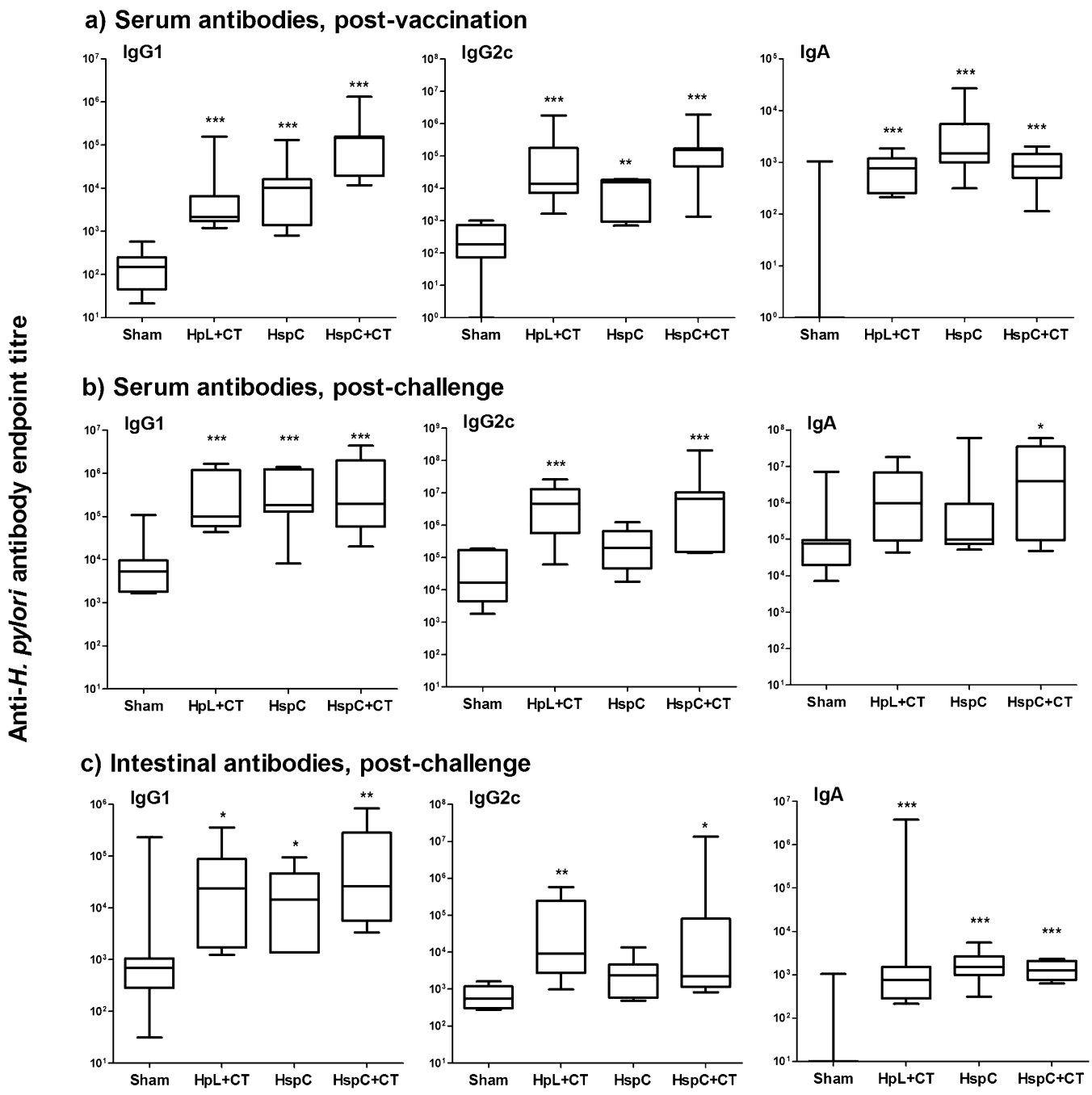

Fig. 3. Antibody response induced by respiratory delivery of *H. pylori* HspC. C57BL/6 mice ($n = 7$) were vaccinated twice by the nasal route with 50 μg *H. pylori* HspC in 30 μL PBS either with or without addition of 10 μg cholera toxin (CT). Negative controls ($n = 8$) were sham treated with PBS while positive controls ($n = 9$) were vaccinated with 50 μg *H. pylori* lysate (HpL) plus CT. Vaccinations were spaced by 3 weeks, and sera collected 1 week post-vaccination (a). Four weeks after the second vaccination, all mice were challenged with *H. pylori* SS1. Four weeks later, post-challenge sera (b) and intestinal scrapings (c) were collected for antibody analyses by ELISA and stomachs for quantification of *H. pylori* colonisation levels by colony-forming assay (d). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ cf sham control (ANOVA).

that induced in positive control groups that received formalin-fixed bacteria or HspC mixed with ISCONOVA adjuvant. ISCONOVA was selected as we have previously shown ISCOMATRIX™-based adjuvants to be effective at inducing protective immunity against *H. pylori* following subcutaneous vaccination [27]. Subcutaneous vaccination with *H. pylori* HspC alone also induced a mucosal antibody response, as demonstrated by a significant increase in intestinal anti-*H. pylori* IgG1, but not IgG2c or IgA, in challenged animals (Fig. 2c).

