Heat shock protein complex vaccines induce antibodies against *Neisseria meningitidis* via a MyD88-independent mechanism

Jia-Xi Han^{1,2}, Garrett Z Ng^{1,2}, Paola Cecchini³, Yok Teng Chionh^{1,2}, Muhammad A Saeed^{1,2}, Lisbeth M. Næss⁴, Michael Joachim,³ Lucy E Blandford,³ Richard A Strugnell^{4,5}, Camilo A Colaco³, & Philip Sutton^{1,2#}

- 1 Mucosal Immunology, Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne, Parkville, VIC 3052, Australia
- 2 Centre for Animal Biotechnology, Faculty of Veterinary and Agricultural Science, The University of Melbourne, Parkville, VIC 3010, Australia
- 3 ImmunoBiology Ltd., Babraham Research Campus, Babraham, Cambridge, UK
- 4 Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway
- 5 Department of Microbiology and Immunology, University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Parkville, Victoria 3010, Australia

#Author for Correspondence:

Tel: +61-3-9936-6751; Fax: +61-3-9348-1391

Email: phil.sutton@mcri.edu.au

ABSTRACT

BACKGROUND:

Neisseria meningitidis are common colonisers of the human nasopharynx. In some circumstances, *N. meningitidis* becomes an opportunistic pathogen that invades tissues and causes meningitis. While a vaccine against a number of serogroups has been in effective use for many years, a vaccine against *N. meningitidis* group B has not yet been universally adopted. Bacterial Heat shock protein complex (HSPC) vaccines comprise bacterial HSPs, purified with their chaperoned protein cargo. HSPC vaccines use the intrinsic adjuvant activity of their Hsp, thought to act via Toll-like receptors (TLR), to induce an immune response against their cargo antigens. This study evaluated HspC vaccines from *N. meningitidis* and the closely related commensal *N. lactamica*.

RESULTS:

The protein composition of *N. lactamica* and *N. meningitidis* HSPCs were similar. Using human HEK-293 cells we found that both HSPCs can induce an innate immune response via activation of TLR2. However stimulation of TLR2 or TLR4 deficient murine splenocytes revealed that HSPCs can activate an innate immune response via multiple receptors. Vaccination of wildtype mice with the *Neisseria* HSPC induced a strong antibody response and a Th1-restricted T helper response. However, vaccination of mice deficient in the major TLR adaptor protein, MyD88, revealed that while the Th1 response to *Neisseria* HSPC requires MyD88, these vaccines unexpectedly induced an antigen-specific antibody response via a MyD88-independent mechanism.

CONCLUSIONS:

N. lactamica and *N. meningitidis* HSPC vaccines both have potential utility for immunising against neisserial meningitis without the requirement for an exogenous adjuvant. The mode of action of these vaccines is highly complex, with HSPCs inducing immune responses via both MyD88-dependent and -independent mechanisms. In particular, these HSPC vaccines induced an antibody response without detectable T cell help.

Keywords: Heat shock protein, Heat shock protein complex vaccine, *Neisseria meningitidis*, meningitis, *Neisseria lactamica*

INTRODUCTION

Neisseria is a large genus of gram-negative, aerobic diplococcal bacteria that colonize various mucosal surfaces in a range of animals. The majority of *Neisseria* species are typically harmless commensals, such as *N. lactamica* which colonize the human nasopharynx [1]. A few however are significant pathogens, including *N. meningitidis* which also occupies the same nasal niche. *N. meningitidis* is a common coloniser of humans, with up to 10% of individuals carrying this organism with no ill-effect. In a small proportion of those infected however, and for reasons that are poorly understood, *N. meningitidis* can turn into an opportunistic pathogen invading the tissues and disseminating to become a major cause of meningitis or septicaemia [2]. The pathogenic effects of *N. meningitidis* infection are far more prevalent in children.

N. meningitidis is separated into serogroups based on its polysaccharide capsule; 13 have been identified with A, B, C, X, W135 and Y being those most commonly associated with disease [3]. A highly successful vaccine that induces antibodies against many of these major serogroup polysaccharides has been available for over ten years, having a major impact on reducing meningococcal disease [4]. Due to similarities between *N. meningitidis* type B and human polysaccharides, it was not possible to develop a similar vaccine against this serogroup. An aluminium hydroxide adjuvanted protein-based type B vaccine recently become available [5, 6], although this vaccine has not yet been universally adopted internationally. There thus remains a need for an effective and economical vaccine against *N. meningitidis* to protect against meningococcal disease.

Proteomic, genetic and immunological analyses have indicated considerable similarity between *N. lactamica* and *N. meningitidis* antigenicity [7-9], and there is evidence that *N. lactamica* colonization can provide some protection against *N. meningitidis* infection [10], possibly either by competing for colonization sites or via the induction of cross-reactive antibodies [11]. This has resulted in *N. lactamica* being studied as a potential vaccine tool for inducing protective immunity against *N. meningitidis* [9, 12-14].

