Phage display revisited: Epitope mapping of a monoclonal antibody directed against Neisseria meningitidis adhesin A using the PROFILER technology

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ABSTRACT
There is a strong need for rapid and reliable epitope mapping methods that can keep pace with the isolation of increasingly larger numbers of mAbs. We describe here the identification of a conformational epitope using Phage-based Representation OF ImmunoLigand Epitope Repertoire (PROFILER), a recently developed high-throughput method based on deep sequencing of antigen-specific lambda phage-displayed libraries. A novel bactericidal monoclonal antibody (mAb 9F11) raised against Neisseria meningitidis adhesin A (NadA), an important component of the Bexsero anti-meningococcal vaccine, was used to evaluate the technique in comparison with other epitope mapping methods. The PROFILER technology readily identified NadA fragments that were capable of fully recapitulating the reactivity of the entire antigen against mAb 9F11. Further analysis of these fragments using mutagenesis and hydrogen-deuterium exchange mass-spectrometry allowed us to identify the binding site of mAb 9F11 (A250-D274) and an adjoining sequence (V275-H312) that was also required for the full functional reconstitution of the epitope. These data suggest that, by virtue of its ability to detect a great variety of immunoreactive antigen fragments in phage-displayed libraries, the PROFILER technology can rapidly and reliably identify epitope-containing regions and provide, in addition, useful clues for the functional characterization of conformational mAb epitopes.

Abbreviations: PROFILER, Phage-based Representation Of ImmunoLigand Epitope Repertoire; NadA, Neisseria meningitidis adhesin A; MenB, serogroup B Neisseria meningitidis; FACS, immunofluorescence flow cytometry; HDX-MS, hydrogen-deuterium exchange mass spectrometry; mAb, monoclonal antibody

Introduction
Neisseria meningitidis adhesin A (NadA) is a trimeric outer membrane protein thought to mediate adhesion to and invasion of epithelial and endothelial barriers in the course of meningococcal infection. The NadA gene is present in approximately 30% of virulent meningococcal isolates and is highly prevalent in 3 of the 4 hypervirulent lineages of serogroup B (MenB) strains.1,2 Sequence analysis of NadA indicates the presence of 6 variants clustering in 2 groups: 1) group I, comprising protein variants NadA1, NadA2 and NadA3; and 2) group II, including protein variants NadA4, NadA5 and NadA6. Group I variants are the most represented and highly cross-protective.3,4 NadA induces high levels of bactericidal antibodies in humans,5-7 and is one of the 3 recombinant proteins that are included in a broadly protective anti-MenB vaccine, designated as Bexsero.8 Like other trimeric autotransporter adhesins, NadA is made of a C-terminal β-barrel, which anchors the protein to the outer membrane, a central α-helical coiled-coil domain (stalk) and an N-terminal “head.”8 The latter is an important component of the adhesin, since it is directly involved in binding to host receptors, including β-1 integrin and heat shock protein Hsp90.9,10 Detailed epitope mapping is crucial for understanding the mechanisms of anti-pathogen immunity. We have previously shown that vaccination with Bexsero induces antibodies that are predominantly directed against the cell binding area of NadA, which comprises the head and the adjacent portion of the central domain.11 By using a novel bactericidal mAb designated 9F11, we describe here the identification of a conformational epitope located in the C-terminal portion of the stalk, away from the region predominantly targeted by polyclonal responses in vaccinated individuals. To map the epitope, we employed an array of different techniques, including the newly described Phage-based Representation Of ImmunoLigand Epitope Repertoire (PROFILER) technology, which is based on next-generation sequencing of antibody-selected lambda phage display libraries.11 This method was
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Epitope mapping using PROFILER

To map the epitope recognized by the murine mAb 9F11 using PROFILER, we employed a previously described nadA gene-specific lambda phage-displayed library. Following 2 rounds of affinity selection using mAb 9F11, we sequenced the inserts of the selected phage population and compared them with those present in the library before selection. A total of 52,613 and 34,129 reads were obtained in the non-selected and in the antibody-selected libraries, respectively. This allowed us to follow the process of antibody-dependent selection by comparing the 2 libraries for various parameters, including: 1) copy number of each unique “natural frame” sequence; 2) ratio of the number of “natural frame” sequences over the total number of sequences (“normalized occurrence”). Fig. 2A shows that library complexity decreased after selection, with rapid convergence toward a relatively limited set of sequences. Moreover, as it can be appreciated from the black areas shown in Fig. 2B, there was a remarkable increase, after selection, in the frequency of “natural frame” sequences, over the total number of sequences. This indicated that the particles expressing authentic antigenic fragments had been selectively enriched by the mAb, while those carrying “not natural frame” sequences (which before selection represented the large majority of NadA gene fragments, because of the cloning strategy used) or no antigenic inserts markedly decreased in numbers. Moreover, in the antibody-selected library, the “natural frame” fragments were not evenly distributed along the entire length of the antigens, but rather clustered in a relatively limited set of sequences.