Most notably, subcutaneous vaccination with HspC alone induced significant protection against challenge with live *H. pylori*, which was at least equivalent to that present in positive control groups, including mice receiving HspC plus adjuvant (Fig. 2d).

3.2. *H. pylori* HspC induces systemic and mucosal antibodies, as well as protective immunity, when delivered via the respiratory route

As *H. pylori* is a mucosal pathogen and we had demonstrated a mucosal antibody response following subcutaneous HspC injection (Fig. 2c), we examined whether mucosal delivery of HspC could induce an effective immune response. The mucosal delivery

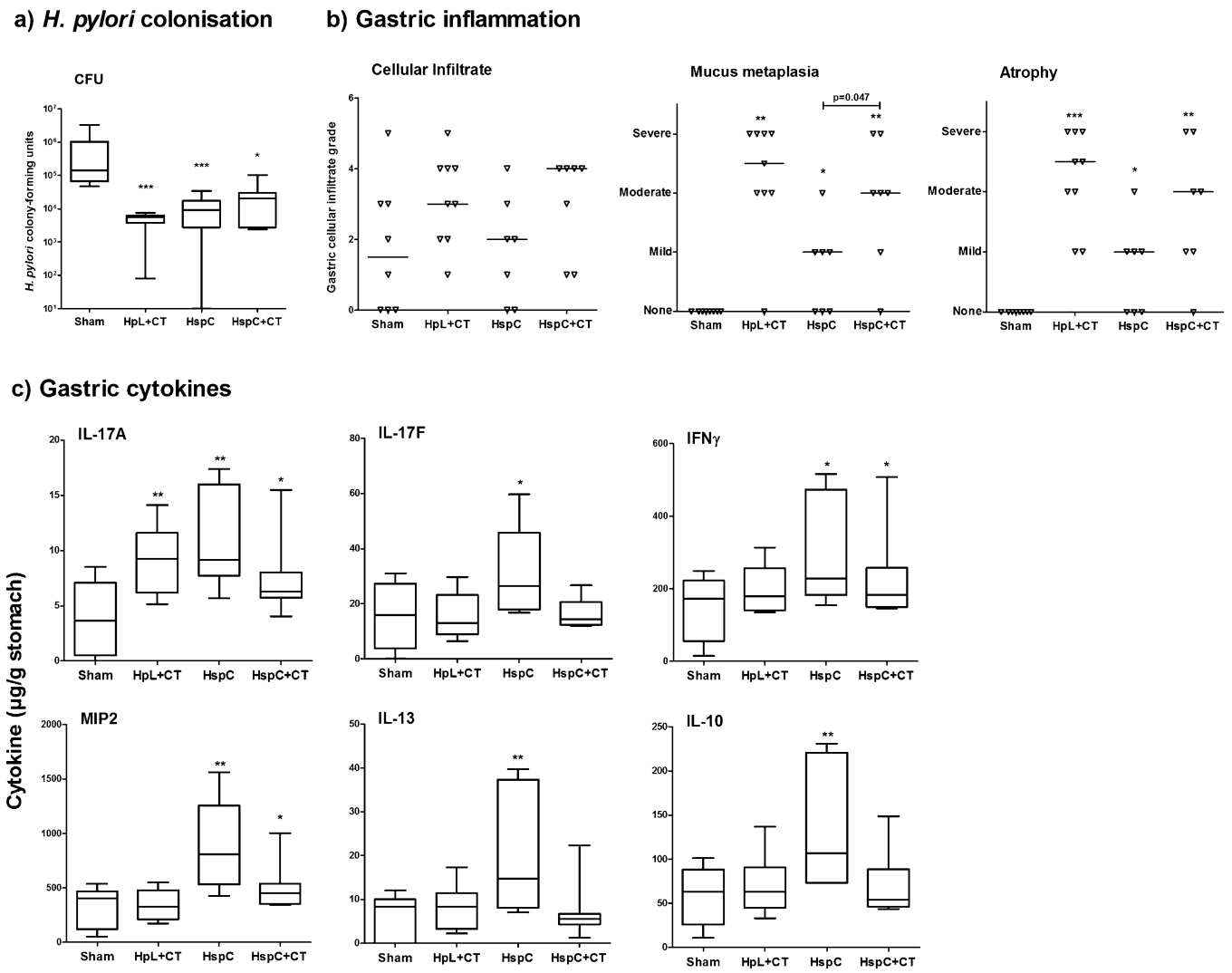

Fig. 4. Protective immunity and gastric response induced by respiratory delivery of *H. pylori* HspC. C57BL/6 mice ($n = 7$) were vaccinated twice by the nasal route with 50 μg *H. pylori* HspC in 30 μL PBS either with or without addition of 10 μg cholera toxin (CT). Negative controls ($n = 8$) were sham treated with PBS while positive controls ($n = 9$) were vaccinated with 50 μg *H. pylori* lysate (HpL) plus CT. Vaccinations were spaced by 3 weeks, and four weeks after the second vaccination, all mice were challenged with *H. pylori* SS1. Four weeks later, stomachs were removed for analysis. (a) *H. pylori* colonisation was quantified by colony-forming assay. (b) Severity of gastritis was assessed by histological assessment of blinded sections. (c) Gastric cytokine levels in homogenised tissues were quantified by ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ cf sham control (ANOVA).

