Evaluation of Truncated NhhA Protein as a Candidate Meningococcal Vaccine Antigen

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Abstract

NhhA (Neisseria hia homologue) is an outer membrane protein from Neisseria meningitidis, the causative agent of meningococcal disease. The protein is surface exposed and its expression in a wide range of meningococcal strains suggests it is a promising vaccine candidate. In addition, immunization of mice with outer membrane vesicles of strains that overexpress NhhA in conjunction with one of TbpA, Omp85 or NspA results in synergistic bactericidal responses. We previously showed that the NhhA sequence is highly conserved between strains, with the majority of the differences localized to four distinct variable regions located in the amino-terminal region of the mature protein. In this study, N. meningitidis strains were constructed that over-express wild-type NhhA. Strains expressing truncated versions of NhhA, with deletions from the amino-terminal region that removed the most variable regions, were also made. These expression strains were also modified so that immunodominant, phase- and antigenically-variable outer membrane proteins were not expressed, truncated lipooligosaccharide (LOS) expression was genetically fixed (no phase variability), and capsular polysaccharide expression abolished. Outer membrane vesicles derived from these strains were used to immunize mice. As previously observed, a synergistic effect involving another antigen, TbpA, was required to demonstrate bactericidal activity. The highest bactericidal response obtained against a heterologous strain was obtained with a truncated variant of NhhA. These results indicate that removal of (a) variable region(s) does not reduce bactericidal responses against NhhA, and that bactericidal targets exist in regions other than the variable N-terminus. This provides the basis for future examination of responses against truncated NhhA in protecting against heterologous NhhA strains, and further evaluation of truncated NhhA as a candidate for inclusion in a vaccine against all serogroups of N. meningitidis.

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Competing Interests: VEW, CF, and JTP are employees of GlaxoSmithKline. JTP was an employee of GSK at the at the time of the study. IRP and MPJ are named inventors on patents relating to this work and therefore have a potential financial interest. The current or former employment of authors, or potential interest through patent inventorships of authors does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Neisseria meningitidis is the causative agent of meningococcal meningitis and septicaemia. Its only known host is the human, and it may be carried asymptomatically by approximately 10% of the population [1]. There is currently no vaccine that is effective in all age groups and against all serogroups, despite extensive research efforts [2] [3]. Candidate antigens for inclusion in a vaccine must be expressed in the majority of strains, be antigenically conserved, and elicit a protective immune response. The sequencing of the genomes of a number of meningococcal strains has facilitated the identification of novel antigens by a bioinformatic approach [4]. Analysis of genes up-regulated after contact with epithelial cells [5] or endothelial cells or serum [1] using micro-arrays has identified more potential vaccine candidates. This is in addition to all the protein and carbohydrate antigens investigated prior to genome sequence data became available [3]. In previous work we reported the sequence of NhhA in N. meningitidis [6], which is a homologue of the adhesins Hia and Hsf of Haemophilus influenzae [7,8,9]. NhhA has also been referred to as Hsf [10] and Msf [11]. This protein prevents complement attack [12] by binding vitronectin [11], and purified NhhA stimulates a proinflammatory response [13]. NhhA is a strong vaccine candidate because of the ubiquitous presence of the nhhA gene in meningococcal strains, its surface exposure and the high level of sequence conservation between strains (amino acid identity 85.3%–99.8%; [6]). The majority of the sequence variation that does exist is limited to four distinct variable regions (V1–V4) located in the first 200 amino acids of the mature protein [6]. Unlike many other outer membrane proteins of N. meningitidis, there are no obvious sequence features such as short tandem DNA repeats in or upstream of the gene that may mediate phase variable expression of NhhA. Furthermore, this protein is recognized by antisera from patients [14] [15], implying that it is expressed and immunogenic in vivo. We now report further...
investigations of NhhA as a vaccine candidate, by characterizing
the murine antibody response against a wild-type NhhA, and
against truncated forms of NhhA and show that removal of the
region of most variation does not prevent production of
bactericidal antibodies.

**Materials and Methods**

**Bacterial culture**

*Escherichia coli* were cultured in LB media, and *N. meningitidis*
were cultured on BHI agar overnight at 37°C. Kanamycin and
ampicillin were added at 100 μg/mL. Tetracycline was added at a
final concentration of 300 μg/mL and 15 μg/mL to select *E. coli*
and *N. meningitidis* respectively. Strains used in this study are listed
in table 1.