Results

Reactivity of mAb 9F11 against different NadA variants

The murine mAb 9F11 was obtained by conventional hybridoma techniques from mice immunized with a recombinant form of NadA3. The mAb was first tested by immunofluorescence flow cytometry (FACS) for reactivity against 4 MenB strains (BZ83, 5/99, NMB and M01-0240320), chosen as representative of different NadA variants. By FACS analysis, 9F11 bound to the surface of strains 5/99 and NMB (expressing NadA2 and 3, respectively), but not to strains BZ83 (NadA1) or M01-0240320 (NadA5; Fig. 1). Similarly, in a serum bactericidal assay (SBA) using rabbit complement, mAb 9F11 effectively killed strains 5/99 and NMB, but not BZ83 or M01-0240320 (Table 1). Collectively, these data indicated that mAb 9F11 bound to NadA2 and 3, but not to NadA1 and 5, expressed on the bacterial surface.

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Table 1. Bactericidal activity of mAb 9F11 against representative strains of N. meningitidis expressing different NadA variants.

| N. meningitidis strains | BZ83 Var. 1 | 5/99 Var. 2 | NMB Var. 3 | M01-0240320 Var. 5 |
|------------------------|-------------|-------------|-------------|-------------------|
| SBA titersa             | <4          | 65,536      | 16,384      | <4                |

a mAb 9F11 was tested in a serum bactericidal assay (SBA) using rabbit complement against different group B strains (BZ83, 5/99, NMB and M01-0240320), chosen as representative of the indicated NadA variants. Bacteria were incubated with serial 2-fold dilutions of mAb 9F11 (from 0.37 mg/ml to 0.023 μg/ml) and 25% baby rabbit complement. SBA titers were defined as the dilution resulting in 50% decrease in colony-forming units/ml. No bacterial killing was observed against strains BZ83 and M01-0240320 at the highest tested concentration of mAb 9F11.
and FrS (E246-T300), are indicated by the arrows in Fig. 3A.

Comparison with conventional sanger sequencing

Next, it was of interest to compare PROFILER analysis with traditional Sanger sequencing of immunoscreening-positive plaques. To this end, we used the same antibody-selected library previously analyzed using PROFILER. As expected, the traditional approach, involving isolation of 166 positive clones, provided a much lower number of unique sequences compared to next-generation sequencing (38 and 1,312, respectively; not shown). Moreover, the sequences identified by immunoscreening were all >102 aa long, and resembled those of the previously categorized “high” and “medium” length families (confront Figs. 3A and 3B). In conclusion, PROFILER allowed the identification of a greater variety of affinity-selected fragments and, particularly, of a set of shorter, C-terminally located fragments, which would have been missed using only the immunoscreening/single-clone sequencing approach.

Comparison with a filamentous phage-displayed library

To rule out any potential bias linked with the nature of the lambda phage vector or library design, we repeated the selection experiments using a different NadA library, constructed with the filamentous phage M13 as a vector. In this system, heterologous peptides are expressed as fusions to the major capsid protein (pVIII). After the M13 library was subjected to mAb-mediated selection and next-generation sequencing, we identified 11,619 “natural frame” reads, belonging to few unique sequences only (Fig. 3C). These fragments also mapped in the C-terminal portion of the stalk and closely resembled the family of short fragments previously identified with the lambda library. For example, the most enriched M13 fragment (A242-A305; Fig. 3C), which accounted for 99% of the “natural frame” reads, largely overlapped with FrS (i.e., E246-T300; Fig. 3A). Therefore, the affinity-selected pVIII library was composed of clones bearing only relatively short inserts, compared to the lambda library. This was expected because of the properties of pVIII-display system, which is known to favor shorter sequences.