of HspC vaccines has not previously been reported. Nasal delivery was selected as it has been shown previously that sterilising immunity is possible following mucosal vaccine delivery against *H. pylori* via this route [28]. However, as the volume of vaccine used in our studies has been shown previously to also go to the lungs of mice when delivered nasally [29], we refer here to this route as respiratory vaccination.

Respiratory vaccination with HspC alone induced a significant antigen-specific serum IgG and IgA response (Fig. 3a) and both serum IgG1 (Fig. 3b) and intestinal IgG1 and IgA (Fig. 3c) after live challenge with *H. pylori*. The addition to HspC of the powerful mucosal adjuvant, cholera toxin (CT), did increase some of these antibody responses over HspC alone. Notably however, the serum and intestinal IgG1 levels in mice vaccinated with HspC alone were, as for subcutaneous vaccination, not different from the positive control groups following infectious challenge.

Quantification of *H. pylori* colonisation levels in challenged animals revealed that mucosal delivery of HspC alone did indeed induce significant protection against this pathogen (Fig. 4a). The level of protection from HspC alone was equivalent to that induced by the positive control group that received whole bacterial lysate plus CT. This indicates that, with respect to protection against *H. pylori*, the intrinsic adjuvant activity of HspC was essentially equivalent to that of the powerful mucosal adjuvant CT.

Vaccine-induced protective immunity against *Helicobacter* in mice has previously been associated with a transient phenomenon termed post-immunisation gastritis, where immunised then challenged mice develop a more severe gastritis than unvaccinated, infected controls [6,7]. To examine the possibility of post-immunisation gastritis in HspC vaccinated mice, we quantified the severity of inflammation in the gastric tissues of these animals. Protection induced by the positive control vaccine which contained CT adjuvant was associated with the development of a median score of moderate-severe mucus metaplasia and atrophic gastritis (Fig. 4b). Mice vaccinated with HspC alone before *H. pylori* challenge however had a median score of mild metaplasia and mild atrophy (Fig. 4b), indicating that while gastritis was still increased, it was less severe than in mice receiving the other vaccines. Cellular infiltration also trended lower in HspC vaccinated mice compared to the other vaccine groups, although this did not reach significance. Of note, this reduction in gastritis was observed despite the induction of equivalent levels of protection (Fig. 4a). The addition of CT as

Please cite this article in press as: Chionh YT, et al. Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.02.051