Heat shock proteins (HSPs) are highly conserved molecules, grouped into families, mostly based on their molecular weight and sequence homology. Main family members include HSP90, HSP70 and HSP60 in mammalian cells, while in bacteria HSP60 (also called GroEL) and HSP70 (DnaK) are generally the most prevalent forms. The predominant role of HSPs is to serve as chaperones, binding and providing

stability to cytosolic proteins, particularly during folding and unfolding. However HSPs also serve a number of other functions, including the ability to act as a link between the innate and acquired immune systems, for example by delivering antigens for immune presentation and acting as damage associated molecular pattern (DAMP) molecules that can activate Toll-Like receptors (TLR) [15]. HSP-induced innate immune signalling has been particularly linked with the activation of TLR2 and TLR4 [16].

The enrichment of HSP from bacteria along with its bound protein cargo (termed the HSP Complex; HSPC) provides a source of immunogen that, because of the intrinsic immunostimulatory activity of the HSP and the range of antigenic molecules it is associated with, can potentially produce an effective vaccine without requiring the addition of an exogenous adjuvant [17-19].

While the mechanism of action of such non-exogenously adjuvanted HSPC vaccine formulations was predicted to be via HSP activation of TLR2 and/or TLR4, this has not previously been experimentally confirmed. In this study, we characterize and compare the antigenic cargo of HSPC from closely-related commensal (*N. lactamica*) and pathogenic (*N. meningitidis*) nasal-colonizing *Neisseria* species and use these formulations to examine the importance of TLR activation in the immune response to HSPC vaccination.

MATERIALS AND METHODS

Preparation of Neisseria HSPC

HSPCs from *N. lactamica* strain Y92/1009 or *N. meningitidis* strain H44/76 were prepared essentially as described [20]. Lysed bacteria (using a Triton X-100 solution; 40 mM Tris, 1 mM MgCl₂, 20 mM NaCl, 0.5% Triton X-100, pH 8.0), were centrifuged at 13,000 rpm, passed through a 0.2 µm filter, and 10 mg of clarified lysate loaded onto a 5mL ion exchange column (Capto Q, GE Healthcare, Uppsala) at 0.5 mL/min. HSPCs 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 (Bradford assay) and HSP60 content by western blot using the anti-HSP60 antibody SPA-875 (Stressgen Bioreagents, Ann Arbor, MI, USA). The final vaccine comprised pooled fractions containing most HSP60, sterile filtered (0.2

µm filter) and diluted to 500 µg/mL in pH8.0 buffer containing 40 mM Tris, 1 mM MgCl₂, 300 mM NaCl and 0.1% Triton X-100.

The reduction of contaminating *Neisserial* lipopolysaccharide (LPS) from *N. lactamica* HSPCs was performed using an EndoTrap[®] HD kit (hyglos, Charleston, USA). LPS levels in the HSPCs used in this study, as quantified by Endosafe[®]-PTST[™] (Charles River, Wilmington, Mass, USA), were determined to be 14,155 (*N. lactamica* HSPC), 66 (*N. lactamica* HSPC LPS-reduced) and 19,679 (*N. meningitidis* HSPC) EU/mg.

Proteomic analysis of Neisseria HSPC

For proteomic analysis, proteins were denatured in urea (Life Technologies, Carlsbad, USA), reduced with 10 mM TCEP (Pierce, Rockford, IL, USA), alkylated with 55 mM iodoacetamide (Sigma-Aldrich, St. Louis, USA) and digested with sequencing grade modified trypsin (Thermo Scientific Pierce) overnight at 37°C. Peptides were acidified with 1% formic acid (Sigma-Aldrich), purified through solid phase extraction with Oasis HLB cartridges (Waters, Milford, USA) and freeze-dried before analysis by liquid chromatography–tandem mass spectrometry (LC–MSMS) as previously described [17]. Data analysis was carried out using Proteome Discoverer (Thermo Scientific version 1.4) with the Mascot search engine (Matrix Science version 2.4) against the Uniprot database. Search results were set to a maximum of 1% false discovery rate (FDR), one missed trypsin cleavage, and text-filtered for *Neisseria*. Proteins were positively identified if they contained at least two unique peptides in at least two of three sample replicates. Percentage molarity was calculated based on the exponentially modified protein abundance index [21].

For protein visualization, 5 µg HSPC was compared with 5 µg lysate on Coomassie Blue stained SDS-PAGE gels as previously described [22].

Cell stimulation assays

HEK293 cells and splenocytes from C57BL/6, *Tlr2*^{-/-} or *Tlr4*^{0/0} mice [23, 24], were cultured in DMEM (high glucose) or RPMI 1640 respectively both containing 10% foetal calf serum, penicillin, streptomycin, glutamine (Life Technologies) and 2.5 µg/mL amphotericin B (Sigma-Aldrich). HEK293 cells expressing TLR2 [25], were kindly provided by Dr Ashley Mansell (Monash Institute of Medical Research). Cells were stimulated with 5 µg/mL *N. lactamica* lysate or *Neisseria* HSPC vaccine, while controls were cultured in media alone, or stimulated with the TLR4 ligand ultrapure

LPS at 100 ng/mL, or the TLR2 ligand Pam3CysSerLys4 (P3C) at 50 ng/mL (Invivogen, San Diego, CA, USA).