**Over expression constructs**

Plasmids used in this study are listed in table 1. To overexpress a
wild-type protein, the nhhAMC21 gene (accession number
AF137611) was amplified using primers HOMP5’ and HOMP-
P5’AN (table 2). The amplifier was digested with *Eag*I and
*Nco*I restriction endonucleases, and ligated into pCO14K, generating
plasmid pCO14K (Fig. 1). To overexpress a protein with most of the
Restriction endonucleases, and ligated into pCO14K, generating
final concentration of 300 μg/mL and 15 μg/mL to select *E. coli*
and *N. meningitidis* respectively. Strains used in this study are listed
in table 1.

**Unmarked and marked deletion of opc**

The plasmid pBE501 contains the opc gene and flanking regions
[16]. *HindIII* cuts pBE501 at −41 relative to ATG start, *SfiI* cuts
570 bp downstream of that (gene is 861 bp). pBE501 was digested with
*HindIII*/*SfiI*, blunted, and self-ligated, deleting the majority of
the gene including the start codon and part of the promoter
region. The resulting plasmid was named pIP14. To create a
marked deletion, pBE501 was digested with *SfiI*, deleting 327 bp,
and the tetracycline resistance determinant was excised from
pGEMTetA [17] and cloned into the blunted *SfiI*-digested
pBE501. The resulting plasmid was named pOpcTet.

**Table 1. Strains and plasmids used in this study.**

| Bacterial strains: | Genotype/relevant characteristic | NhhA phenotype | Reference |
|--------------------|----------------------------------|----------------|----------|
| PMC21 | *N. meningitidis*, serogroup C | Expresses wild-type NhhA | This study |
| c3 | *N. meningitidis*, derived from MC58, acapsulate (ΔOpa2erm), Opa - variant | Expresses wild-type levels NhhAMC21 | [23] |
| 2A | *N. meningitidis*, c3 derivative, nhhA:kan | NhhA expression abolished | [6] |
| c3lgtA | c3 derivative, lgtA:kan | Expresses wild-type levels of NhhAMC21 | [18] |
| 7G2 | *N. meningitidis*, c3lgtA derivative LOS phenotype fixed LB | Expresses wild-type levels of NhhAMC21 | This study |
| P6 | 7G2 derivative, porA replaced by nhhA | Over-expression of NhhA, wild-type levels of NhhAMC21 | This study |
| P6ΔOpcA | P6 derivative, Δopc | Over-expression of NhhA, wild-type levels of NhhAMC21 | This study |
| P5 | 7G2 derivative, porA replaced by nhhA | Over-expression of NhhA (Bgl II deletion) wild-type levels of NhhAMC21 | This study |
| P5ΔΔOpcA | P5 derivative, Δopc | Over-expression of NhhA, wild-type levels of NhhAMC21 | This study |
| P5O1 | 7G2 derivative, porA replaced by nhhA | Over-expression of NhhA, wild-type levels of NhhAMC21 | This study |
| P5O1.17A | P5O1 derivative, Δopc: tet | Over-expression of NhhA (splice-overlap deletion) wild-type levels of NhhAMC21 | This study |

**Plasmids**

| Plasmids | Genotype/relevant characteristic | Reference |
|----------|----------------------------------|----------|
| pCO14K | porA gene with kanamycin resistance gene cloned downstream | [24] |
| pIP52(PMC21) | nhhA cloned into pCO14K | This study |
| pIP52(PMC21BglI) | PIP52(PMC21) derivative: BglI deletion of nhhA | This study |
| pIP51 | Splice overlap deletion of nhhA cloned into pCO14K | This study |
| pBE501 | Plasmid contains opc gene | [16] |
| pIP14 | opc deletion plasmid: HindIII/Styl digest of pBE501 | This study |
| pOptet | pBE501 derivative, opc: tetM | This study |
| pT7lgtAG7 | Contains lgtABE locus | This study |

<ref>Table 1. Strains and plasmids used in this study.</ref>
Fixing LOS expression

In order to fix the expression of the phase variable lgtA gene to “off”, so that the L8 immunotype was expressed, the homopolymeric tract of the lgtA gene was altered so that only 7 G residues remained in the homopolymeric tract region. This results in a frame shift mutation and no expression of LgtA activity (the wild type strain, MC58, has 14 G; [18]). Using primers Lic31ext and lgtAG7 in PCR with Neisseria meningitidis strain MC58 chromosomal DNA as template the region encompassing the poly-G tract to be altered was amplified. The lgtAG7 primer incorporated the change in the lgtA sequence from 14G to 7G. The resulting amplimer was cloned into pT7Blue (Novogen), to create plasmid pT7lgtAG7. To reconstitute the complete lgtA gene so that the plasmid could be used to transform the new allele into Neisseria meningitidis, a BssHII fragment from plasmid pMJ1b11 [18] was cloned into the BssHII site of pT7lgtAG7 in the correct