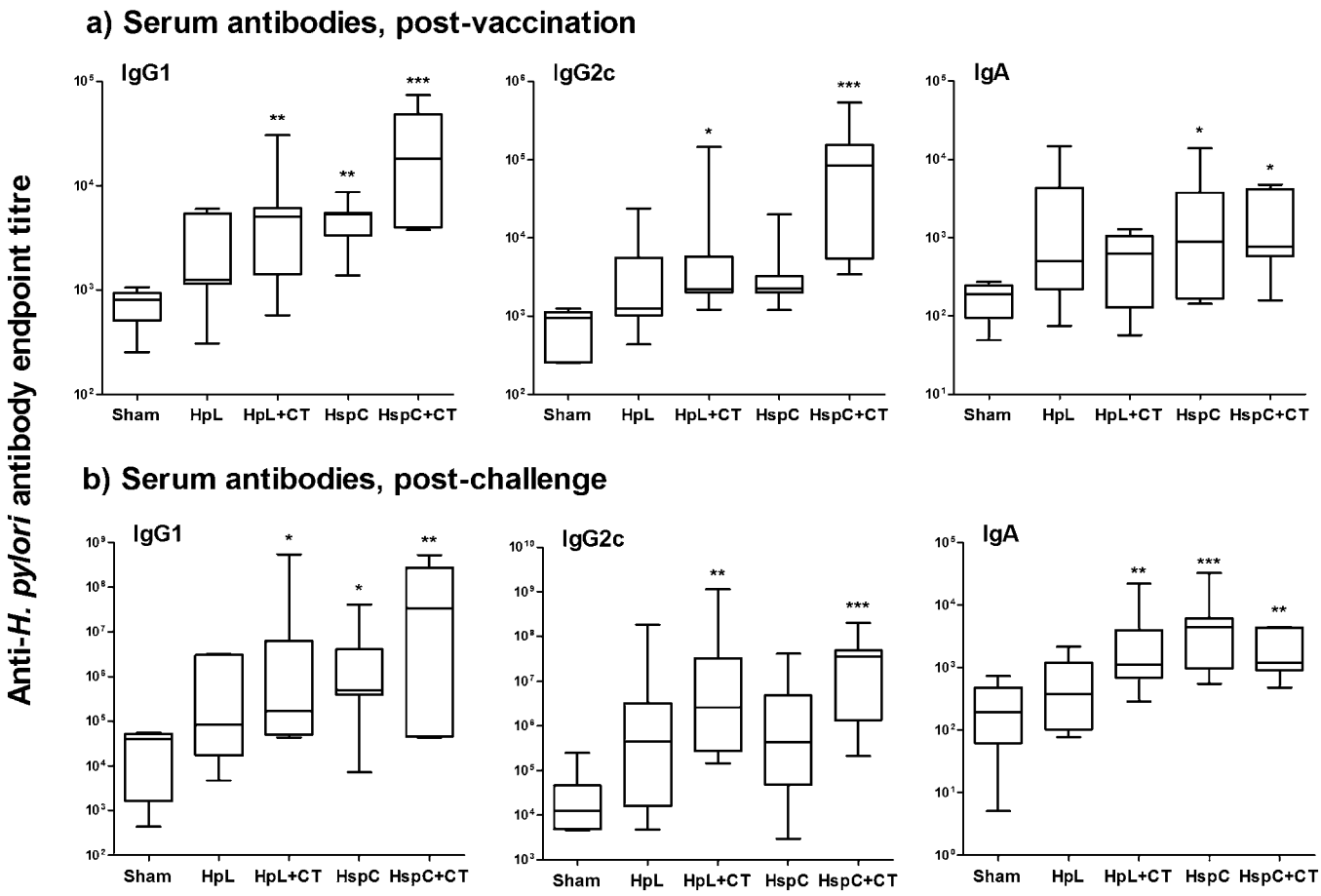

Fig. 5. Antibody response induced by respiratory delivery of *H. pylori* HspC and *H. pylori* lysate. C57BL/6 mice were vaccinated twice by the nasal route with either 50 μg *H. pylori* HspC in 30 μL PBS either with or without addition of 10 μg CT ($n = 7$) or 50 μg *H. pylori* lysate ($n = 8$). Negative controls ($n = 8$) were sham treated with PBS while positive controls ($n = 9$) were vaccinated with *H. pylori* lysate (HpL) plus CT. Vaccinations were spaced by 3 weeks, and sera collected 1 week post-vaccination (a). Four weeks after the second vaccination, all mice were challenged with *H. pylori* SS1. Four weeks later, post-challenge sera (b) were collected for antibody analyses by ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ cf sham control (ANOVA).

an adjuvant to the HspC resulted in a significant increase in gastritis with no concomitant increase in protection against challenge.

To further study this observation, we examined the gastric cytokine levels of these animals. Despite the less severe gastritis, mice vaccinated with HspC alone had the highest gastric cytokine levels, with significantly increased levels of all cytokines measured, including IL-17, IFNγ and MIP2 (Fig. 4c). Notably, in HspC vaccinated mice, the secretion of these pro-inflammatory cytokines appeared to be balanced by a significant increase in production of the anti-inflammatory IL-10 and IL-13 (Fig. 4c).

3.3. *H. pylori* HspC is more protective than whole cell lysate without addition of exogenous adjuvant

As *H. pylori* lysate contains a range of antigens and bacterial products, including HspC, we examined whether purification of HspC was actually necessary and conferred any advantage over lysate alone. Groups of mice were vaccinated via the respiratory route with *H. pylori* lysate alone or HspC alone, or both these antigen preparations adjuvanted with CT.

Without adjuvant, vaccination with *H. pylori* lysate via the respiratory route produced a trend towards increased anti-*H. pylori* antibodies, although this did not reach significance, either before or after bacterial challenge (Fig. 5a and b). In contrast *H. pylori* HspC alone, again induced a significant serum IgG1 and IgA response (Fig. 5a and b) demonstrating an improved ability of HspC to induce an antibody response over whole bacterial lysate.

This improved immunogenicity of HspC over bacterial lysate resulted in improved protection against live challenge as assessed by quantification of bacterial burden. Although vaccination with *H. pylori* lysate alone did induce a small but significant reduction in colonisation upon subsequent challenge, as compared to the sham-immunised controls, the protection observed was significantly less than that induced by HspC alone (Fig. 6a). While the protection induced by *H. pylori* lysate was improved by the addition of CT adjuvant, it was notable that this was not significantly different from the protection induced by the non-adjuvanted HspC vaccine (Fig. 6a). As previously observed, the addition of CT adjuvant did not significantly improve upon the level of protection induced by the HspC vaccine alone.