Evaluation of cytokine levels

Cytokine concentrations were determined by enzyme linked immunosorbent assay (ELISA). 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated with purified anti-mouse MIP2 (100 ng/well), IL-10 (200 ng/well), TNF α (40 ng/well)(R&D Systems, Minneapolis, MN, USA), IL-6, IL-13, IL-17A (50 ng/well)(eBioscience, San Diego, CA, USA), IFN γ (100 ng/well)(BD Biosciences, San Jose, CA, USA), or anti-human IL-8 (200 ng/well)(R&D Systems) at 4°C overnight in bicarbonate coating buffer, pH 9.6. Plates were blocked with 1% BSA (Sigma-Aldrich) in PBS (blocker buffer) for one hour prior to addition of samples in duplicate at 4°C overnight. Captured cytokines were then labelled with biotinylated anti-mouse MIP2 (3.9 ng/well), IL-10 (2.5 ng/well), TNF α (15 ng/well)(R&D Systems), IL-6 (25ng/well), IL-13 (25 ng/well) IL-17A (25 ng/well)(eBioscience), IFN γ (50 ng/well)(BD Biosciences) or anti-human IL-8 (1 ng/well)(R&D Systems) in blocker buffer for one hour prior to the addition of 50 μ L horseradish peroxidase conjugated streptavidin (Pierce) 1/5000 in blocker buffer for 30 min. Colour was developed with 100 μ L of TMB solution prepared as 1% of 10 mg/mL TMB (Sigma-Aldrich) in DMSO and 0.006% hydrogen peroxide in phosphate-citrate buffer, pH 5.0, and the reaction stopped with an equal volume of 2M sulphuric acid prior to reading absorbance at 450 nm. Sample concentration was determined against a standard curve of recombinant cytokine (same manufacturers as antibodies).

Mouse vaccination experiments

Animal experimentation was performed under institutional guidelines and with approval from the University of Melbourne Animal Ethics Committee. Groups of age-matched, C57BL/6 or *Myd88*^{-/-} mice (Walter and Eliza Hall Institute, Melbourne, Australia) [26], were injected subcutaneously with either 100 μ g of HSPC (vaccinated) or an equal volume of sterile phosphate buffered saline (controls). Mice were vaccinated twice, four weeks apart, then euthanized and tissues collected for analysis one week after the second immunization.

Evaluation of serum antibody responses

Blood was collected by cardiac puncture and left to coagulate prior to collection of sera. Levels of specific antibodies were quantified by ELISA. Maxisorp

immunoplates (Nunc) were coated with 5 µg/well of *N. lactamica* lysate at 4°C overnight in bicarbonate coating buffer, pH 9.6. Plates were blocked for one hour at room temperature then 1/20 serially diluted sera in blocker added in duplicate for one hour at room temperature. Captured antibodies were labelled with 4 ng/well horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Pierce), 8 ng/well horseradish peroxidase-conjugated goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL, USA) or 10 ng/well horseradish peroxidase-conjugated goat anti-mouse IgG2c (Immunology Consultants Laboratory, Portland, OR, USA) in blocker for one hour. Colour was developed and read as above prior to calculation of endpoint titres. Serum bactericidal activity against *N. meningitidis* was performed as previously described [27].

Statistics

For statistical analyses, all data were log-transformed then compared by analysis of variance (ANOVA) with Dunnett's post-hoc analysis, using SPSS software, version 21.0.

RESULTS

Characterization of Neisseria lactamica HSPC

N. lactamica and *N. meningitidis* HSPCs were made using an optimized and standard procedure [20], originally developed to produce and analyse HSPCs from other bacteria [17]. The composition of *N. lactamica* and *N. meningitidis* HSPCs were examined by proteomic analysis and the twenty most abundant proteins are listed in Table 1. The antigenic compositions of the HSPCs from these two nasal *Neisseria* species were notably similar. In addition to the expected HSPs (HSP60 and HSP70), many of the most highly abundant *Neisseria* HSPC components were ribosome-associated proteins.

TLR2 and TLR4 signalling in the cytokine response to N. lactamica and N. meningitidis HSPC

Some HSPs induce an innate immune response by activation of TLR2. We therefore examined whether *Neisseria* HSPC were also capable of stimulating a response via this receptor. Culture of HEK-293 cells that express TLR2 with HSPC from *N. lactamica* or *N. meningitidis* induced the secretion of IL-8, in contrast to control

HEK-293 cells which were unresponsive to both HSPC and TLR2 activation (Figure 1). No response to LPS was observed for any cells, demonstrating that the HSPC induced secretion of IL-8 was not related to contaminating endotoxin. Hence HSPC from both *Neisserial* species could trigger an innate immune response via TLR2.