Immunoreactivity of NadA fragments identified by PROFILER

To verify whether the antigenic fragments identified by phage display reacted against the 9F11 mAb, we recombinantly expressed, as fusions to glutathione S-transferase (GST), the representative FrL, FrM and FrS fragments indicated in Fig. 3A. As a negative control, we used a polypeptide (GST-FrI) consisting of a fragment the irrelevant antigen Neisserial Heparin Binding Antigen (not shown). The recombinant fragments were then compared to the entire antigen for binding to mAb 9F11 in a sensitive ELISA inhibition assay in which NadA was used as the coating antigen. Fig. 4A shows that both GST-FrL and GST-FrM were capable of

![Figure 2. Diversity of the NadA lambda phage-displayed library before and after affinity selection with the 9F11 mAb. (A) abundance of unique "natural frame" sequences in the unselected (left panel) and affinity-selected (right panel) libraries. Each point represents the number of unique sequences (vertical axis) displaying the number of copies indicated in the horizontal axis. (B) Enrichment of "natural frame" NadA phage inserts after affinity selection with the 9F11 mAb. Each graph reports the cumulative occurrence, per single amino acid position, of predicted "natural frame" sequences before (left panel) and after (right panel) affinity selection with the 9F11 mAb. The horizontal axis reports the amino acid sequence of the NadA gene used to engineer the library. The occurrence of each "natural frame" sequence was normalized over the total number of sequences, as described in the text (see Materials and Methods). Colored bars in the horizontal axis refer to NadA domains, black, leader peptide (LP); yellow, globular head (GH); green, coiled-coil stalk (CCS); blue, linker region (LR). The area indicated by the red curly bracket corresponds to cell binding region of NadA.](image-url)
recapitulating the immunoreactive properties of the soluble NadA protein, since these 3 constructs were equally capable of competing with immobilized NadA for mAb 9F11 binding. Interestingly, even the shortest fragment (GST-FrS) was capable of inducing some binding inhibition, although it was considerably less potent in this activity than GST-FrL or GST-FrM. Therefore, although the E246-T300 (FrS) sequence was sufficient for binding to mAb 9F11, the additional presence of a C-terminal aa stretch (e.g., R301-Y347 in FrM) was required to fully reconstitute the epitope. To further characterize this requirement, we engineered 2 C-terminally truncated forms of FrM, designated as GST-A245-E336 and GST-A245-E306. When tested for their ability to compete with Nad-A-His for mAb 9F11 binding, only GST-A245-E336 retained the inhibitory activity of FrM (Fig. 4B). In contrast, GST-A245-E306 showed some inhibitory activities only at high doses.

To further confirm these data, mAb 9F11 was tested in a protein microarray containing 41 recombinant fragments (GST or His or TRX tagged) covering the entire length of the NadA3 protein. All of the polypeptides that strongly reacted with mAb 9F11 contained sequences extending to H312 or beyond at the C-terminus (e.g., R301-Y347 in FrM) was required to fully reconstitute the epitope. To further characterize this requirement, we engineered 2 C-terminally truncated forms of FrM, designated as GST-A245-E336 and GST-A245-E306. When tested for their ability to compete with Nad-A-His for mAb 9F11 binding, only GST-A245-E336 retained the inhibitory activity of FrM (Fig. 4B). In contrast, GST-A245-E306 showed some inhibitory activities only at high doses.

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**Epitope mapping using HDX-MS**

To more precisely identify the binding site of mAb 9F11, we used hydrogen-deuterium exchange mass spectrometry (HDX-MS), which is based on the differential rate of deuterium incorporation by an antigen when it is free or bound to the antibody following incubation in appropriate deuterated solvents. When the antigen-antibody complex forms, the interface between the binding partners can occlude solvent accessibility, reducing the exchange rate. Epitope mapping by HDX-MS was carried out in 2 parallel steps: deuterium incorporation was performed on NadA alone (reference experiment) and on the antigen-antibody complex, as previously described. Both samples were digested by pepsin and deuterium uptake was monitored for 52 peptides covering 98% of NadA sequence. In Fig. 5A, the HDX-MS results have been simplified reporting the extent of deuterium uptake for only 19 sequential peptide fragments covering the
entire peptide map. HDX-MS revealed that H-D exchange was reduced in presence of mAb 9F11 for 2 of the 19 NadA fragments, corresponding to the 2 overlapping peptides spanning A250-D274 and A250-V275 residues, respectively. None of the other NadA fragments displayed any difference in deuterium uptake between the protein alone and the mAb 9F11-bound form. Since the difference in deuterium uptake is the same for both segments, these data suggested that the minimum sequence recognized by the mAb 9F11 is comprised in the A250-D274 portion of NadA.