**Figure 1. Generation of overexpression constructs.** pIP52(PMC21): nhhAPMC21 was amplified using primers HOMPS’ (H5 in this figure) and HOMPS’AN (H3), and cloned into the Eagl/NcoI sites of pCO14K. pIP52(PMC21Bgl): the C1 region was amplified using primers H5 and NH3’BG (N, incorporating a BglII site) and cloned into the Eagl/BglII sites of pIP52(PMC21). pIPSO1: the C1 region was amplified from plasmid pMJ1b11 [18] and the C5 region was amplified using primers SO-D and H3. The two products were annealed, then amplified using primers H5 and H3. The resulting amplimer was cloned into the Eagl and NcoI sites of pCO14K. Conserved regions (C1–5) and variable regions (V1–V4) as defined in (19). doi:10.1371/journal.pone.0072003.g001

**Table 2.** Oligonucleotides used for generation of variant NhhA, and lgtA amplifications.

| Oligonucleotide | Sequence                                           | Description                                                                 |
|-----------------|----------------------------------------------------|-----------------------------------------------------------------------------|
| HOMP5’          | 5’-CAA TTA ACG GCC GAA TAA AAG GAA GCC GAT ATG AAC AAA ATA TAC CGC ATC-3’ | This contains an Eagl restriction site (underlined) and the sequence encoding the first 7 (seven) amino acids of NhhA (bold type) |
| HOMPS’AN        | 5’-TGG AAT CCA TGG AAT CGC CAC CCT TCC CTT C-3’    | This contains a NcoI restriction site (underlined) and the reverse complement of sequence 48–61 nucleotides past the end of the nhhA open reading frame of PMC21 (bold type) |
| NH3’BG          | 5’-GCT CAG ATC TGT TTC ATT GTG AGC ACT TGC-3’      | This contains a BglII restriction site (underlined) and the reverse complement of sequence encoding amino acids 13, 14, (double underlined) and 49–54 of wild-type PMC21 NhhA (bold type). |
| SO-C            | 5’-GAC GAA ATC AAG GTT CTT AGC ACT TGC CTG AAC CGT TGC-3’ | Reverse complement of sequence encoding amino acids 237–241 at the start of the C5 region (underlined) and amino acids 45–52 at the end of the C1 region (bold type) of wild-type NhhA of strain PMC21. |
| SO-D            | 5’-AAC GTT GAT TTC GTC CGC ACT TAC-3’              | encodes amino acids 237–244 at the start of C5 (underlined indicates reverse complement of Primer SO-C) |
| Lic31ext        | 5’- CCT TTA GTC AGC GTA TTG ATT TGC G-3’           | Used to mutate poly-G tract of lgtA and to amplify mutant lgtA for transformation |
| lgtAG7          | 5’-ATC GGT GCG GGC AAT ATA TCC CCC CCC GA CTT TGC CAA TTC ATC – 3’ | Used to mutate poly-G tract of lgtA |
| Lic16ext        | 5’- CGA TGA TGC TGC GGT CTT TTT CCA T -3’         | To amplify mutant lgtA for transformation |
orientation. Nucleotide sequence analysis confirmed the correct orientation of the gene and that the sequence segment was identical to the corresponding section of the wild-type lgtA gene apart from the alteration of the homopolymeric tract from 14 to 7 G residues. In order to transfer the lgtAG7 mutation to the chromosome of Neisseria meningitidis to make a mutant strain, the plasmid pF7lgtAG7 was linearized and used to transform Neisseria meningitidis strain c3lgtA (containing an lgtA:kan mutation, [10]). Confirmation of the transfer of the lgtAG7 allele to the chromosome in kanamycin sensitive colonies obtained from the transformation was confirmed by PCR of the relevant section of the lgtA gene using primers Lic31ext and Lic16ext, followed by nucleotide sequencing with the same set of primers.

Nm transformation and screening

The plasmids were linearised by restriction digestion and used to transform N. meningitidis using the method described by Janik et al. [19]. Transformants were selected by overnight incubation at 37°C in 5% CO₂ on solid media containing antibiotic as appropriate.