To extend this observation, as other HSP have been shown to activate both TLR2 and TLR4, we stimulated splenocytes from wildtype, *Tlr2*^{-/-} and *Tlr4*^{0/0} mice with *N. lactamica* or *N. meningitidis* HSPC. While neither HSPC induced secretion of anti-inflammatory IL-10, both induced the secretion of innate proinflammatory cytokines (IL-6, MIP2, TNF α) equivalent to or greater than the levels induced by the positive controls (LPS for *Tlr2*^{-/-} cells and P3C for *Tlr4*^{0/0} cells) (Figure 2). It was notable that the cytokine response of *Tlr4*^{0/0} cells to *N. lactamica* HSPC was significantly greater than that induced by *N. meningitidis* HSPC, suggesting a possibly greater role for TLR4 activation in the response to *N. meningitidis* HSPC.

Hence these data indicated that *Neisseria* HSPC stimulated the secretion of innate cytokines via a mechanism that was not solely dependent on either TLR2 or TLR4. This raised the possibility that these HSPCs were activating both TLR2 and TLR4, although signalling via TLR4 might play a larger role in the response induced by *N. meningitidis* HSPC.

MyD88-dependent pathways in the response to Neisseria HSPC vaccination

As our data suggested that *Neisseria* HSPC might activate multiple TLR pathways, we vaccinated mice lacking MyD88 (*Myd88*^{-/-}) with HSPC from *N. lactamica*. MyD88 is an adaptor protein essential for most TLR-dependent signalling pathways. In addition, to determine whether contaminating LPS may contribute to the functionality of such a vaccine, we also immunized an additional group of mice with *N. lactamica* HSPC depleted of 99.5% of the endotoxin by repeated passage over an EndoTrap[®] HD resin column. One dimensional SDS-PAGE comparisons demonstrated that LPS depletion had no effect on the antigenic composition of *N. lactamica* HSPC (Figure 3a).

Serum antibodies play a critical role in the protective response to pathogenic *Neisserial* infection [4, 28]. Vaccination with *N. lactamica* HSPC induced a large antigen-specific serum IgG antibody response in both wildtype and *Myd88*^{-/-} mice against both *N. lactamica* and *N. meningitidis* (Figure 3b). The levels of this antibody

response in these two strains of mice was indistinguishable, indicating the serum IgG response to *N. lactamica* HSPC vaccine occurred independently of MyD88-signalling.

A substantial *Neisseria*-specific serum IgG response also developed in mice receiving the LPS-reduced vaccine, although in the response to *N. lactamica*, this was significantly lower in mice receiving HSPC containing endotoxin (Figure 3b). This suggests the antibody response was partially, though not entirely, adjuvanted by LPS present in the formulation. As this effect was present in both wildtype and *Myd88*^{-/-} mice, any effect of LPS on the resulting antibody response occurred via a MyD88-independent mechanism.

To examine the adaptive T cell memory response induced by these vaccinations, splenocytes from these mice were stimulated *in vitro* with *N. lactamica* lysate. Vaccination with *N. lactamica* HSPC induced a strong memory Th1-type response as demonstrated by significant secretion of IFN γ by antigen-stimulated spleen cells from immunized wildtype mice (Figure 3c). A similar level of response was observed in cells from mice vaccinated with the LPS-depleted formulation. Furthermore, in contrast to the antibody response, induction of the IFN γ recall response was completely dependent on MyD88 as no IFN γ was secreted by antigen-stimulated splenocytes from *Myd88*^{-/-} mice. No significant IL-17A (Th17) or IL-13 (Th2) secretion was induced by *N. lactamica* stimulation of splenocytes from any group (Figure 3c).

This experiment was repeated using *N. meningitidis* HSPC with similar results. Vaccination of wildtype mice with *N. meningitidis* HSPC also induced a very strong serum IgG antibody response. We thus quantified the IgG subclasses and found that the antibody response comprised both IgG1 and IgG2c and, as for *N. lactamica* HSPC, was not significantly different between wildtype and *Myd88*^{-/-} mice (Figure 4a).

Stimulation of splenocytes from these vaccinated mice with *N. meningitidis* HSPC induced a MyD88-dependent IFN γ response, without either IL-17A or IL-13 production (Figure 4b), as seen with *N. lactamica* HSPC vaccination. In contrast to *N. lactamica* HSPC however, this occurred as a basal response of naïve cells, with no evidence of a Th1-type memory response, as shown by a lack of increased IFN γ secretion by splenocytes from *N. meningitidis* HSPC vaccinated, as compared to naïve (unvaccinated) animals (Figure 4b).

These observations indicate that the antibody and Th1 cellular response to *Neisseria* HSPC vaccination were induced by two distinct mechanisms, which were MyD88-independent and dependent respectively.

