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Understanding and exploiting lipoprotein translocation in Gram negative pathogens for improved vaccine antigen delivery platforms

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Abstract

Lipoproteins of pathogenic Gram-negative bacteria are involved in different biological processes due to their highly immunogenic nature; and have proven to be good vaccine antigens. The lipoprotein translocation machinery of model organisms such as *Escherichia coli* is well characterized but unlikely *E. coli*, *N. meningitidis* displays many lipoproteins on its surface and recently an additional translocation component, Surface lipoprotein assembly modulators (Slam1 and Slam2), involved in the surface exposure of specific *N. meningitidis* lipoproteins, has been identified. Several aspects were investigated: first of all the sorting signals for Neisserial lipoproteins localization with a particular focus on the outer membrane crossing and surface localizations and the sorting differences of *N. meningitidis* with respect to the *E. coli* model were pointed out. The role of Slam1 for lipoproteins surface exposure was better characterized. Our results described a new role for Slam1 in outer membrane translocation of NHBA (Neisserial Heparin Binding Antigen) and Mip (Macrophage infectivity potentiator). fHbp as Slam1 substrate was confirmed but also a new role for Slam1 in fHbp expression stabilization was elucidated. Surface expression of *N. meningitidis* lipoproteins in the heterologous *E. coli* background was attempted, by taking advantage of Slam1 component which we clearly demonstrate to be necessary to actively translocate NHBA and fHbp on bacterial surface. Finally, the immunogenicity of OMVs with different lipoproteins surface display modulated by Slam1 expression was evaluated in homologous or heterologous systems. The immunogenicity studies suggested that Slam1 is able to drive bactericidal activity in *N. meningitidis* OMVs through modulating lipoprotein surface localization and furthermore other Slam-dependent bactericidal surface lipoproteins, different from fHbp and NHBA, might be present in *N. meningitidis*. We clearly demonstrated the importance of the co-expression of surface lipoproteins with Slam1 for the correct display of antigens and we showed that, depending on the presence of Slam1, heterologous *E. coli* OMVs, enriched with *N. meningitidis* SLPs, elicit different antibody responses.

Disclaimer

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Contents

1 Introduction

1.1 Gram negative bacteria

Bacterial cell envelope is a complex multilayered structure that serves to protect bacteria from the environment. Cell envelopes classify bacteria into two major groups, Gram positive and Gram negative bacteria. Gram negative bacteria present a thin peptidoglycan cell wall and an external envelope membrane, called outer membrane, containing lipopolysaccharides. On the contrary, Gram positive bacteria have a thicker peptidoglycan cell wall and the outer membrane is missing [1]. Gram negative cell envelope is principally divided in three layers: the cytoplasmic or Inner Membrane (IM); the periplasm (an aqueous cellular compartment) and the Outer Membrane (OM) [1, 2]. Bacterial IM surrounds the cytoplasm and is made of a symmetrical phospholipid bilayer and membrane proteins that have key role in energy production/transduction, lipid biosynthesis, protein secretion and signal transduction [3].

The peptidoglycan (PG) is located in the periplasmic space and its principal roles are correlated to cell integrity and support for anchoring other cell envelope components [4]. Outside the periplasm, the aqueous layer between bacterial membranes, there is OM protecting bacteria from the environment [5]. The OM is an asymmetrical bilayer composed by phospholipids and lipopolysaccharides (LPS), in the inner and outer leaflet respectively [2, 6].

In the OM two major classes of protein are found: Outer Membrane Proteins (OMP), characterized by transmembrane domains which span the membranes [7] and Lipoproteins (LP) anchored to outer membrane thanks to lipid chains at their N-terminal domain [8].

1.1.1 Neisseria meningitidis

Neisseria meningitidis is a Gram negative β-proteobacterium. An aerobic diplococcus, non-motile and non-sporulating which can be either capsulated or un encapsulated; and humans are the unique reservoir of this bacterium.

The *N. meningitidis* genome is around 2.1 Mb [9] and the species is classified into 13 serogroups based on the immunogenicity and the structure of the polysaccharide capsule [10], Among these groups only six (A, B, C, W-135, X, Y) cause life-threatening disease [10]. *N. meningitidis* is usually carried asymptomatically at the level of the mucosal epithelium of the respiratory tract and spreads by respiratory droplets [11]. Menigoccoccus, infrequently, can cross the epithelial barrier resulting in access to the bloodstream and fulminant sepsis or focal infections such as meningitis [12]. The switch from the commensal to pathogenic state is poorly understood, but thanks to *N. meningitidis* genome plasticity, it is able to evolve strategies to efficiently survive in both situations [13].