Unlike in the previous experiment (Fig. 4c), gastric IL-17A levels were only significantly increased in *H. pylori* lysate vaccinated mice (Fig. 6b), which were not significantly protected against bacterial challenge. No other cytokines were significantly elevated, except for IL-10 which was again increased in HspC vaccinated and challenged mice (Fig. 6b). Therefore, as all three vaccines induced protection, none of the cytokines measured correlated with vaccine-mediated protective immunity in this study.

4. Discussion

The development of an effective vaccine against *H. pylori* has been hindered by several obstacles, including the identification of a suitable mucosal adjuvant for use in a human vaccine. In this study

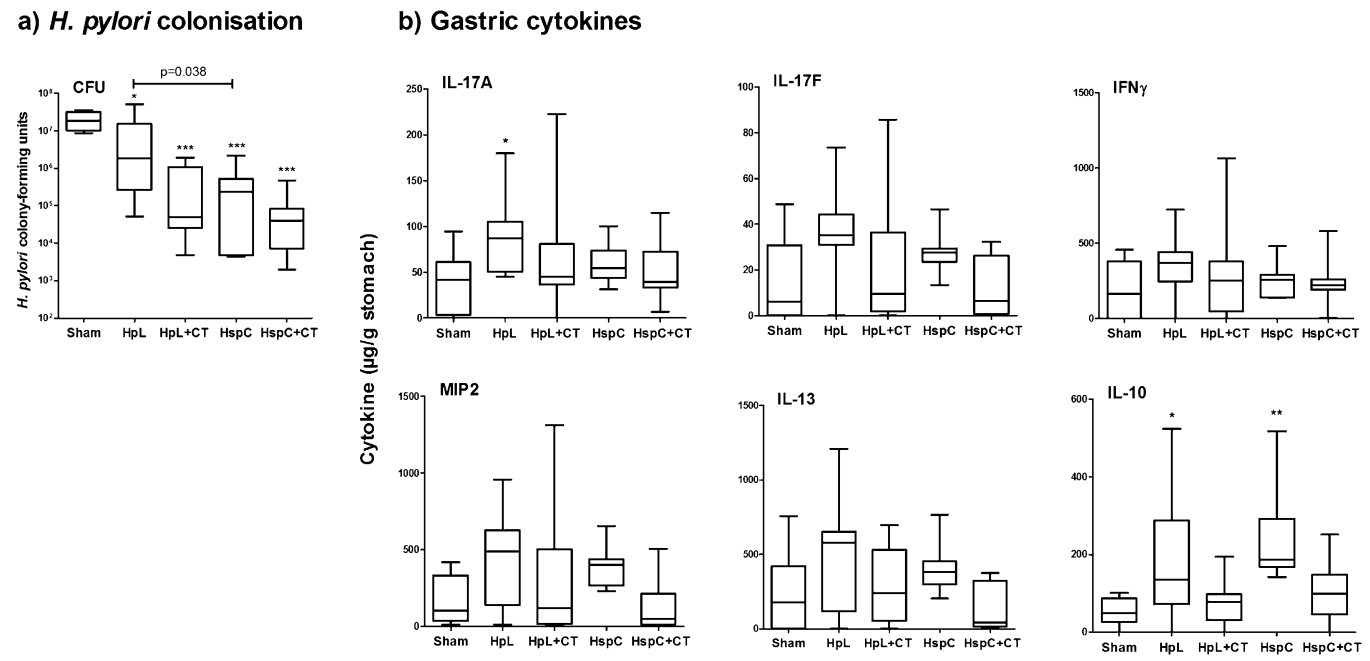

Fig. 6. Protective immunity and gastric cytokines induced by respiratory delivery of *H. pylori* HspC and *H. pylori* lysate. C57BL/6 mice were vaccinated twice by the nasal route with either 50 μg *H. pylori* HspC in 30 μL PBS either with or without addition of 10 μg CT ($n = 7$) or 50 μg *H. pylori* lysate ($n = 8$). Negative controls ($n = 8$) were sham treated with PBS while positive controls ($n = 9$) were vaccinated with *H. pylori* lysate (HpL) plus CT. Vaccinations were spaced by three weeks and four weeks after the second vaccination, all mice were challenged with *H. pylori* SS1. Four weeks later, stomachs were removed for analysis. (a) *H. pylori* colonisation was quantified by colony-forming assay. (b) Gastric cytokine levels in homogenised tissues were quantified by ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ cf sham control (ANOVA).

we demonstrate for the first time that a vaccine containing HspC produced from *H. pylori*, can induce protective immunity without the addition of an exogenous adjuvant. This protective effect could be elicited by *H. pylori* HspC delivery either by subcutaneous injection or mucosally via the respiratory route. To our knowledge, this is the first time that an HspC vaccine has been shown to be efficacious when delivered mucosally [9,10]. *H. pylori* lysate alone also induced a small but significant level of protection, when delivered via the respiratory route without adjuvant, although this was less protective than HspC. As *H. pylori* lysate would contain HspC, albeit at lower levels, we speculate that the protection induced by this lysate may in fact be due to the HspC that it contains.