Multiple sequence alignment analysis

Next, the sequences of the above-identified epitope-containing region were compared in different NadA variants. Fig. 6 shows that there is a high degree of conservation, in this region, between NadA2 and NadA3, the 2 variants displaying specific binding to mAb 9F11. Notably, both NadA1 and Nad5, which are not recognized by mAb9F11, have a large deletion encompassing part of the binding site, as identified by HDX-MS, and the entire adjoining sequence that was previously found to be required for optimal binding to mAb 9F11. Thus, the results of sequence alignment analysis were fully compatible with those of our epitope mapping approach.

Discussion

Data presented here indicate that the combined use of gene-specific libraries and deep sequencing, as exemplified by the PROFILER technology, can significantly empower phage display-based epitope mapping methods and usefully complement the results of more sophisticated techniques, such as HDX-MS. In this respect, our results are in general agreement with those obtained with other methods based on next-generation sequencing of surface-displayed libraries, including phage, yeast, or ribosome display.
Figure 5. Epitope mapping by HDX-MS. (A) time course of deuterium incorporation by peptic peptides covering the entire NadA3 sequence. The blue curves derive from the NadA-mAb 9F11 complex, while the red curves derive from NadA alone. The two peptides showing a significant difference in deuterium uptake between the free and bound NadA forms are highlighted with red boxes. (B) in-silico model of NadA3 built from the X-ray structure of the recently solved variant 5.17 Dashes show regions of unknown secondary structure. The red and orange bar indicates the A250-H312 sequence that was found to be sufficient, in the present study, to fully recapitulate the reactivity of NadA against the mAb. Shown in red is the A250-V275 subsequence containing the binding site identified by HDX-MS. Shown in orange is the adjoining V275-H312 subsequence required for the full functional reconstitution of the epitope.

Figure 6. Multiple sequence alignment of the C-terminal region of NadA variants. The dashed pink box indicates the mAb 9F11 binding site as identified by HD-XMS, while the dashed orange box indicates the adjoining region that is also required for optimal binding. Red letters indicate mutations using the NadA3 sequence for reference. Asterisk (*), positions with a single, fully conserved residue; colon (:), conservation of residues with strongly similar properties (≥ 0.5 in the Gonnet PAM 250 matrix); period (.), conservation of residues with weakly similar properties (< 0.5 in the Gonnet PAM 250 matrix).
Our previous experience has indicated that lambda phage systems, such as the one employed in the PROFILER technology, can be used to efficiently display fragments of a wider variety of antigens compared with the more commonly used filamentous phage display systems.\textsuperscript{12,13,22} Since filamentous phage assembly occurs at or within the membrane of \textit{Escherichia coli}, residues inhibiting proper protein insertion into this membrane may block the assembly and extrusion of phage particles, thereby preventing the display of the corresponding proteins. On the other hand, the capsid of lytic bacteriophages, such as lambda, is assembled in the cytoplasm of bacteria, and the display of fusion proteins does not depend on their ability to translocate across the membrane. Moreover, lambda phages also show an increased ability, over filamentous phages, to display proteins or peptides that may be toxic to the cell.\textsuperscript{23} Nevertheless, since any display system has drawbacks, in addition to advantages, it cannot be excluded from our data that some proteins might be less efficiently displayed on lambda phages than the NadA antigen used in this study.

The selection conditions (including the number of selection rounds) utilized here were chosen to provide the most informative phage pools in terms of their complexity and sequence variability. This, in conjunction with the ability of PROFILER to analyze thousands of different fragments for their degree of antibody-mediated enrichment, allowed the identification of a wider variety of affinity-selected polypeptides, compared with conventional techniques. For example, traditional immunoscreening was unable to identify the shorter and less immunoreactive fragments contained in the affinity-selected library. These shorter sequences are often useful in epitope mapping to identify structures that are still sufficient for antibody binding. On the other hand, the M13/pVIII NadA library that was also tested here could identify only short antigen fragments, which were unable to fully reconstitute the epitope. Indeed, when representative short antigen fragments, which were unable to fully reconstitute the epitope. NadA on live bacteria was determined by FACS analysis as follows. The ability of mAb 9F11 to bind surface-exposed variants of NadA on live bacteria was determined by FACS analysis in conjunction with the ability of PROFILER to analyze thousands of different fragments for their degree of antibody-mediated enrichment. This mAb was used to validate the ability of a recently developed phage display/deep sequencing platform to expedite mapping and functional characterization of complex conformational epitopes.