Different virulence factors are expressed by *N. meningitidis* and are mainly involved in host-pathogen interactions, immune evasion and nutrients uptake [13]. For example, the polysaccharide capsule represents a barrier against the host immune system [14] while LPS, referred to as lipooligosaccharide (LOS) due to the lack of an O-antigen, is required for resistance of *N. meningitidis* to serum complement [15]. Other virulence factors such as pili, consisting of protein subunits that protrude from the bacterial capsule; and Opa and Opc, which are OM-associated transmembrane proteins, important in adhesion and host cell interaction [16, 17]. Finally, lipoproteins involved in adhesion, immune evasion and iron acquisition [18-20] play also a key role in meningococcal pathogenesis.

1.2 Lipoprotein translocation mechanism

1.2.1 Lipoproteins

Lipoproteins are molecules both present in Gram-positive and -negative bacteria involved in various processes ranging from outer membrane stabilization, and interaction with host cells to nutrient acquisition [18-24]. Many lipoproteins from pathogenic bacteria, have been selected as vaccine antigens because of their ability to stimulate the immune system, both via the acquired and innate responses [25-27]. Lipoproteins (**Figure 1**) are associated to membranes thanks to a characteristic lipid moiety at the N-terminal which facilitates the anchoring of the hydrophilic domain to the membrane, allowing the protein to function in an aqueous environment [28].

Figure 1 Schematic representation of lipoproteins. Lipobox motif with the conserved Cysteine is highlighted in red. Arrow indicated the cleavage site of the signal peptide.

Lipoproteins' signal peptide has the peculiarity of a conserved motif at the C-terminus called the lipobox, composed by four amino acids L(A/S)(G/A)C, and the cysteine is the first amino acid of the mature lipoprotein where diacylglycerol and fatty acyl chains are added [29, 30]
Looking at the bacteria genome for the presence of coding sequences including the characteristic lipobox motif, it is evident that most bacteria usually encode approximately 100 to 200 putative lipoproteins [31].

Lipoproteins are synthetized in the cytosol as a precursor and translocated across the inner membrane via the Sec or Tat machinery [32, 33]. As immature proteins, with the type II signal peptides [34], they reach the outer leaflet of the inner membrane and undergo sequential reactions of maturation including lipid modification and signal peptide cleavage.

Three different enzymes (Lgt, SpII and Lnt) are involved in these post-translational modifications and they appear to be essential in most of the Gram-negative bacteria (**Figure 2**) [35, 36].

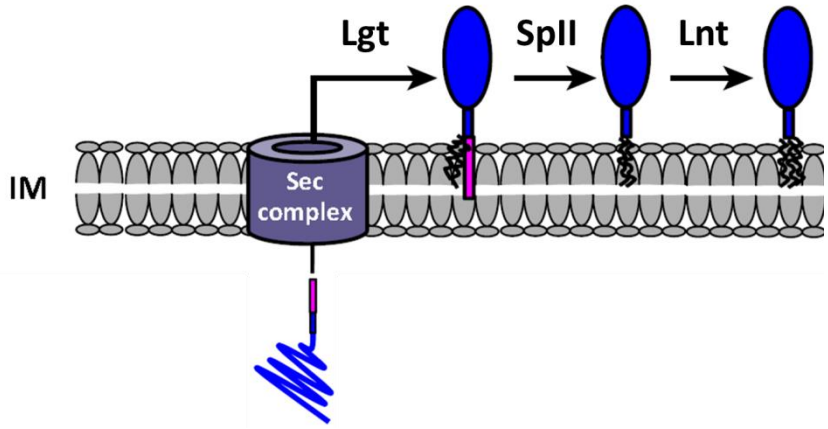

Figure 2 Lipoproteins maturation process. Prolipoprotein cross the inner membrane with Sec machinery. Lgt enzyme catalyzes transfer of a diacylglyceryl group on the cysteine; SpII cleaves the signal peptide and Lnt adds a third acyl group on the cysteine (adapted from *Wilson et al*. *2016*)

Upon membrane insertion of the immature lipoprotein, prolipoprotein, Lgt (diacylglyceryltransferase) transfers the diacylglceryl group, from the phosphatidylglycerol to the sulfidryl group of the conserved cysteine [37]. This intermediate is then recognized by SpII (Signal peptidase II) which cleaves the signal peptide leaving the acylate cysteine as the first amino acid of the mature lipoprotein. Lnt (N-acyltrasferase) is the last enzyme involved and is able to attach an acyl group to the N-terminal residue of the cysteine creating the mature triacylated lipoprotein [30, 38].

1.2.2 Surface exposed lipoproteins

Lipoproteins are usually found to be anchored to the membrane but facing the periplasm however in the past years some lipoproteins were found to be displayed on the outer leaflet of the outer membrane. This new class of lipoproteins called surface lipoproteins (SLP) is highly heterogeneous and no common features were identified to date, suggesting the existence of many different ways by which lipoproteins might reach cell surface and consequently, the presence of various mechanisms for lipoprotein translocation among bacteria [39, 40].