A second potential impediment to the development of a *H. pylori* vaccine is post-immunisation gastritis. Hence the induction of a protective effect by HspC vaccination, with reduced gastritis compared to other adjuvanted vaccines, is a noteworthy observation. Interestingly, this protection with reduced gastritis was, perhaps counter-intuitively, accompanied by increased levels of normally pro-inflammatory cytokines in the gastric mucosa. However, HspC vaccination was also associated with an increase in gastric levels of both IL-10 and IL-13 cytokines, which would therefore appear to have suppressed the inflammatory response to the vaccination and infection, without interfering with the resulting protective immune response.

As expected, mass spectrometric analysis of the antigenic content of the HspC revealed that the most abundant proteins were Hsp themselves, and that a range of other antigens were also present. Two of these, urease subunit B and alkyl hydroperoxide reductase, have previously been demonstrated to be partially protective antigens when delivered to mice with adjuvant [30,31]. Hence it is logical that these are important antigens against which the *H. pylori* HspC vaccine induces an effective immune response, although other proteins present within the HspC may also be targeted.

The mechanism by which vaccines impact upon *H. pylori* colonisation in mouse models remains unclear, but has been shown to typically not involve antibodies [32–34] and to require CD4+ T-cells [32,35], possibly via a mechanism involving Th17 cells and/or IL-17, although that remains controversial [36–40]. While there was no correlation between gastric cytokine levels (including IL-17) and protection in this study, this does not rule out a potential role for these cytokines in protective immunity.

Hsp have an intrinsic ability to activate TLR [9], which is thought to be a significant component of the mechanism by which HspC vaccines induce immunity to co-delivered antigens without addition of an adjuvant. In order to facilitate long term colonisation of its host, *H. pylori* has undergone considerable modifications to minimise TLR activation. For example, *H. pylori* LPS has very low biological activity, related to a modified lipid A that has reduced ability to activate TLR4 [41,42], and *H. pylori* flagellin is similarly less potent at activating TLR5 [43]. As *H. pylori* HspC vaccines appear highly immunostimulatory this would suggest that, unlike LPS and flagellin, the biological activity of *H. pylori* Hsp may not be modified to reduce TLR binding or activation. This may reflect the highly conserved nature of Hsp in general, or that, unlike *H. pylori* LPS and flagellin, Hsp from these bacteria may not reach the host immune cells and so there was no evolutionary requirement for modification. Alternatively, the *H. pylori* HspC vaccine may be acting via a non-TLR mediated mechanism and it is interesting to note that *H. pylori* Hsp60 has previously been reported to induce IL-6 expression by macrophages via a MyD88-independent mechanism [44]. Another possibility is that detergents used during production of the HspC result in the formation of liposomes using *H. pylori* phospholipids, which help adjuvant the vaccine.

In conclusion, *H. pylori* HspC vaccines have the potential to overcome some of the key issues preventing the development of a human vaccine against this pathogen. Our studies show that they can induce protective immunity without requiring the addition of an adjuvant. Moreover, this protection can be achieved by inducing a strong immune response in the gastric mucosa, but without the induction of a severe inflammatory response. Finally the

Please cite this article in press as: Chionh YT, et al. Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.02.051

ability to deliver, without adjuvant, by injection or via a mucosal route provides flexibility for product development and could potentially avoid the requirement for needles.

However, the protection induced, at least in mice, was not optimal as sterilising immunity was not obtained in this animal model. Though further work may be necessary to optimise the HspC vaccine formulation, it should be noted that mouse results frequently do not directly translate to human efficacy, so the level of protection achieved may not truly be known until tested in humans.

Acknowledgements

This work was supported by the Victorian Government's Operational Infrastructure Support Program, ARC Linkage Grant LP120100226 from the Australian Research Council and by ImmunoBiology Limited. PS is supported by a Senior Research Fellowship from the National Health and Medical Research Council of Australia.

Conflict of interest statement: EW, CE, CAC and SM are employees of ImmunoBiology Limited, a company developing vaccines targeted to dendritic cells using Heat shock proteins. The work is partially funded by ImmunoBiology Limited.