Screening for NhHA overexpression and other protein electrophoresis

Following transformation with linearised pIP52/PMC21, pIP52/PMC21Bgl, or pIPSO1, kanamycin resistant colonies were selected, subcultured overnight and screened for over-expression of NhHA by separating total cell proteins electrophoretically on duplicate 10% acrylamide gels, followed by coomassie Blue staining or were Western blotted to nitrocellulose (BioRad) before block in skim milk/PBST followed by sequential incubation in rabbit polyclonal anti-NhHA sera [6], goat anti-rabbit alkaline-phosphatase, and detection with NBT/BCIP (BioRad). The nhha allele was sequenced to confirm replacement of porA with the PMC21 or truncated allele. For observing overexpression, sarkosyl-insoluble proteins were separated electrophoretically using Bis-Tris buffer system and 4–12% precast 8 cm gels with sarkosyl-insoluble proteins were separated electrophoretically on duplicate 10% acrylamide gels, followed by coomassie Blue staining or were Western blotted to nitrocellulose (BioRad) before block in skim milk/PBST followed by sequential incubation in rabbit polyclonal anti-NhHA sera [6], goat anti-rabbit alkaline-phosphatase, and detection with NBT/BCIP (BioRad). The nhha allele was sequenced to confirm replacement of porA with the PMC21 or truncated allele. For observing overexpression, sarkosyl-insoluble proteins were separated electrophoretically using Bis-Tris buffer system and 4–12% precast 8 cm gels with MOPS buffer (Invitrogen), or for western immunoblot proteins were separated using Tris-Acetate 3–8% pre-cast gels (Invitrogen).

Screening for removal of Opc expression

Antisera containing Opc-specific polyclonal rabbit antibodies were raised by immunizing rabbits with sarkosyl-insoluble OMCs of strain P6 (Opc-expressing strain). Serum was adsorbed against strain P6ΔOpcA as previously described [20], removing most antibodies except those recognizing Opc. Following transformation with linearised pIP14 (unmarked deletion construct of opc), bacteria were plated at low density. After overnight incubation, colonies were transferred to nitrocellulose (BioRad) and immunoblotted using rabbit Opc-specific sera, and antibody binding was visualized using goat anti-rabbit-Alkaline phosphatase and colorimetric detection with NBT/BCIP. Colonies that had lost Opc reactivity were identified and subcultured, and analysed by immunoblot. Southern blot analysis (using DIG-labelled HindIII/ SphI fragment or opc gene from pBE501 as a probe) was used to confirm that the lack of expression was due to the introduced mutation and not to the inherent phase-variation of this gene, and PCR confirmed the presence of the deleted opc allele. Anti-Opc sera were also used to confirm loss of Opc expression following transformation with pOpcTet and selection with tetracycline.

Protein sequencing

Sarkosyl-insoluble proteins (enriched for outer-membrane proteins) were separated by SDS-PAGE (7.5% acrylamide) and transferred to PVDF membrane. The PVDF membrane was stained with Coomassie Blue and the high molecular weight protein excised and subjected to N-terminal sequencing in a PE Biosystems 492LC protein sequencer.

Culture and preparation of OMVs

A vial of frozen N. meningitidis (recombinant or not) was thawed and streaked onto modified Frantz medium agar plate which was then incubated at 37°C for 18 h. Colonies were resuspended and added to a flask containing modified Frantz medium supplemented with the appropriate antibiotic, and incubated at 37°C for 16 h under shaking. The cells were separated from the culture broth by centrifugation at 5,000 g at 4°C for 15 min. OMVs were isolated using deoxycholate as described previously [21].

Mice and immunizations

Outbred OF1 mice (Charles River, Lyon, female, 6–8 wks of age, also known as CF1) received three injections with OMVs via intramuscular route on days 0, 21 and 28. With each injection of 50 μl, 10 μg of antigen formulated onto 100 μg of Al(OH)₃ (aluminum hydroxide) was administered. Control mice received adjuvant only. Blood samples were collected 14 days after the third injection.

Ethics. Mice were provided with food and water ad libitum and were monitored for adverse events following injections. Mice were anaesthetized prior to collection of blood from carotid artery prior to cervical dislocation under continuing anaesthesia. The experiments have complied with the relevant national guidelines of Belgium and institutional policies of GlaxoSmithKline Biologicals and all protocols were approved by GSK Biologicals ethics committee.

Antibody assays

The NhHA derived from strain H44/76 was expressed and purified from E. coli as C-terminally His tagged protein. ELISA plates were coated with NhHA. The assays were performed as described previously [22].

Complement-dependent bactericidal antibody assays

Bactericidal assays were performed as described previously [10]. Briefly, bacteria from an overnight on Mueller Hinton agar plates were inoculated in tryptic soy broth with iron chelator and grown in shaking flasks for 3 h at 37°C. The culture was diluted in order to reach an OD₆₀₀ nm of 0.4 (bacterial suspension). The sera were heat inactivated (40 min at 56°C) and subsequently diluted in PBS-glucose. In microtiter plates, diluted test serum was mixed with baby-rabbit complement and bacterial suspension. Serial dilutions of test sera were tested similarly. Controls included bacteria plus complement, bacteria plus heat-inactivated complement, and test serum plus bacteria plus heat-inactivated complement. The microtiter plates were sealed and incubated while shaking (520 rpm) for 75 min at 37°C without CO₂. Agar was added to each well and after an overnight incubation at 33°C in 5% CO₂, the colonies were counted. Bactericidal titers are defined as the reciprocal of the serum dilution yielding 50% killing. Additive or synergistic effects of antibodies directed against different over-expressed minor OMPs were studied by using sera pooled from mice immunized with each vaccine preparation. For mixing experiments, equal volumes of pooled sera of each relevant treatment group were combined and subsequently tested in the bactericidal assay.
Results