As example PulA of *K. pneumoniae* is a lipoprotein found either secreted or anchored to the outer leaflet of the outer membrane. PulA reaches the surface thanks to the type II secretion system, that acquires its substrate from the IM [41]. Autotransporters use Type Va secretion system for passing the outer membrane. Their N-terminal domain can reach the bacterial surface thanks to the C-terminal translocator domain and lipidated passenger domain is then anchored at the outer leaflet of the OM [42-44]. *B. burgdorferi*, member of the spirochete family, has the most abundant number of surface lipoproteins (SLPs) [45]. These bacteria exhibit an incomplete Lol system and *Schulez et al.* suggested that lipoproteins are translocated onto the surface by default and outer membrane proteins can help in the translocation to the surface [46, 47]. It was also described that in *E. coli* RcsF shows a transmembrane topology with the C-terminal domain that remains in the periplasm, while its lipidated N-terminal domain is displayed on the outer leaflet of the OM thanks to the interaction with the Bam complex [48]. In summary, surface lipoproteins show the necessity to translocate across the OM and, as described above, some of them require OMP to cross the phospolipid bilayer.

In *N. meningitidis,* different SLPs were found on the bacterial surface [49] and most of them are virulence factors. Well known lipoproteins displayed on the surface are fHbp (factor H binding protein) [19], NHBA (Neisserial Heparin Binding Antigen) [50], TbpB (Transferrin Binding Protein B), LpbB (Lactoferrin Binding Protein B), HpuA (hemoglobin-haptoglobin utilization protein A) [51-53] and NalP (Neisserial autotransporter lipoprotein) [42]. With the exception of NalP that, as mentioned above is an autotrasporter, all the other SLPs require the support of outer membrane proteins for their surface localization.

1.2.3 Lipoproteins Outer membrane localization system

Mature lipoproteins are localized either in the inner or the outer membrane depending on sorting signals. In *E. coli*, and in general in the *Enterobacteriacee* family, the signal for IM retention is the presence of an aspartate in position +2 after the lipidated cysteine (**Figure 3**.A) [54, 55]. This signal is not conserved across bacterial families and for example in *P. aeruginosa* the amino acid in position +3 and +4 play a role in the localization [56].

In *E. coli*, and in general in all Gram negative bacteria, mature lipoproteins are translocated to the OM by the Lol (Lipoprotein outer membrane localization) pathway [57] (**Figure 3**.A). Even if the signal for the correct localization of the lipoproteins is not completely elucidated, the Lol pathway is highly conserved among bacteria. In *N. meningitidis*, homologous proteins to the *E. coli* Lol system were identified [58] but, contrarily to *E. coli*, lipoproteins can reach bacteria surface thanks the newly identified translocator component Slam (Surface lipoprotein assembly modulator) [59] (**Figure 3**.B). The signals required for lipoproteins localization in *N. meningitidis* are yet to be understood.

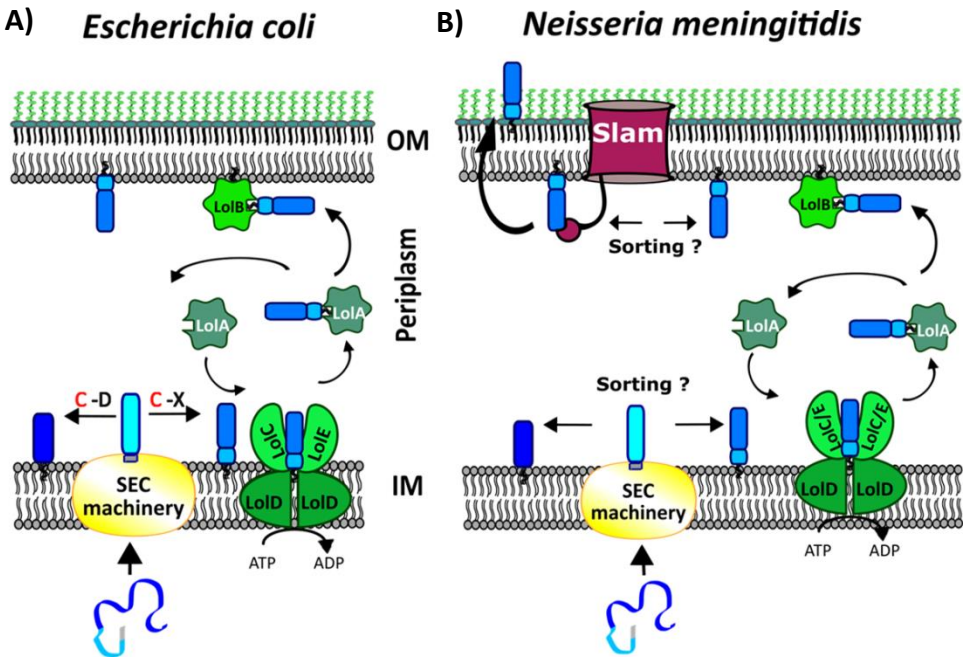

Figure 3 Lipoprotein outer membrane mechanisms. Schematic representation of the differences between *E. coli* and *N. meningitidis*.