References

[1] Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, et al. *Helicobacter pylori* infection and the development of gastric cancer. The New England Journal of Medicine 2001;345(September (11)):784–9.
[2] Wotherspoon AC, Doglioni C, Diss TC, Pan LX, Moschini A, Deboni M, et al. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. Lancet 1993;342(8871):575–7.
[3] Graham DY, Klein PD, Opekun AR, Boutton TW. Effect of age on the frequency of active *Campylobacter pylori* infection diagnosed by the [13C]urea breath test in normal subjects and patients with peptic ulcer disease. The Journal of Infectious Diseases 1988;157(April (4)):777–80.
[4] Sutton P, Chionh YT. Why can't we make an effective vaccine against *Helicobacter pylori*? Expert Review of Vaccines 2013;12(April (4)):433–41.
[5] Michetti P, Kreiss C, Kotloff KL, Porta N, Blanco JL, Bachmann D, et al. Oral immunization with urease and *Escherichia coli* heat-labile enterotoxin is safe and immunogenic in *Helicobacter pylori*-infected adults. Gastroenterology 1999;116(April (4)):804–12.
[6] Ermak TH, Ding R, Ekstein B, Hill J, Myers GA, Lee CK, et al. Gastritis in urease-immunized mice after *Helicobacter felis* challenge may be due to residual bacteria. Gastroenterology 1997;113(October (4)):1118–28.
[7] Sutton P, Danon SJ, Walker M, Thompson LJ, Wilson J, Kosaka T, et al. Post-immunisation gastritis and *Helicobacter* infection in the mouse: a long term study. Gut 2001;49(4):467–73.
[8] Prohaszka Z, Fust G. Immunological aspects of heat-shock proteins-the optimum stress of life. Molecular Immunology 2004;41(May (1)):29–44.
[9] McNulty S, Colaco CA, Blandford LE, Bailey CR, Baschieri S, Todryk S. Heat-shock proteins as dendritic cell-targeting vaccines–getting warmer. Immunology 2013;139(August (4)):407–15.
[10] Calderwood SK, Murshid A, Gong J. Heat shock proteins: conditional mediators of inflammation in tumor immunity. Frontiers in Immunology 2012;3:75.
[11] Henderson B, Calderwood SK, Coates AR, Cohen I, van Eden W, Lehner T, et al. Caught with their PAMPs down? The extracellular signalling actions of molecular chaperones are not due to microbial contaminants. Cell Stress & Chaperones 2010;15(March (2)):123–41.
[12] Zhao Y, Yokota K, Ayada K, Yamamoto Y, Okada T, Shen L, et al. *Helicobacter pylori* heat-shock protein 60 induces interleukin-8 via a Toll-like receptor (TLR)2 and mitogen-activated protein (MAP) kinase pathway in human monocytes. Journal of Medical Microbiology 2007;56(February (Pt 2)):154–64.
[13] Engstrand L, Scheynius A, Pahlson C. An increased number of gamma/delta T-cells and gastric epithelial cell expression of the groEL stress-protein homologue in *Helicobacter pylori*-associated chronic gastritis of the antrum. The American Journal of Gastroenterology 1991;86(August (8)):976–80.
[14] Suerbaum S, Thiberge JM, Kansau I, Ferrero RL, Labigne A. *Helicobacter pylori* hspA-hspB heat-shock gene cluster: nucleotide sequence, expression, putative function and immunogenicity. Molecular Microbiology 1994;14(December (5)):959–74.
[15] Du RJ, Ho B. Surface localized Heat Shock Protein 20 (HslV) of *Helicobacter pylori*. Helicobacter 2003;8(August (4)):257–67.
[16] Ferrero RL, Thiberge JM, Kansau I, Wuscher N, Huerre M, Labigne A. The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. Proceedings of the National Academy of Sciences of the United States of America 1995;92(July (14)):6499–503.
[17] Yamaguchi H, Osaki T, Taguchi H, Sato N, Toyoda A, Takahashi M, et al. Effect of bacterial flora on postimmunization gastritis following oral vaccination of mice with *Helicobacter pylori* heat shock protein 60. Clinical and Diagnostic Laboratory Immunology 2003;10(September (5)):808–12.
[18] Lee A, O'Rourke J, De Ungria MC, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology 1997;112(April (4)):1386–97.
[19] Salim KS, Colaco C, inventors; Method of purifying protein complexes patent WO2012010/026432. 2010.
[20] Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. The Journal of Biological Chemistry 1977;252(February (3)):1102–6.
[21] Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Molecular & Cellular Proteomics: MCP 2005;4(September (9)):1265–72.
[22] Harbour SN, Every AL, Edwards S, Sutton P. Systemic immunization with unadjuvanted whole *Helicobacter pylori* protects mice against heterologous challenge. Helicobacter 2008;13(December (6)):494–9.
[23] Stent A, Every AL, Ng GZ, Chionh YT, Ong LS, Edwards SJ, et al. *Helicobacter pylori* thiolperoxidase as a protective antigen in single- and multi-component vaccines. Vaccine 2012;(October).
[24] Chionh YT, Wee JL, Every AL, Ng GZ, Sutton P. M-cell targeting of whole killed bacteria induces protective immunity against gastrointestinal pathogens. Infection and Immunity 2009;77(July (7)):2962–70.