Expression Strains

In order to remove confounding factors in analysis of immune responses to NhhA, a number of phase-variable and/or immunodominant components of the meningococcus were fixed or abrogated: the starting strain was c3, an acapsulate, Opa-negative derivative of serogroup B strain MC38 [23]. Phase variation of lacto-N-neotetraose in LOS was abolished by a making an unmarked frameshift mutation in the tgt4 gene, resulting in a strain expressing an L8 immunotype LOS structure. Transformation with the NhhA overexpression constructs resulted in deletion of porA, and subsequent manipulation (as described in material and methods, and below) abolished Opc expression.

NhhA Overexpression constructs

Our previous observations suggested that NhhA is not strongly expressed in vivo, so plasmids were constructed containing alleles to express NhhA at high levels when introduced into the N. meningitidis chromosome. As nhha exhibits some sequence variation, (mostly confined to the region encoding the predicted amino-terminal 200 amino acids of the predicted mature protein), alleles were also constructed with some or all of this region deleted (See materials and methods).

The strategy of gene replacement was used: the recombinant nhha allele was placed under the control of the strong porA promoter, using plasmid pCO14K. The plasmid pCO14K contains porA (promoter and coding region) with the selectable kanamycin resistance determinant downstream [24]. Alleles of nhha were cloned into convenient restriction sites in pCO14K downstream of the porA promoter, replacing the majority of the porA gene (Fig. 1). Transformation of N. meningitidis with these plasmids results in reciprocal exchange by homologous recombination resulting in expression of the nhha gene under the control of the porA promoter, and abrogation of porA expression.

For overexpression of the wild-type allele, nhha was amplified from serogroup C strain PMC21 and cloned to create plasmid pIP52(PMC21). Plasmids with deletions of the most variable regions were also constructed. Plasmid pIP52(PMC21Bgl) contains a recombinant allele of nhha, encoding amino acids 1–54 and 134–591 of NhhaPMC21, a truncation of 81 amino acids relative to wild-type. This amino acid sequence, NhhaPMC21, has the majority of the V1 region (most variable), all of the V2 and C2 regions, and part of the C3 region removed relative to the parental NhhaPMC21 protein (Fig. 1). Plasmid pIPSO1, constructed by a PCR strategy, encodes a further truncated allele, nhhaBgl, encoding amino acids 1–52 and 337–591 (i.e. lacking 285 amino acids relative to wild-type NhhaPMC21) with V1–V3 and C2–C4 deleted (Fig. 1).

Transformation and Over-Expression analysis

The plasmids pIP52(PMC21), pIP52(PMC21Bgl), and pPSO1 were linearised and individually transformed into N. meningitidis strain 7G2 (acapsulate, Opa+ L8 immunotype fixed expression, see table 1). In each case, approximately 20–30 kanamycin resistant clones were screened to find one clone in which the double cross-over integration event had occurred to include both the kanamycin resistance determinant and the recombinant nhha allele, replacing porA and expressing NhhA at a higher level compared with the parental strain. To confirm overexpression of NhhA and replacement of porA in resulting kanamycin resistant clones, outer-membrane proteins were prepared and separated electrophoretically, prior to visualization of proteins by coomassie stain.

Deletion of opc

In order to further reduce confounding factors in our analysis of the vaccine potential of NhhA, we abolished Opc expression from our strains. Plasmids pIP14 and pOpcTet contain a deleted allele of opc, the latter with insertion of tetM resistance cassette (see methods). Opc is an outer membrane protein that is immunodominant, but is not present in all strains [25]. Plasmid pIP14 or pOpcTet was linearised and transformed into strains overexpressing NhhA variants. Colonies were screened by immunoblot using Opc-specific polyclonal rabbit sera (results not shown). Colonies that did not express Opc were analysed by immunoblot, PCR, and Southern blot to confirm that the lack of expression was due to the introduced mutation and not to the inherent phase-variation of this gene (results not shown). Thus strains used for immunogenicity studies overexpressed NhhA variants, expressed no PorA or Opc, had LOS fixed in the L8 immunotype, and these characteristics were introduced into a previously defined acapsulate, Opa-negative strain [23].