In general the Lol system is composed by three different components: an ABC transporter (Lol CDE), a chaperonine (LolA) and a lipoprotein (LolB) [30].

The ABC transporter LolCDE complex is involved in the first step of the translocation process, and is present at the level of the inner membrane. In *E. coli,* LolC and LolE are integral membrane proteins, encoded by two different genes with high levels of homology and form a heterodimer. On the contrary, in *N. meningitidis,* only one gene encoded for the *LolC/E* gene homolog was identified, therefore the complex is composed by a LolC/E homodimer instead a LolC/LolE dimer [58]. This complex captures the lipoprotein from the IM through conformational changes. The complex LolCE-lipoprotein interacts with LolD, which hydrolyzes ATP to support the transfer of the lipoprotein from LolCE to the hydrophobic cavity of the chaperonin LolA and the consequent release in the periplasm of the LolA:Lipoprotein complex [60].

LolA and LolB show a similar structure, they are folded in an incomplete β-barrel which forms a hydrophobic cavity that provides a binding site for lipoproteins acyl chains [61], this symmetrical structure of LolA and LolB are important for transferred lipoprotein. When the LolA:Lipoprotein complex reaches the OM the acyl chains are then transferred to LolB which mediates the anchoring of the lipoprotein to the inner leaflet of the OM [62].

1.2.4 Surface Lipoprotein assembly modulator family

Recently *Hooda et al.* have identified the new class of translocator component localized in the outer membrane involved in the localization of specific meningococcal SLPs [59] (**Figure 3**.B). This new class of translocator component, the Slam (Surface lipoprotein assembly modulator) family, was identified throughout the Proteobacteria phylum and in some bacteria more than one Slam component were identified [63].

Slam proteins are characterized by the presence C- terminal domain folded with β-strands in a β-barrel which spans the outer membrane and an N-terminal soluble domain containing TPR-repeats [59]. Often, Slam genes are localized close to lipoprotein genes. In *N. meningitidis* two different Slam were identified, Slam1 and Slam2 which show different specificity for lipoprotein translocation. *Slam2* gene was found in proximity of *HpuA* gene, and further analysis revealed that Slam2 specifically translocates only HpuA on bacterial surface. Conversely, no lipoproteins were found in proximity of the *Slam1* gene and many different substrates were identified. fHbp, TbpB and LpbB were specifically translocated on bacterial surface by Slam1 [59].

All the Slam family substrates identified so far are characterized by a common predicted structural folding consisting of a β-barrel domain and a β-rich handle domain [63].

1.3 Outer membrane vesicles

Outer membrane vesicles (OMVs) are small spherical blebs naturally released by Gram-negative bacteria, both pathogenic and commensal [64]. Generally described as vesicles with 20-250 nm diameters, OMVs are produced during active growth. Their generation depends on the detachment of the OM from the underlying peptidoglycan structure, so that a portion of OM can extrude and finally be released from the cell. Being liberated from the outer membrane they are mainly formed by Outer Membrane Proteins (OMPs), lipopolysaccharide (LPS), phospholipids, periplasmic proteins and lipoproteins and only few cytoplasmic proteins were found [65, 66] (**Figure 4**), suggesting that OMVs biogenesis is a deliberate process rather than a stochastic event due to cell lysis [67].

OMV production was reported both *in vitro* as well as in *in vivo* conditions [68, 69] and, in the past ten years, the biological role of OMVs was deeply investigated. Yet, for the moment, there is not a single picture that explains all the functional or mechanistic aspects of OMVs production.