\section*{Materials and methods}

\subsection*{Reagents}

Chemicals and reagents were purchased from Sigma Aldrich unless otherwise stated.

\subsection*{Generation on mAb 9F11}

The mAb used in this work, a murine IgG2b designated as 3509F11, was produced by conventional hybridoma techniques from the spleen cells of CD1 mice immunized with NadA variant 3, as described.\textsuperscript{14,17} Procedures involving mice were conducted in accordance to the guidelines of the European Union for the handling of laboratory animals. The mAb was purified 355 from culture supernatants by Protein G affinity columns (GE Healthcare, cat. n. Seventeen-0618-01) and the sub-class determined with a mouse mAb isotyping kit (AbD Serotec, cat. n. MMT1) as described.\textsuperscript{14,17}

\subsection*{Bacterial strains and culture conditions}

\textit{N. meningitidis} strains were grown on chocolate agar (Biomentieux cat. n. 43101) at 37°C, 5% CO\textsubscript{2} overnight. For liquid cultures, colonies from an overnight growth were used to inoculate 7 ml cultures in Mueller Hinton broth (Difco, cat. n. 275730) supplemented with 0.25% glucose to an optical density at 600 nm (OD\textsubscript{600}) of 0.05. The culture was incubated for approximately 1.5 to 2.5 h at 37°C with shaking until early log phase (OD\textsubscript{600} of 0.25) or mid-log phase (OD\textsubscript{600} of 0.5).

\subsection*{Fluorescence-activated cell sorting (FACS) analysis}

The ability of mAb 9F11 to bind surface exposed variants of NadA on live bacteria was determined by FACS analysis as follows. One ml of a mid-log phase culture of bacteria (OD\textsubscript{600} of ~0.5) was centrifuged and the bacterial pellet suspended in 1 ml of phosphate-buffered saline (PBS)-1% bovine serum albumin (BSA; Sigma, cat. n. A7030). Then, 50 µl of suspended bacteria were incubated for 1 h with 10 µg/ml mAb in a reaction volume of 100 µl. The binding of mAb 9F11 was detected using an anti-mouse IgG (whole-molecule) fluorescein isothiocyanate (FITC) conjugated antibody (Sigma, cat. n. F9006) at a 1:10 dilution. All incubations were performed at room temperature. Prior to flow cytometry analysis, bacteria were fixed with 0.5% formaldehyde (Carlo Erba, cat. n. 415694) in PBS.
buffer for 1 h. Bacteria incubated with PBS-1% BSA and secondary antibody were used as negative control.

**Complement-mediated serum bactericidal activity**

SBA was determined as described. Briefly, early-log phase cultures of bacteria were diluted in Dulbecco’s PBS containing 1% BSA and 0.1% glucose (assay buffer) at a working dilution of 10^4 - 10^5 CFU/ml. Bacteria were incubated with serial 2-fold dilutions of mAb 9F11 (from 0.37 mg/ml to 0.023 μg/ml), and 25% baby rabbit complement. The total volume in each well was 50 μl, with 25 μl of serial 2-fold dilutions of test mAb, 12.5 μl of bacteria at the working dilution, and 12.5 μl of baby rabbit complement. SBA titers were defined as the serum dilution resulting in 50% decrease in colony-forming units/ml after a 60 min incubation of bacteria with the reaction mixture compared with the control colony forming units per milliliter at time zero.