Analysis of high molecular weight NhhA species

As NhhA is a surface expressed protein [6], each of the overexpressed NhhA proteins were engineered to include the signal peptide (amino acids 1–51) predicted by SIGNALP (not shown). In each of the resulting strains, NhhA expression upregulated relative to the non-transformed parental strain, and PorA expression was abolished. In order to determine the level of recombinant NhhA overexpression relative to the endogenous NhhA, serial two-fold dilutions and Western immunoblot of sarkosyl insoluble proteins was conducted with detection by rabbit polyclonal anti-NhhA sera [6]. Expression of NhhA is increased at least 25-fold (Fig. 2) for the full-length NhhA-overexpressing strain P6AOpAc. Reactivity with NhhA-specific sera is equivalent between 25 μg sarkosyl-insoluble protein of strain 7G2, and <1 μg protein of strain P6AOpAc.

It is possible that the high molecular weight species expressed by NhhA overexpressing strains represent either stable multimers of NhhA (Fig. 3), or complexes of NhhA with other protein(s), or other proteins alone. To confirm the identity of the high molecular weight species, and to confirm the cleavage of the predicted signal peptide, the overexpressed proteins were N-terminally sequenced. Amino acid sequence for the two truncated NhhA proteins indicated in each case that the high molecular weight complex is comprised essentially of NhhA. The N-terminal sequence XXETDLTSGVT was obtained for protein isolated from

Figure 2. Overexpression of NhhA. Whole cell lysates were separated on 3–8% Tris-Acetate buffered PAGE, transferred to nitrocellulose, and immunoblotted with rabbit polyclonal anti-NhhA sera. Lane 1 Benchmark Prestained Marker, approximately 180 kDa marker indicated. Lane 2, 25 μg protein of strain 2A (NhhA expression abolished); Lanes 3 and 11, 25 μg protein of strain 7G2 (parental strain expressing wild type levels of NhhA). Lane 4, 25 μg protein of strain P6AOpAc. Lane 5, 12.5 μg protein of strain P6AOpAc; Lane 6, 6.25 μg protein of strain P6AOpAc; Lane 7 3.125 μg protein of strain P6AOpAc; Lane 8, 1.56 μg protein of strain P6AOpAc; Lane 9, 0.78 μg protein of strain P6AOpAc; Lane 10, 0.39 μg protein of strain P6AOpAc. doi:10.1371/journal.pone.0072003.g002
PAΔOpcA, which corresponds to predicted amino acids 52 to 62 of the truncated non-mature allele. The N-terminal sequence XNVXFVRTY was obtained for the PSO1.17A-derived protein, corresponding to amino acids 52–60. These data confirm the presence and cleavage site for the long signal peptide (aa 1–51), and that it is cleaved when expressed in N. meningitidis. The predicted molecular weight of the mature forms (i.e. after cleavage of the predicted signal peptide) of NhhAPMC21, NhhAAbg, and NhhAASO1, is 56.6, 47.6, and 36.7 kDa respectively. We had previously noted that NhhA migrates on SDS-PAGE at a size equivalent to >250 kDa [6] and NhhA has recently been described as a trimer when expressed in E. coli [26]. Overexpression in N. meningitidis of NhhAPMC21, and of the truncated proteins NhhAAbg, and NhhAASO1, results in migration at a size implying that NhhA exists as a stable multimeric form (Fig. 2) and that the truncations did not remove any structural features necessary for oligomerisation or stability.

Immunogenicity
In order to assess the antibody response to the NhhA variants, outer membrane vesicles were prepared. The OMV preparations were adsorbed onto Al(OH)₃ and injected into mice on days 0, 21 and 28. On day 42, the mice were bled and sera pooled from animals in each group. Each vaccine preparation elicited a similar antibody response, as assessed by ELISA using purified recombinant NhhAHH4/76 (Fig. 4). Mice immunized with OMV produced from a NhhA null strain did not elicit anti-NhhA antibodies (data not shown).

As it has previously been observed that over-expression of more than one minor protein is required for high-titer bactericidal activity [10], sera from mice inoculated with OMVs obtained from strains over-expressing the different NhhA variants (NhhA-OMVs) were mixed with sera from mice immunized with OMVs produced from a TbpA over-expressing strain (TbpA-OMVs) [10] and serum bactericidal assays were performed against H44/76 and CU385 (Table 3).