[25] McGuckin MA, Every AL, Skene CD, Linden SK, Chionh YT, Swierczak A, et al. Muc1 mucin limits both *Helicobacter pylori* colonization of the murine gastric mucosa and associated gastritis. Gastroenterology 2007;133(October (4)):1210–8.
[26] Sutton P. Considering increased mouse stomach mass, when calculating prophylactic vaccine efficacy against *Helicobacter pylori*. Helicobacter 2007;12:210–2.
[27] Skene CD, Doidge C, Sutton P. Evaluation of ISCOMATRIX and ISCOM vaccines for immunisation against *Helicobacter pylori*. Vaccine 2008;26(July (31)):3880–4.
[28] Garhart CA, Redline RW, Nedrud JG, Czinn SJ. Clearance of *Helicobacter pylori* infection and resolution of postimmunization gastritis in a kinetic study of prophylactically immunized mice. Infection and Immunity 2002;70(July (7)):3529–38.
[29] Visweswaraiah A, Novotny LA, Hjemdahl-Monsen EJ, Bakaletz LO, Thanavala Y. Tracking the tissue distribution of marker dye following intranasal delivery in mice and chinchillas: a multifactorial analysis of parameters affecting nasal retention. Vaccine 2002;20(August (25–26)):3209–20.
[30] O'Riordan AA, Morales VA, Mulligan L, Faheem N, Windle HJ, Kelleher DP. Alkyl hydroperoxide reductase: a candidate *Helicobacter pylori* vaccine. Vaccine 2012;30(June (26)):3876–84.
[31] Nedrud JG, Bagheri N, Schon K, Xin W, Bergroth H, Eliasson DG, et al. Subcomponent vaccine based on CTA1-DD adjuvant with incorporated UreB Class II peptides stimulates protective *Helicobacter pylori* immunity. PLoS ONE 2013;8(12):e83321.
[32] Ermak TH, Giannasca PJ, Nichols R, Myers GA, Nedrud J, Weltzin R, et al. Immunization of mice with urease vaccine affords protection against *Helicobacter pylori* infection in the absence of antibodies and is mediated by MHC class II-restricted responses. Journal of Experimental Medicine 1998;188(December (12)):2277–88.
[33] Sutton P, Wilson J, Kosaka T, Wolowczuk I, Lee A. Therapeutic immunization against *Helicobacter pylori* infection in the absence of antibodies. Immunology and Cell Biology 2000;78(February (1)):28–30.
[34] Garhart CA, Nedrud JG, Heinzel FP, Sigmund NE, Czinn SJ. Vaccine-induced protection against *Helicobacter pylori* in mice lacking both antibodies and interleukin-4. Infection and Immunity 2003;71(June (6)):3628–33.
[35] Pappo J, Torrey D, Castriotta L, Savinainen A, Kabok Z, Ibraghimov A. *Helicobacter pylori* infection in immunized mice lacking major histocompatibility complex class I and class II functions. Infection and Immunity 1999;67(January (1)):337–41.
[36] Delyria ES, Nedrud JG, Ernst PB, Alam MS, Redline RW, Ding H, et al. Vaccine-induced immunity against *Helicobacter pylori* in the absence of IL-17A. Helicobacter 2011;16(June (3)):169–78.
[37] DeLyria ES, Redline RW, Blanchard TG. Vaccination of mice against *H pylori* induces a strong Th-17 response and immunity that is neutrophil dependent. Gastroenterology 2009;136(January (1)):247–56.
[38] Velin D, Favre L, Bernasconi E, Bachmann D, Pythoud C, Saiji E, et al. Interleukin-17 is a critical mediator of vaccine-induced reduction of *Helicobacter* infection in the mouse model. Gastroenterology 2009;136(June (7)), 2237.e1–46.e1.
[39] Flach CF, Ostberg AK, Nilsson AT, Malefyt Rde W, Raghavan S. Pro-inflammatory cytokine gene expression in the stomach correlates with vaccine-induced protection against *Helicobacter pylori* infection in mice: an important role for interleukin-17 during the effector phase. Infection and Immunity 2011;79(February (2)):879–86.
[40] Ding H, Nedrud JG, Blanchard TG, Zagorski BM, Li G, Shiu J, et al. Th1-mediated immunity against *Helicobacter pylori* can compensate for lack of Th17 cells and can protect mice in the absence of immunization. PLoS ONE 2013;8(7):e69384.
[41] Moran AP. Lipopolysaccharide in bacterial chronic infection: insights from *Helicobacter pylori* lipopolysaccharide and lipid A. International Journal of Medical Microbiology: IJMM 2007;297(September (5)):307–19.

Please cite this article in press as: Chionh YT, et al. Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.02.051

[42] Muotiala A, Helander IM, Pyhala L, Kosunen TU, Moran AP. Low biological activity of *Helicobacter pylori* lipopolysaccharide. Infection and Immunity 1992;60(April (4)):1714–6.
[43] Gewirtz AT, Yu Y, Krishna US, Israel DA, Lyons SL, Peek Jr RM. *Helicobacter pylori* flagellin evades toll-like receptor 5-mediated innate immunity. The Journal of Infectious Diseases 2004;189(May (10)):1914–20.
[44] Gobert AP, Bambou JC, Werts C, Balloy V, Chignard M, Moran AP, et al. *Helicobacter pylori* heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. The Journal of Biological Chemistry 2004;279(January (1)):245–50.

Please cite this article in press as: Chionh YT, et al. Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.02.051