**Construction and selection of phage-displayed libraries**

For epitope mapping using the PROFILER technology, a previously described library, expressing fragments of the NadA3 antigen fused to the capsid protein D on the surface of lambda phage λKM4 particles, was subjected to 2 rounds of selection using 10 μg/ml of mAb 9F11. Antibody-bound phage particles were separated by incubation with protein G-coated magnetic beads (Thermo Fisher Scientific cat. n. 88847), as described. Next, the inserts from the selected phage population were amplified and fused to sequencing adaptors and index oligonucleotides by extension PCR, exactly as described. For the construction of the NadA-specific M13 display library, DNA inserts with an average size of 150 nt in length were prepared as previously described, and cloned into the phagemid vector pFl1, a derivative of pC89, in the region encoding for the N-terminus of the pVIII capsid protein. The total number of independent clones was 1 x 10⁴ and the phage-displayed library was prepared by superinfection with M13K07 helper phage. Affinity selection of the library with mAb 9F11 was performed as previously described. Briefly, 100 μl of magnetic beads linked to Protein G (Dynabeads Protein-G, Thermo Fisher Scientific, cat. n. 10004D) were incubated for 1 hour at RT with 10 μg/ml mAb in 1 ml of 1X PBS. Beads were then incubated with 10^6 pfu recombinant phages and agitated for 3 hours at RT; then, they were washed 10 times with 1 ml of 1X PBS/0.05% Tween20. Bound phages were eluted with 500 μl 0.1 M HCl and adjusted to pH 2.2 with glycine and 10 mg/ml BSA.

**Sequencing**

After two cycles of affinity selection, pools of affinity selected phages were prepared for Illumina MiSeq sequencing as previously described. The following primers were used to amplify the inserts of the M13 phage-displayed library and to add Illumina adaptor subsequences: #295: 5’-TCTCGGAGACTGCTGTCGGGCGG-3’ and #296: 5’-GTCTCGGGGCTCGGAGATGTGTTATAAGAGA-CAGGGCTTGCAGGGAGT-3’. A further amplification step was performed to add index sequences, using the Nextera® Kit Index (Illumina, cat. n. FC-121-1011), according to manufacturer’s instructions, as previously described. Purity, concentration and length of PCR products were evaluated using a LabChip® XT system (PerkinElmer, cat. n. PN760541). The normalized libraries were then denatured prior to MiSeq sequencing using the MiSeq Reagent Nano kitv2 and paired end, 150 bp-long reads were obtained (Illumina, cat. n. MS-103-1001). The insert sequences were then analyzed by a dedicated software that identifies the sequences predicted to be expressed on the phage surface as authentic antigen fragments. These “natural frame” sequences are then translated into amino acid sequences and aligned along the length of the antigen as described previously.

Sanger sequencing of immunoreactive clones isolated for the lambda library was performed as previously described. Briefly, immunopositive plaques were picked, amplified in bacteria and then subjected to polyethylene glycol/NaCl precipitation. Phage suspensions were subjected to PCR amplification using the following primers K47 5’-GGGCACTCAGCCGAATTATCG-3’ and K48 5’-GTATGAGCCGGGTACACTGT-3’ in order to amplify inserts. PCR products were subjected to Sanger sequencing using capillary electrophoresis, as described. Inserts from immunopositive clones were then aligned along the amino acid sequence of NadA protein.

**Expression of isolated fragments**

To assess whether the antigenic fragments identified by phage display reacted against the 9F11 mAb in a molecular context different from that of the capsid proteins, the 3 representative antigenic fragments FrL (D165-S341), FrM (A245-Y347) and FrS (E246-T300) were recombinantly expressed as fusions to GST, along with a polypeptide corresponding to an irrelevant antigenic fragments FrL (D165-S341), FrM (A245-Y347) and FrS (E246-T300) were recombinantly expressed as fusions to GST, along with a polypeptide corresponding to an irrelevant antigen (FrL) as a negative control, using the Gateway® Cloning System (Thermo Fisher Scientific, cat. n. 12535-019), according to manufacturer’s instructions. C-terminally truncated (GST-A245-E336 and GST-A245-E306) and N-terminally truncated (GST-V252-E336, GST-A257-E336 and GST-V286-E336) forms of FrM were also expressed as fusions to GST using the Gateway® System. Briefly, the DNA sequences of interest were amplified using the primers containing attB sites -3’ shown in Table S2. Next, the amplified products were purified and used to obtain expression clones ready for gene expression according to manufacturer’s instructions (Thermo Fisher Scientific). After induction of the GST-fusion proteins, recombinant fragments were purified from soluble lysates of bacterial cells by affinity chromatography as previously described.