The mix of pooled sera from mice immunized with TbpA-OMVs and NhhAPMC21-OMVs had higher bactericidal titers than mixed sera from control mice (immunized with adjuvant alone) and from mice immunized with TbpA-OMVs. These results are in line with results obtained previously showing additive or synergistic impact of antibodies against more than one minor component for bactericidal activity [10]. The highest bactericidal titers were measured on the mix of anti-TbpA-OMV sera and NhhAAbg-OMVs (NhhAAbg lacking the variable regions V1 and V2). The enhanced bactericidal activity of NhhAAbg was seen against both the strains used in SBA. By comparison, the deletion of the four variable regions of NhhA (NhhAASO1) clearly had a negative impact on the induction of bactericidal antibodies as the bactericidal titers measured on the mixed anti-TbpA sera were lower than those obtained either with the wild type NhhAPMC21 or with NhhAAbg.

Discussion
Many outer membrane proteins of N. meningitidis exhibit strain to strain sequence variation, presumably as a result of selection by the host immune system. This is one of the reasons no effective cross-protective vaccine is available. The most variable region of PorA, for example, are in the longest, surface exposed loops which are highly immunogenic, and in part responsible for the strain specificity of previous vaccine formulations. NhhA has a number of variable regions, but the longest and most variable region (V1) is confined to the amino-terminus of the mature protein [6], Fig. 1. It has been previously noted that sera from meningococcal disease patients recognize NhhA. Van Ulsen et al. [14] suggest the reactivity of patient sera implies that cross-reactive antibodies are elicited in vivo despite the infecting strain being different in each case [14]. Another study indicated that convalescent sera of meningococcal disease patients (age 0.2–4 yrs) also recognise NhhA (referred to as NMB0992, [15]). However, whether the antibodies were bactericidal, and the allele of nhhA in the infecting strain was not investigated. Healthy carriers also had antibodies that recognized NhhA [14], which may be the result of asymptomatic colonisation by N. meningitidis. NhhA expression levels are variable between strains [6,27], but the mechanisms for this variable expression are unclear.

We over-expressed the wild-type protein to assess immunogenicity and protective potential against the autologous strain. We also over-expressed two truncated alleles to assess whether the conserved regions could elicit a protective immune response. As a consequence of the method of over-expression, these strains were deficient for PorA production. In addition, we deleted the strongly immunogenic protein Opc, in a strain background that was acapsulate, Opa−, and phase variation of terminal LOS was abolished. The resulting phenotype, lacking several immunodominant OMPs enabled assess the potential of NhhA for inclusion in a vaccine.

As NhhA is expressed at low levels in vitro in many strains, we placed NhhA under the control of the porA promoter resulting in strong expression of NhhA, as previously reported [10]. NhhA shares sequence similarity with other autotransporter proteins [6], particularly the adhesins Hia and Hsf of H. influenzae. One of the features of autotransporters is a long signal peptide: we confirmed that the mature protein has a signal peptide that is cleaved after amino acid 51. We also observed that for each of the overexpressed NhhA proteins and truncations, NhhA migrated on SDS-PAGE at much greater than the predicted molecular weight. This demonstrated that removal of N-terminal domains did not affect trimerisation and stability. The stability and correct trimerisation of the recombinant NhhA described in this study is important, as expression of monomeric NhhA (due to mutation of
the translocator domain) results in reduced susceptibility to NhhA-specific bactericidal killing [27].

The autotransporters YadA (Yersinia sp.) and UspA1 (Moraxella catarrhalis) form a membrane anchored oligomeric “lollipop” [28] and the C-terminal 76 amino acids of Hia form a trimer in the outer-membrane and are necessary and sufficient for export of the passenger domain [29]. Surana et al. also showed that the C-terminal 119 amino acids of NhhA could act to surface localize the Hap passenger domain [29]. More recently, Scarselli et al., demonstrated that the transporter domain of NhhA could be further localized to the C-terminal 72 amino acids [26].

By analogy with the structure of related autotransporters such as YadA [30], the N-terminal domain of the mature NhhA protein is predicted to form the tip region of a filamentous structure, and the most variable region of nhhA encodes this N-terminal domain. The deletion of variable regions (V1 to V2 or V1 to V4) did not reduce the immunogenicity of the truncated variants when presented in OMVs obtained from NhhA over-expressing strains, as assessed by ELISA to wild-type recombinant NhhA. This indicates that proteins of the OMVs are processed in such a way to elicit antibodies to epitopes other than the variable regions, and that these epitopes are accessible on recombinant E. coli-derived NhhA. Previous studies using another vaccine candidate, FrpB, also demonstrated that removal of immunogenic domains does not reduce production of antibodies recognizing wild-type FrpB when assessed by ELISA [31]. Crucially, in the FrpB study, the conserved cryptic epitopes were not accessible when the wild-type protein is expressed.