DISCUSSION

While *N. lactamica* and *N. meningitidis* are related bacterial species that occupy the same niche in their human host, there is a major difference in their ability to cause disease. A number of studies have compared these organisms seeking to explain this difference in pathogenicity. At the genetic level there is some uncertainty as to whether commensal *N. lactamica* and pathogenic *N. meningitidis* differ greatly with respect to their expression of virulence factors [8, 29, 30]. Several previous studies have also compared the proteomes of these bacteria, however such studies have typically focused on their outer membrane components, motivated predominantly as strategies to identify candidate vaccine antigens [7, 31].

This study provides the first analysis and comparison of the HSPC, the chaperoned proteins associated with the heat shock proteins, of *N. lactamica* and *N. meningitidis* as vaccine candidates. Proteomic analysis revealed no obvious major differences between the composition of HSPC from commensal *N. lactamica* and HSPC from the pathogen *N. meningitidis* B. While only a crude comparison is possible, this does not point to any clear features that contribute to pathogenicity. This antigenic similarity importantly supports the utility of HSPCs from either *N. lactamica* or *N. meningitidis* in a meningitis vaccine. Furthermore, HSPC from both *N. lactamica* and *N. meningitidis* were shown to be capable of 1) activating cells via TLR2-dependent signalling in the human HEK293 cell line, and 2) inducing cytokine secretion by mouse immune cell, via a mechanism that requires MyD88 but is not entirely dependent on either TLR2 or TLR4. This is consistent with HSPC signalling via multiple innate receptors including both TLR2 and TLR4. It is well documented that *Neisseria* can induce TLR2-signalling via their porin PorB [32-35]. However our proteomic analysis revealed a complete absence of porins in both HSPC, indicating TLR2 activation by these vaccines did not involve PorB.

It was interesting to note that the mouse cytokine response to *N. lactamica* HSPC was at least equivalent to that induced by *N. meningitidis* HSPC. This contrasts with the poor immune response to *N. lactamica*, relative to *N. meningitidis*, that has been reported in studies using whole bacteria [36, 37]. Hence the poor immunogenicity of

N. lactamica does not appear to be related to a lower immune-activating potential of their HSPs. HSPCs from both *Neisserial* species induced a Th1 but not a Th2 or Th17 response and such a Th1-polarizing effect has previously also been reported for HSP from both humans and other bacteria [38, 39]. In contrast, TLR4-deficient cells stimulated with *N. meningitidis* and *N. lactamica* HSPC secreted different levels of innate cytokines. This indicates important differences in the pathways by which individual HSPCs, even from closely related bacteria, activate an immune response with TLR4 appearing to play a greater role in the response to *N. meningitidis* HSPC.

Protective immunity against *N. meningitidis* is believed to be antibody mediated. While an analysis of the bactericidal activity of this response was beyond the aims of this study, in a previous experiment performed by ImmunoBiology Limited, subcutaneous vaccination of CD1 mice (n=10) with 50 µg of *N. meningitidis* HSPC induced serum bactericidal activity (titre >1:512) against the homologous H44/76 strain, in contrast to no activity in sera from sham-vaccinated controls. ~~Tand~~ the ability of HSPs to activate TLRs is a key mechanism by which HSPC induce an immune response to co-delivered antigens [18]. An important and surprising finding of the current study therefore, was that *Neisseria* HSPC potently induced an effective antibody response, even without addition of an exogenous adjuvant, in MyD88-deficient mice. This result was unexpected and indicates these HSPC vaccines induce a humoral response via a currently unidentified MyD88-independent mechanism. Perhaps related to this mechanism are recent demonstrations that MyD88 is also not essential for the generation of an antibody response against adenoviral capsid protein and bacterial flagellin [40, 41].

In conclusion, the type and similarity of the immune response mounted against vaccination with *N. lactamica* and *N. meningitidis* HSPC, both with respect to cell-mediated immunity and in the production of a specific antibody response, suggests that either formulation has potential utility in a meningococcal vaccine. Our examination of the mode of action of these vaccines has revealed a high level of complexity, with HSPC inducing immune responses via a number of mechanisms, including a MyD88-dependent cellular response with subtle differences in the use of innate receptors, and a MyD88-independent mechanism that induces an antibody response without any detectable T helper cell response. Further studies are required to identify the mechanism by which HSPC induce this apparently T helper cell-independent antibody response.