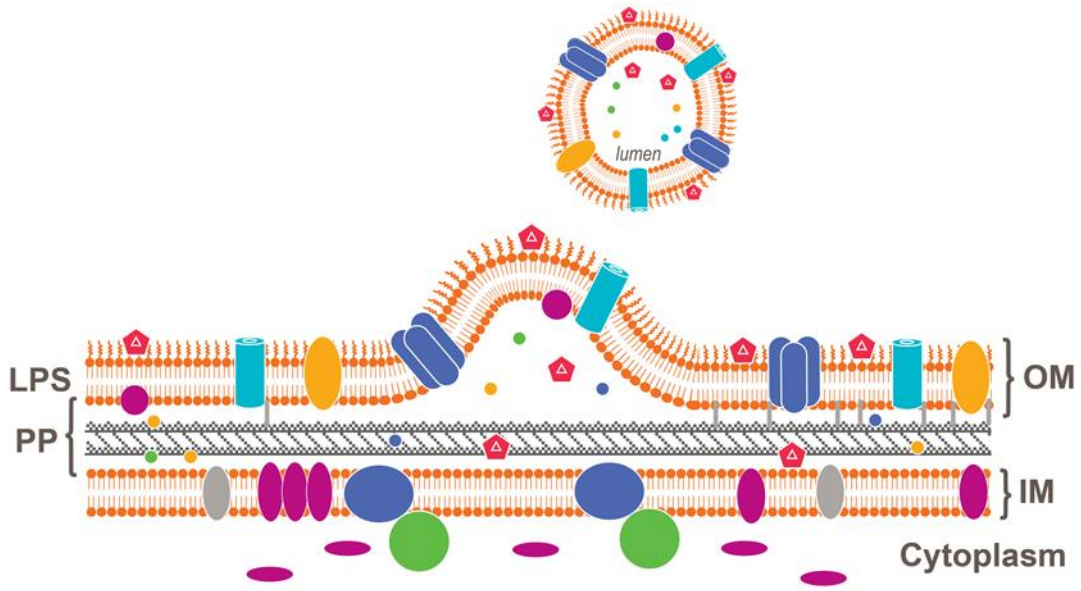

Figure 4 Model of Outer Membrane Vesicles (OMV) biogenesis. Outer membrane protrusion liberates the membrane from the underlying peptidoglycan layer and OMVs vesicles are release. Vesicles are proteoliposomes consisting of OM phospholipids and LPS, a subset of OM proteins and periplasmic (luminal) proteins. (LPS) Lipopolysaccharide; (Pp) periplasm; (OM) outer membrane; (IM) inner membrane.

OMVs are indispensable for bacteria and contribute either to bacterial survival or virulence. In the literature, various roles of OMVs in bacterial physiology and pathogenesis were reported [70]. They can be utilized from the bacterium to improve bacterial survival or to induce changes in the environment, including the host and the surrounding bacterial community.

It is reported that under stress conditions, bacteria increase OMVs production and this mechanisms could help preventing the accumulation of misfolded proteins and their toxic effect [71]. OMVs contribute to innate bacterial defenses conferring protection against antibiotics and antimicrobial peptides [72]. OMVs are also identified so far as important delivery systems. We can list different types of molecules found on OMVs ranging from proteins to saccharides and nucleic acids [67, 73, 74]. Last but not least, OMVs released from pathogenic bacteria play important roles in the delivery of toxins and virulence factors and can also be involved in nutrient acquisitions and interaction with host cells [75, 76]. They are often considered a non-replicative representation of the parent bacterium. OMVs contain innate immuno-stimulatory properties able to facilitate activation of the immune system [77], and OMVs purified from several pathogens are able to induce protective immune responses against the pathogen from which they are purified[74, 76-79]. Furthermore, thanks to the ability to genetically engineer the OMVs-producing bacteria, this system proves to be versatile and can be exploited as a vaccine platform [80, 81].

2 Aims

The main aim of this project is the characterization of *N. meningitidis* lipoprotein translocation system; with a particular focus on the outer membrane crossing and surface localization.

In particular this work is based on the investigation of different topics:

- Investigation of Neisserial lipoproteins distribution to identify possible sorting signals involved in lipoproteins localization.
- Investigation of whether different lipoproteins can be translocated onto the Neisserial surface in a Slam1-dependent manner.
- Evaluation of the immunogenicity in mice of OMVs obtained by modulating SLPs surface exposure in *N. meningitidis*.
- Employment of *E. coli* as a heterologous system to express Neisserial immunogenic lipoproteins on the surface and evaluation of the ability of the resulting OMVs to induce a functional immune response.

Chapter One

Slam1 translocates NHBA and Mip onto the meningococcal surface and influences OMV immunogenicity

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The Neisseria genus contains *N. meningitidis* and *N. gonorrhoeae ,* two strictly human pathogens present at level of mucosal surface such as the nasopharynx or genital epithelium [82]. In *N. meningitidis* and *N. gonorrhoeae* eight different lipoproteins were experimentally identified on the bacterial surface [49] and most of them are virulence factors, including fHbp (only surface exposed in meningococcus) [19], NHBA [50], TbpB , LpbB , Mip (Macrophage infectivity potentiator) [83] and HpuA [51-53]. Many of these SLPs, except for Mip, show a conserved β-barrel domain in the C-terminal portion, suggesting the presence of a common ancestor [84-86]. Recently a class of OMP, named Surface lipoprotein assembly modulator (Slam), was identified as essential for the surface localization of specific SLPs in *N. meningitidis* [59]. This new class of translocator was identified throughout the *Proteobacteria* phylum and in some of them more than one Slam were identified using Slam1 sequence from MC58 as query [63] .