To assess whether antibodies raised against truncated NhhA could access the same epitope on wild-type proteins, bactericidal assays were performed. This was done by mixing anti-NhhA-OMV sera with anti-TbpA-OMV sera because it was previously observed that NhhA-OMVs alone elicited reduced or undetectable serum bactericidal antibody titers in mice [10]. When high levels of NhhA are expressed in a target strain, however, polyclonal NhhA-specific sera are bactericidal [27]. The results presented here confirmed the previous observation that antibodies directed against different minor OMPs work either in addition or in synergy to mediate the complement killing of bacteria [10], as the bactericidal titers measured on the pooled sera from mice

| Mix of sera | H44/76 SBA | CU385 SBA |
|-------------|------------|-----------|
| a-TbpA OMVs+ control negative pooled sera | 778 | 532 |
| a-TbpA OMVs+a-NhhA<sub>PMC21</sub> OMVs | 2595<sup>a</sup> | 1438 |
| a-TbpA OMVs+a-NhhA<sub>Bgl</sub> OMVs | 4383 | 2891 |
| a-TbpA OMVs+a-NhhA<sub>SO1</sub> OMVs | 1568 | 742 |

<sup>a</sup>S BA = Serum Bactericidal Assay. Geometric mean titers for 50% killing.
<sup>b</sup>NhhA<sub>PMC21</sub>, wild type NhhA; NhhA<sub>Bgl</sub>, NhhA without variable regions 1&2; NhhA<sub>SO1</sub>, NhhA without variable regions 1 to 4.

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immunized with TbpA-OMVs were systematically lower than the titers observed for the different NhhA-TbpA mixed sera.

In contrast to results obtained for FrpB truncations, removal of variable regions 1 and 2 of NhhA did not reduce bactericidal activity induced by OMVs containing the truncated NhhA<sub>Bgl</sub>-NhhA<sub>Bgl</sub>. Indeed, the bactericidal titer induced by NhhA<sub>Bgl</sub>-OMVs against both tested strains was higher than that elicited by NhhA<sub>MC221</sub>-OMVs expressing wild-type, full length NhhA, NhhA<sub>ACu385</sub>, or NhhA<sub>CLR312</sub>, despite being being geographically distinct (from epidemics in Norway and Cuba respectively) contain NhhA that differed by only one amino acid located in the signal peptide. The differences in bactericidal titers against these two strains may be explained by differences in TbpA (amino acid sequence is 92% identical), in differences in other minor antigens within the OMVs, or in levels of capsular polysaccharide produced. Nevertheless, in both cases the highest titers were with the NhhA<sub>Bgl</sub>-OMVs, confirming that antibodies against this truncated protein can act synergistically with anti-TbpA to mediate complement activation and killing. As removal of the variable regions did not reduce functional antibody responses, it is tempting to speculate that this truncated variant would elicit a response against strains expressing NhhA whose sequence differs within the variable domain but this remains to be formally examined.

Further truncation of NhhA (NhhA<sub>SOCl</sub>) resulted in a reduced bactericidal response against wild-type target strains, but not a reduction in immunogenicity assessed by ELISA. A similar effect has been reported when variable loops were selectively deleted from the Envelope protein of HIV: removal of variable loops resulted in an immune response capable of neutralizing strains containing variant loops whereas further truncations abolished neutralizing activity [32]. The fact the deletion of V1 to V2 or V1 to V4 regions of NhhA in our study does not impact on the immunogenicity of the OMV vaccine (as observed in ELISA) but does affect the induction of bactericidal antibodies suggests either that some protective epitopes are localized in the C3 domains or that the larger truncation of the N terminal part of the protein impact on the conformation of the NhhA passenger domain. Another possibility is that removal of these variable domains of NhhA reveals another protein that is responsible for the synergistic protection, although this is considered less likely. It is also notable that the larger truncation of NhhA involves the first of two consensus sequences between N-CAMS and NhhA, domains which may be involved in the potential adhesin function of NhhA [33]. NhhA also inhibits complement-mediated killing via vitronectin binding [11], but which domains of the protein mediate this is currently not elucidated.

Taken together, our studies suggest that the strategy of removing the most variable region of this protein still elicits an effective antibody response. In this study, we demonstrated that an OMV vaccine incorporating NhhA with the most variable region deleted elicits an higher bactericidal antibody titer than one containing the the wild-type protein. Removing this region results in an antigen with potential to elicit responses effective against strains with variant NhhA sequence, thus truncated NhhA represents a promising candidate for inclusion in an OMV based vaccine formulation for prevention of meningococcal disease.

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Author Contributions
Conceived and designed the experiments: IRP MPJ JTP. Performed the experiments: IRP YNS VEW CF. Wrote the paper: IRP MPJ VEW.

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