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

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ABSTRACT

*Background: Neisseria meningitidis* are common colonizers 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 HEK293 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.



## 1. 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 *Neisseria lactamica* which colonize the human nasopharynx [1]. A few however, are significant pathogens, including *Neisseria meningitidis* which also occupies the same nasal niche. *N. meningitidis* is a common colonizer 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.

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*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 10 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.

## 2. Materials and methods

### 2.1. 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 $MgCl_2$, 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 5 mL 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 $MgCl_2$, 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®-PTS™ (Charles River, Wilmington, Mass, USA), were determined to be 14,155 EU/mg (*N. lactamica* HSPC), 66 EU/mg (*N. lactamica* HSPC LPS-reduced) and 19,679 EU/mg (*N. meningitidis* HSPC).

### 2.2. 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].

### 2.3. 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).

### 2.4. 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), TNFα (40 ng/well)(R&D Systems, Minneapolis, MN, USA), IL-6, IL-13, IL-17A (50 ng/well)(eBioscience, San Diego, CA, USA), IFNγ (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 1 h 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