In *N. meningitidis* two different Slam were identified Slam1 which can translocate different SLPs as fHbp TbpB and LpbB on bacterial surface; and Slam2 which specifically translocate Neisserial HpuA on bacteria surface [59].

One of the aims of this work was to elucidate sorting signals for lipoprotein localization in *N. meningitidis*. To this end Neisseria lipoproteins were predicted in MC58 *N. meningitidis* strain and analyzed in order to identify signals for IM/OM localization or necessary for surface translocation. Secondly, we investigated if other meningococcal SLPs are translocated by Slam1 onto the bacterial surface and finally we evaluate, how different SLPs exposure, through Slam1 modulation, can affect immunogenicity of meningococcal OMVs in mice.

3 Results

3.1 Neisseria species require different signals, compared to *E. coli*, for the correct lipoprotein distribution

In other bacterial species lipoproteins are localized either to the inner membrane or the outer membrane depending on the presence of signals within the amino acid sequence [29, 56].

In particularly *E. coli* signals involved in correct localization of lipoproteins have been deeply investigated in the past. Based on the presence of the lipobox motif, 2% of the coding sequence are predicted to be lipoproteins in K-12 *E. coli* reference strain [31]. An aspartate in position +2 after the lipidated cysteine was found to be responsible for the retention to the IM [87] in *E. coli*, the retention signals were further investigates and the "+2 rule" was confirmed as guide principle although is not universal in Gram negative bacteria [57].

In *N. meningitidis* signals involved in lipoproteins localization were not yet investigated therefore to elucidate possible signals we performed a systematic analysis of all predicted lipoproteins within the MC58 Neisserial genome by using Lipo P, which is an online server that produces a prediction of lipoproteins based on their specific signal peptide sequence and can discriminate it from other signal peptides [34]. In *N. meningitidis* the number of lipoproteins found (Table 1), represents 3.3 % of all predicted ORFs which is a higher percentage of lipoprotein content in this species as compared to *E. coli* in which the percentage is around 2% either pathogenic (EHEC 0157:H7 1,84%) and non-pathogenic (K-12 2.02 %) strains [31].

Table 1: List of predicted lipoproteins In table the predicted list of lipoproteins with the amino acid sequences long more than 100 amino acids

	GENE number	Gene	Product
1	NMB0032	-	hypothetical protein
2	NMB0033	Mlt-A	membrane-bound lytic murein transglycosylase A
3	NMB0035	-	hypothetical protein
4	NMB0044	pilB	trifunctional thioredoxin/methionine sulfoxide reductase A/B protein
5	NMB0071	ctrA	capsule polysaccharide export outer membrane protein
6	NMB0086	-	hypothetical protein
7	NMB0204		lipoprotein, putative smpA
8	NMB0278	dsbA-1	thiol:disulfide interchange protein DsbA
9	NMB0294	dsbA-2	thiol:disulfide interchange protein DsbA
10	NMB0375	mafA-1	mafA protein
11	NMB0460	tbp2	transferrin-binding protein B
12	NMB0532	htrA	protease Do
13	NMB0550	dsbC	thiol:disulfide interchange protein DsbC
14	NMB0563	apbE	thiamine biosynthesis lipoprotein ApbE
15	NMB0623	potD-2	spermidine/putrescine ABC transporter substrate-binding protein
16	NMB0652	mafA-2	mafA protein
17	NMB0703	comL	competence lipoprotein
18	NMB0707	-	rare lipoprotein B
19	NMB0787	-	amino acid ABC transporter substrate-binding protein
20	NMB0841	-	hypothetical protein
21	NMB0873	lolB	outer membrane lipoprotein LolB
22	NMB0923	-	cytochrome c
23	NMB1010	-	hypothetical protein
24	NMB1017	sbp	sulfate ABC transporter substrate-binding protein
25	NMB1047	-	hypothetical protein
26	NMB1107	-	hypothetical protein
27	NMB1124/1162	-	hypothetical protein
28	NMB1125/1163	-	hypothetical protein
29	NMB1126/1164	-	hypothetical protein
30	NMB1162/1124	-	hypothetical protein
31	NMB1163/1125	-	hypothetical protein
32	NMB1164/1126	-	hypothetical protein
33	NMB1213	-	lipoprotein