**ELISA inhibition assay**

The immunoreactivity of NadA antigenic fragments against the 9F11 mAb was assessed using a previously described ELISA inhibition assay. Briefly, the 9F11 mAb (0.1 μg/ml) was pre-incubated with nanomolar concentrations of recombinant protein fragments used as competitive inhibitors prior to the addition to microtiter wells sensitized with the whole antigen (NadA-His, 5 μg/ml). Alkaline phosphatase-conjugated goat anti-mouse IgG (cat. n. A7434) was then added at a 1:5,000
Epitope mapping by HDX-MS

Sample preparation, local HDX-MS analysis and mass spectra acquisition and interpretation, were performed as previously described. Briefly, the HDX experiments, with and without the mAb, were performed in the same experimental session. The antigen/mAb complex was formed by adding 250 pmols of NadA antigen to the mAb, using a molar ratio of 1:1 and incubated for 30 min at RT. Labeling was initiated by adding the proper deuterated buffer (PBS with a pD of 7.4), reaching a deuterium excess of 79% and was performed at RT. Over the time course of the experiment (ranging from 30 sec to 1 hour), samples were removed for quenching and dissociation of the antigen/mAb complex, and immediately flash frozen in liquid nitrogen. In parallel, a control experiment without mAb was performed using the same conditions except that PBS buffer was used instead of the antibody preparation. Labeled samples were thawed rapidly to 0°C and injected into a Waters nano-ACQUITY ultra-performance liquid chromatographic system with HDX technology. Protein samples were online digested using a Poroszyme Immobilized Pepsin Cartridge (Life Technologies, cat. n. Two-3131-00) and the generated peptides were immediately trapped, concentrated, desalted and then separated on a ACQUITY UPLC BEH C18 reverse-phase column (Waters cat. n. 186002346) as previously described. Mass spectra were acquired in resolution mode (m/z 100–2,000) on a Waters Synapt G2 mass spectrometer equipped with a standard ESI source. The identity of each peptide was confirmed by mass spectrometry elevated energy, as described. Data were processed using Protein Lynx Global Server 2.5 (Waters), and each fragmentation spectrum was manually inspected to confirm the assignment. The DynamX software (Waters) was used to select the peptides considered for the analysis and to extract the centroid mass of each of them and for each charge state as a function of the labeling time. Only the peptic peptides present in at least 3 repeated digestions of the unlabeled proteins were considered for the analysis.

Epitope mapping by protein and peptide microarrays

A protein array of recombinant NadA3 fragments was generated as previously described. Briefly, gene fragments were expressed in E. coli as either glutathione S-transferase- or His-tagged fusions or TRX fusions and purified from the cytoplasmic fraction as soluble forms, as previously described. Recombinant antigens were spotted on nitrocellulose-coated slides (FAST slides, Kerafast, cat. n. BC4182) using the no-contact Marathon Spotter (Arrayjet). Peptide microarrays were produced using a panel of 15-mer overlapping peptides with an offset of 4 aa, representing the complete sequence of NadA1, 2, 3 and 4. Chemically synthesized peptides were immobilized on glass slides via a flexible linker generating microarrays displaying directed and covalently attached peptides (JPT Technologies GmbH). Non-specific binding was minimized by preincubating protein or peptide microarray slides with a blocking solution (BlockIt, ArrayIt, cat. n. BKT) for 1 hour. mAb 9F11 was diluted 1:2,000 or 1:200 in BlockIt and overlaid on the protein and peptide arrays, respectively, for 1 h at room temperature. AlexaFluor® 647-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch cat. n. 115-605-062) was added for 1 h at room temperature in the dark, before proceeding with slide scanning. Fluorescence signals were detected by using the PowerScanner confocal laser scanner (Tecan Trading AG, Switzerland) and the 16-bit images were generated with the PowerScanner software v1.2 at a 10 μm/pixel resolution. Images were processed using the ImaGene 9.0 software (Biodiscovery Inc., CA). Elaboration and analysis of image raw fluorescence Intensity (FI) data was performed using an in-house developed R scripts. Signals were considered as positive when their MFI value was higher than 5,000, corresponding to the MFI of protein spots after detection with AlexaFluor® 647-conjugated anti-mouse IgG alone, plus 10 standard deviation values.

Disclosure of potential conflicts of interest

Veronica Lanza Caricco, Maria Dominia and Concetta Beninati own shares of Scylla Biotech Srl, a spin-off of the University of Messina that has filed a patent application (RM2014A000185) on the PROFILER technology. Agnese Valeri, Marco Bruttni, Erika Bartolini, Marzia Monica Giuli, Laura Santini, Brunella Brunelli, Nathalie Norais, Erica Borgogni and Vega Masignani are full-time employees of GSK Vaccines. The other authors declare no competing financial interests.

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