ACKNOWLEDGEMENTS

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

REFERENCES

- [1] Olsen SF, Djurhuus B, Rasmussen K, Joensen HD, Larsen SO, Zoffman H, et al. Pharyngeal carriage of *Neisseria meningitidis* and *Neisseria lactamica* in households with infants within areas with high and low incidences of meningococcal disease. *Epidemiology and infection*. 1991;106:445-57.
- [2] Miller F, Lecuyer H, Join-Lambert O, Bourdoulous S, Marullo S, Nassif X, et al. *Neisseria meningitidis* colonization of the brain endothelium and cerebrospinal fluid invasion. *Cellular microbiology*. 2012.
- [3] Read RC. *Neisseria meningitidis*; clones, carriage, and disease. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*. 2014;20:391-5.
- [4] McIntyre PB, O'Brien KL, Greenwood B, van de Beek D. Effect of vaccines on bacterial meningitis worldwide. *Lancet*. 2012;380:1703-11.
- [5] Serruto D, Bottomley MJ, Ram S, Giuliani MM, Rappuoli R. The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens. *Vaccine*. 2012;30 Suppl 2:B87-97.
- [6] Andrews SM, Pollard AJ. A vaccine against serogroup B *Neisseria meningitidis*: dealing with uncertainty. *The Lancet infectious diseases*. 2014;14:426-34.
- [7] Abel A, Sanchez S, Arenas J, Criado MT, Ferreiros CM. Bioinformatic analysis of outer membrane proteome of *Neisseria meningitidis* and *Neisseria lactamica*. *International microbiology : the official journal of the Spanish Society for Microbiology*. 2007;10:5-11.
- [8] Snyder LA, Saunders NJ. The majority of genes in the pathogenic *Neisseria* species are present in non-pathogenic *Neisseria lactamica*, including those designated as 'virulence genes'. *BMC genomics*. 2006;7:128.

- [9] Troncoso G, Sanchez S, Criado MT, Ferreiros CM. Analysis of *Neisseria lactamica* antigens putatively implicated in acquisition of natural immunity to *Neisseria meningitidis*. FEMS immunology and medical microbiology. 2002;34:9-15.
- [10] Oliver KJ, Reddin KM, Bracegirdle P, Hudson MJ, Borrow R, Feavers IM, et al. *Neisseria lactamica* protects against experimental meningococcal infection. Infection and immunity. 2002;70:3621-6.
- [11] Andrade JR, Marques Mda C, de Santa Rosa MR. Nasal secretions of *Neisseria lactamica* carriers have an inhibitory effect on *Neisseria meningitidis* attachment to human oroepithelial cells. Memorias do Instituto Oswaldo Cruz. 1986;81:453-7.
- [12] Gorringe AR, Taylor S, Brookes C, Matheson M, Finney M, Kerr M, et al. Phase I safety and immunogenicity study of a candidate meningococcal disease vaccine based on *Neisseria lactamica* outer membrane vesicles. Clinical and vaccine immunology : CVI. 2009;16:1113-20.
- [13] Li Y, Zhang Q, Winterbotham M, Mowe E, Gorringe A, Tang CM. Immunization with live *Neisseria lactamica* protects mice against meningococcal challenge and can elicit serum bactericidal antibodies. Infection and immunity. 2006;74:6348-55.
- [14] Sanchez S, Troncoso G, Criado MT, Ferreiros C. *In vitro* induction of memory-driven responses against *Neisseria meningitidis* by priming with *Neisseria lactamica*. Vaccine. 2002;20:2957-63.
- [15] Osterloh A, Breloer M. Heat shock proteins: linking danger and pathogen recognition. Medical microbiology and immunology. 2008;197:1-8.
- [16] Colaco CA, Bailey CR, Walker KB, Keeble J. Heat shock proteins: stimulators of innate and acquired immunity. Biomed Res Int. 2013;2013:461230.
- [17] Chionh YT, Arulmuruganar A, Venditti E, Ng GZ, Han JX, Entwisle C, et al. Heat shock protein complex vaccination induces protection against *Helicobacter pylori* without exogenous adjuvant. Vaccine. 2014;32:2350-8.
- [18] McNulty S, Colaco CA, Blandford LE, Bailey CR, Baschieri S, Todryk S. Heat-shock proteins as dendritic cell-targeting vaccines--getting warmer. Immunology. 2013;139:407-15.
- [19] Colaco CA, Bailey CR, Keeble J, Walker KB. BCG (Bacille Calmette-Guerin) HspCs (heat-shock protein-peptide complexes) induce T-helper 1 responses and protect against live challenge in a murine aerosol challenge model of pulmonary tuberculosis. Biochemical Society transactions. 2004;32:626-8.