	GENE number	Gene	Product
34	NMB1279	-	membrane-bound lytic murein transglycosylase B
35	NMB1309	-	fimbrial biogenesis and twitching motility protein
36	NMB1335	creA	creA protein
37	NMB1369	-	hypothetical protein
38	NMB1433	-	hypothetical protein
39	NMB1434	plpD	phopholipase D-family protein
40	NMB1468	-	hypothetical protein
41	NMB1470	-	hypothetical protein
42	NMB1483	NlpD	lipoprotein NlpD
43	NMB1533	-	outer membrane protein
44	NMB1541	lbpB	lactoferrin-binding protein
45	NMB1567	mip	macrophage infectivity potentiator
46	NMB1578	-	hypothetical protein
47	NMB1592	-	lipoprotein
48	NMB1594	potD-3	spermidine/putrescine ABC transporter substrate-binding protein
49	NMB1612	-	amino acid ABC transporter substrate-binding protein
50	NMB1620	-	hypothetical protein
51	NMB1623	pan1	major anaerobically induced outer membrane protein
52	NMB1674	-	GDSL lipase
53	NMB1714	mtrE	multidrug efflux pump channel protein
54	NMB1716	mtrC	membrane fusion protein
55	NMB1811	pilP	pilP protein
56	NMB1870	fHbp	hypothetical protein
57	NMB1880	-	ABC transporter substrate-binding protein
58	NMB1898	mlp	lipoprotein
59	NMB1946	-	outer membrane lipoprotein
60	NMB1949	-	soluble lytic murein transglycosylase
61	NMB1969	NalP	serotype-1-specific antigen
62	NMB1989	-	iron ABC transporter substrate-binding protein
63	NMB2091	-	hemolysin
64	NMB2132	NHBA	transferrin-binding protein-like protein
65	NMB2139	-	hypothetical protein

In order to see if any specific amino acid motifs emerged the first 50 amino acids, including the signal peptide, from the sequences in the predicted list of lipoproteins were aligned around the conserved cysteine (+1) of the lipobox and a consensus sequence was visualized using WebLogo (**Figure 5**) [88]. As expected, the Leucine (L) and the Cysteine (C) of the lipobox motif were conserved in all sequences while, interestingly, looking at the residue in position +2 Glycine (G) and Serine (S) were the most abundant residues among the analyzed sequences.

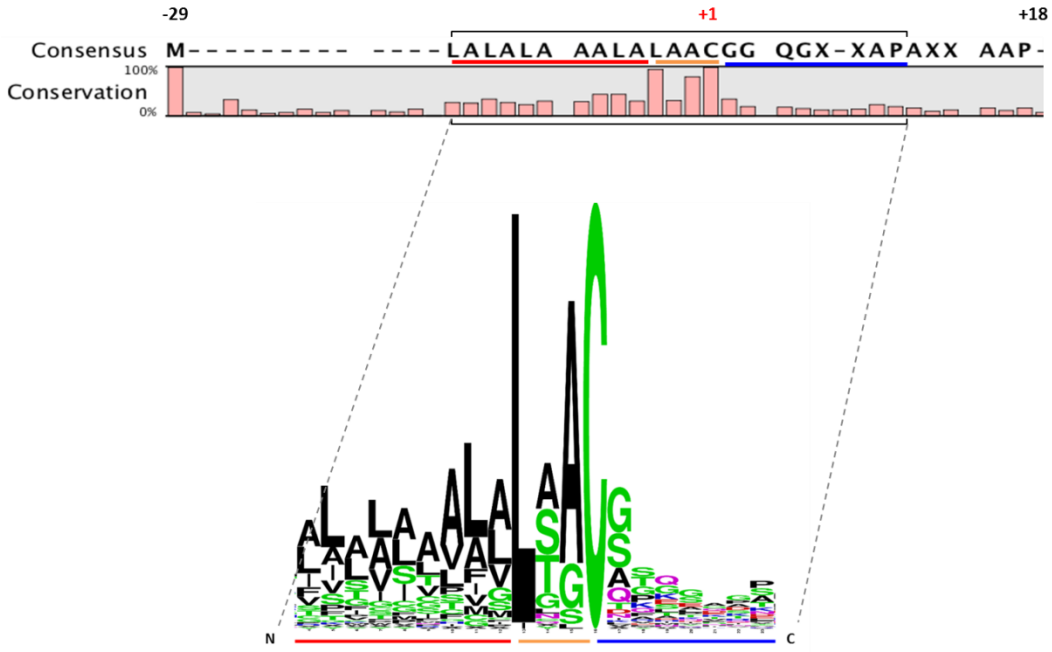
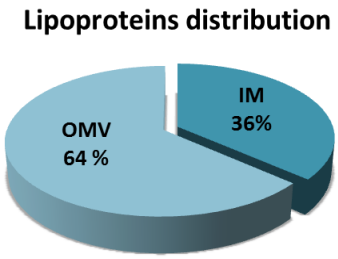


Figure 5 Lipoproteins alignment. **A)** The first 50 amino acids of *N. meningitidis* predicted lipoprotein were aligned and a LOGO was generated by using WebLogo. Sequence conservation, frequency and characteristics of each amino acid (aa) at each position are shown. Colors are representative of the aa characteristics: Green, polar (G, S, T, Y, C, Q, N); Blue ,basic (K, R, H); Red, acid (D, E); Black, hydrophobic (A, V, L, I, P, W, F, M).