- [20] Colaco C, Salim K. Method for purifying protein complexes. 2010.
- [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;4:1265-72.
- [22] 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;252:1102-6.
- [23] Kupz A, Guarda G, Gebhardt T, Sander LE, Short KR, Diavatopoulos DA, et al. NLRC4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8(+) T cells. *Nature immunology*. 2012;13:162-9.
- [24] Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*. 1999;11:443-51.
- [25] Latz E, Visintin A, Lien E, Fitzgerald KA, Monks BG, Kurt-Jones EA, et al. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *The Journal of biological chemistry*. 2002;277:47834-43.
- [26] Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*. 1999;11:115-22.
- [27] Sandbu S, Feiring B, Oster P, Helland OS, Bakke HS, Naess LM, et al. Immunogenicity and safety of a combination of two serogroup B meningococcal outer membrane vesicle vaccines. *Clinical and vaccine immunology : CVI*. 2007;14:1062-9.
- [28] Jarvis GA. Recognition and control of neisserial infection by antibody and complement. *Trends in microbiology*. 1995;3:198-201.
- [29] Stabler R, Hinds J. The majority of genes in the pathogenic *Neisseria* species are present in non-pathogenic *Neisseria lactamica*, including those designated as virulence genes: response. *BMC genomics*. 2006;7:129.
- [30] Stabler RA, Marsden GL, Witney AA, Li Y, Bentley SD, Tang CM, et al. Identification of pathogen-specific genes through microarray analysis of pathogenic and commensal *Neisseria* species. *Microbiology*. 2005;151:2907-22.
- [31] Vaughan TE, Skipp PJ, O'Connor CD, Hudson MJ, Vipond R, Elmore MJ, et al. Proteomic analysis of *Neisseria lactamica* and *Neisseria meningitidis* outer membrane vesicle vaccine antigens. *Vaccine*. 2006;24:5277-93.

- [32] Platt A, MacLeod H, Massari P, Liu X, Wetzler L. *In vivo* and *in vitro* characterization of the immune stimulating activity of the neisserial porin PorB. *PloS one*. 2013;8:e82171.
- [33] Chiavolini D, Weir S, Murphy JR, Wetzler LM. *Neisseria meningitidis* PorB, a Toll-like receptor 2 ligand, improves the capacity of *Francisella tularensis* lipopolysaccharide to protect mice against experimental tularemia. *Clinical and vaccine immunology : CVI*. 2008;15:1322-9.
- [34] Liu X, Wetzler LM, Nascimento LO, Massari P. Human airway epithelial cell responses to *Neisseria lactamica* and purified porin via Toll-like receptor 2-dependent signaling. *Infection and immunity*. 2010;78:5314-23.
- [35] Liu X, Wetzler LM, Massari P. The PorB porin from commensal *Neisseria lactamica* induces Th1 and Th2 immune responses to ovalbumin in mice and is a potential immune adjuvant. *Vaccine*. 2008;26:786-96.
- [36] Vaughan AT, Gorringe A, Davenport V, Williams NA, Heyderman RS. Absence of mucosal immunity in the human upper respiratory tract to the commensal bacteria *Neisseria lactamica* but not pathogenic *Neisseria meningitidis* during the peak age of nasopharyngeal carriage. *J Immunol*. 2009;182:2231-40.
- [37] Fowler MI, Yin KY, Humphries HE, Heckels JE, Christodoulides M. Comparison of the inflammatory responses of human meningeal cells following challenge with *Neisseria lactamica* and with *Neisseria meningitidis*. *Infection and immunity*. 2006;74:6467-78.
- [38] Wan T, Zhou X, Chen G, An H, Chen T, Zhang W, et al. Novel heat shock protein Hsp70L1 activates dendritic cells and acts as a Th1 polarizing adjuvant. *Blood*. 2004;103:1747-54.
- [39] Saygili T, Akincilar SC, Akgul B, Nalbant A. *Aggregatibacter actinomycetemcomitans* GroEL protein promotes conversion of human CD4+ T cells into IFN γ IL10 producing Tbet+ Th1 cells. *PloS one*. 2012;7:e49252.
- [40] Rogers GL, Suzuki M, Zolotukhin I, Markusic DM, Morel LM, Lee B, et al. Unique roles of TLR9- and MyD88-dependent and -independent pathways in adaptive immune responses to AAV-mediated gene transfer. *J Innate Immun*. 2015.
- [41] López-Yglesias AH, Zhao X, Quarles EK, Lai MA, VandenBos T, Strong RK, et al. Flagellin Induces Antibody Responses through a TLR5- and Inflammasome-Independent Pathway. *The Journal of Immunology*. 2014;192:1587-96.

FIGURE LEGENDS

Figure 1: *Neisserial* HSPCs induce TLR2-mediated signalling

HEK293 cells expressing TLR2, or wildtype control HEK293 cells which lack this receptor, were cultured in media alone or stimulated for 24 hours with either *N. lactamica* (NI) HSPC, *N. meningitidis* (MenB) HSPC, the TLR4 ligand LPS or the TLR2 ligand P3C (n=6 replica wells per stimulation). HEK293 TLR2 expression was confirmed by responsiveness to P3C. HSPCs from both *Neisseria* induced IL-8 secretion, only in cells expressing TLR2. ***p<0.001 compared to media control; ANOVA.

Figure 2: Role of immune cell TLR2 and TLR4 in the cytokine response to *Neisserial* HSPCs

Splenocytes from wildtype C57BL/6 (n=13), *Tlr2*^{-/-} (n=11) or *Tlr4*^{0/0} mice (n=10) were cultured for 1 day either in media alone or with *N. lactamica* (NI) or *N. meningitidis* (MenB) HSPC. Controls were stimulated with P3C (TLR2 ligand) or LPS (TLR4 ligand). Cytokine levels in supernatants were determined by ELISA. As expected, *Tlr2*^{-/-} splenocytes were non-responsive to P3C and *Tlr4*^{0/0} splenocytes were non-responsive to LPS. *p<0.05, **p<0.01, ***p<0.001; significantly greater than media alone (ANOVA).

Figure 3: Immune response induced by vaccination of mice with *N. lactamica* HSPC

a) The protein composition of *N. lactamica* HSPC was effectively unchanged by LPS-reduction, when compared by SDS-PAGE. 1) *N. lactamica* lysate; 2) *N. lactamica* HSPC; 3) *N. lactamica* HSPC, LPS-reduced.

b-c) Wildtype C57BL/6 and *Myd88*^{-/-} mice (n=6) were vaccinated twice with *N. lactamica* HSPC or LPS-depleted *N. lactamica* HSPC (↓LPS). Naïve controls (n=6) were left unvaccinated. Sera and spleens were collected for analysis one week after the second vaccination. b) Serum anti-*N. lactamica* and anti-*N. meningitidis* IgG levels were quantified by ELISA. Both vaccines increased antigen-specific antibody levels compared to naïve controls (**p<0.01, ***p<0.001; ANOVA) but titres obtained with the LPS-reduced formulation were significantly lower (ANOVA). There was no difference in IgG levels between wildtype and *Myd88*^{-/-} mice. c) Splenocytes were cultured in media with or without *N. lactamica* lysate (NL) for 1 or 4 days, then cytokine levels in culture supernatants quantified by ELISA. Splenocytes from vaccinated wildtype but not *Myd88*^{-/-} mice secreted IFN γ in response to *N. lactamica* lysate stimulation. **p<0.01; ***p<0.001 compared to unvaccinated control (ANOVA). n.s. = not significantly different (ANOVA).

Figure 4: Immune response induced by vaccination of mice with *N. meningitidis* type B HSPC

Wildtype C57BL/6 (n=8) and *Myd88*^{-/-} mice (n=7) were either left unvaccinated or vaccinated twice with *N. meningitidis* HSPC (MenB). Sera and spleens were collected for analysis one week after the second vaccination.

a) Serum anti-*N. lactamica* levels were quantified by ELISA. Both vaccines increased antigen-specific antibody levels compared to naïve controls (ANOVA). There was no difference in IgG levels between wildtype and *Myd88*^{-/-} mice.

b) Splenocytes were cultured in media with or without MenB HSPC for 1 or 4 days, then cytokine levels in culture supernatants quantified by ELISA. Splenocytes from wildtype but not *Myd88*^{-/-} mice secreted IFN γ in response to MenB HSPC stimulation (ANOVA). There was no significant difference in the response of splenocytes from vaccinated or wildtype mice (ANOVA).

Table 1 Proteomic analysis of *Neisseria* HspC vaccines

<i>Neisseria lactamica</i> HspC			<i>Neisseria meningitidis</i> HspC	
Protein		% mol	Protein	% mol
1	Elongation factor Tu	7.110	Oxidoreductase, zinc-binding dehydrogenase family protein	6.271
2	Alcohol dehydrogenase	5.456	(R,R)-butanediol dehydrogenase	5.139
3	GroEL (Hsp60)	3.306	Elongation factor G	2.837
4	50S ribosomal protein L4	2.563	Trigger factor 2	2.821
5	Universal stress protein	2.459	Phosphate acetyltransferase	2.685
6	Elongation factor G	2.344	Glutamate dehydrogenase	2.603
7	Peroxiredoxin 2 family protein/glutaredoxin	2.278	Antioxidant, AhpC/TSA family/glutaredoxin	2.424
8	Ribosomal protein S7	1.992	GroEL (Hsp60)	2.405
9	Trigger factor	1.975	Elongation factor Ts	2.340
10	30S ribosomal protein S9	1.942	Acetate kinase 1	2.335
11	30S ribosomal protein S1	1.876	30S ribosomal protein S1	2.144
12	Homoprotocatechuate degradation operon regulator	1.805	NifU-like protein	2.097
13	50S ribosomal protein L10	1.794	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	2.017
14	Identified by MetaGeneAnnotator	1.726	Oligopeptidase A	1.981
15	Succinyl-CoA ligase [ADP-forming] subunit beta	1.689	Succinyl-CoA ligase [ADP-forming] subunit beta	1.978
16	NifU-like protein	1.670	Ribosomal protein S7	1.815
17	50S ribosomal protein L16	1.657	Phosphoglycerate kinase	1.645
18	Phosphate acetyltransferase	1.653	Cell division protein ftsZ	1.637
19	Glyceraldehyde 3-phosphate dehydrogenase	1.630	DnaK (Hsp70)	1.605
20	DnaK (Hsp70)	1.629	ATP synthase subunit beta	1.595

Figure 1

Figure 2

Figure 4