For some lipoproteins the localization was known to be in the outer membrane, but for the major part of the lipoproteins the localization was not described in literature. To further understand the possible distribution of lipoproteins between the inner or outer membranes, we combined the proteomic analysis available in the literature for purified OM and spontaneously released OM blebs [66]. Based on their presence in OMVs or OM preparations, we presumed that these lipoproteins were anchored to the OM and hence, by exclusion, we assumed other lipoproteins were retained in the IM. As is shown in **Figure 6**, the majority (64%) of the lipoproteins could be found in the OM preparations.

Looking at the aminoacid in position +2 it appears that the "+2 rule" is not applicable in *N. meningitidis*, as an aspartate (D) was present at the +2 position only in two lipoprotein sequences and they were detected either in the IM or OM. Secondly we were not able to assign and correlate a specific aminoacid in position +2 with the localization in the IM or OM. All the amino acids in position +2 were found both in the IM and OM, suggesting that other signals in other position of the sequences were involved in the retention to the inner membrane.



AA in position +2	Number of proteins	OMV positive	IM positive
Glycine	26	14	12
Serine	15	10	5
Alanine	7	5	2
Glutamine	6	4	2
Threonine	3	3	0
Aspartate	2	1	1
Leucine	2	2	0
Asparagine	1	1	0
Isoleucine	1	1	0
lysine	1	1	
Valine	1	1	
TOT	**65**	**42**	**23**

Figure 6: Lipoproteins distribution in *N. meningitidis*. Lipoprotein distribution based on proteomic data were summarized in the pie chart. In the table is reported the frequency of the amino acid in position +2 and relative distribution among the inner membrane (IM) or outer membrane as OMVs positive (OMVs).

3.2 The C- Term β-barrel domain is well conserved among *N. meningitidis* SLPs, and seems to be important for the surface localization

As mentioned above in the introduction, in *N. meningitidis* some lipoproteins are known to be surface exposed, including HpuA, TbpB, LpbB, fHbp and NHBA. Despite the low sequence similarity, these SLPs show conserved structure at the C-terminal, which is stably folded in an 8-stranded β-barrel domain. Since the MC58 *N. meningitidis* strain does not express HpuA only 4 SLPs structures were reported (**Figure 7**).

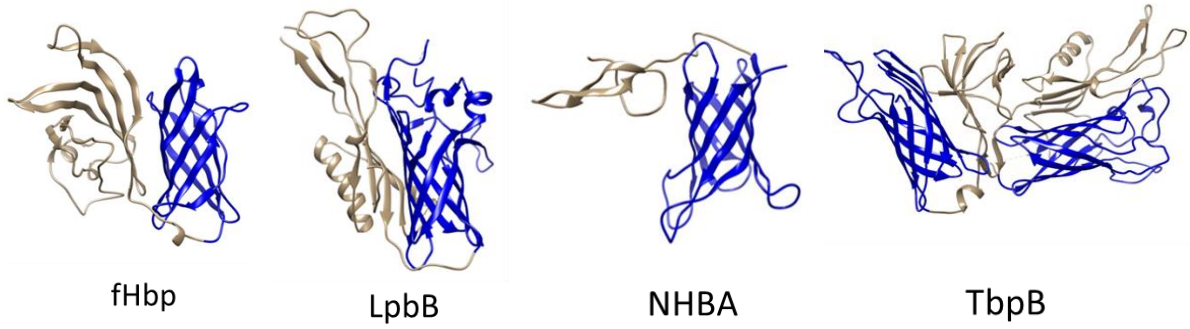


Figure 7 Structures of known MC58 SLPs. Modeled structures of four *N. meningitidis* SLPs were represented. Using Pymol program, β- barrel domains were highlighted in blue. TbpB and LpbB possess two copies of β-barrel domains, but for LpbB only the C-lobe was reported.

To investigate if this recurrent β-barrel domain was important for the surface localization we used NHBA as a model to study the surface translocation. It has been described that NHBA contains two cleavage sites upstream of the C-terminal domain and as such this protein is naturally present on the surface as two truncated forms lacking the C-terminus [50]. We generated a set of recombinant strains in which the genes encoding either the wildtype (WT) protein or two truncated forms of NHBA lacking the C-terminal domain (S1TGA1 and S2TGA2), generated by mutagenesis of the serine cleavage sites with a stop codon (**Figure 8.A**) were expressed under the control of an IPTG-inducible promoter, as described in the material and method session (7.2.1). These constructs were then inserted into the chromosome of the MC58 *ΔNHBA* background by *ex-locus* complementation. Bacteria were grown in GC medium with 0,1mM IPTG until exponential phase, then bacterial total lysates and fixed bacteria were collected and analyzed for NHBA expression and surface localization by Western blot and FACS, respectively (**Figure 8.B** and **C**).

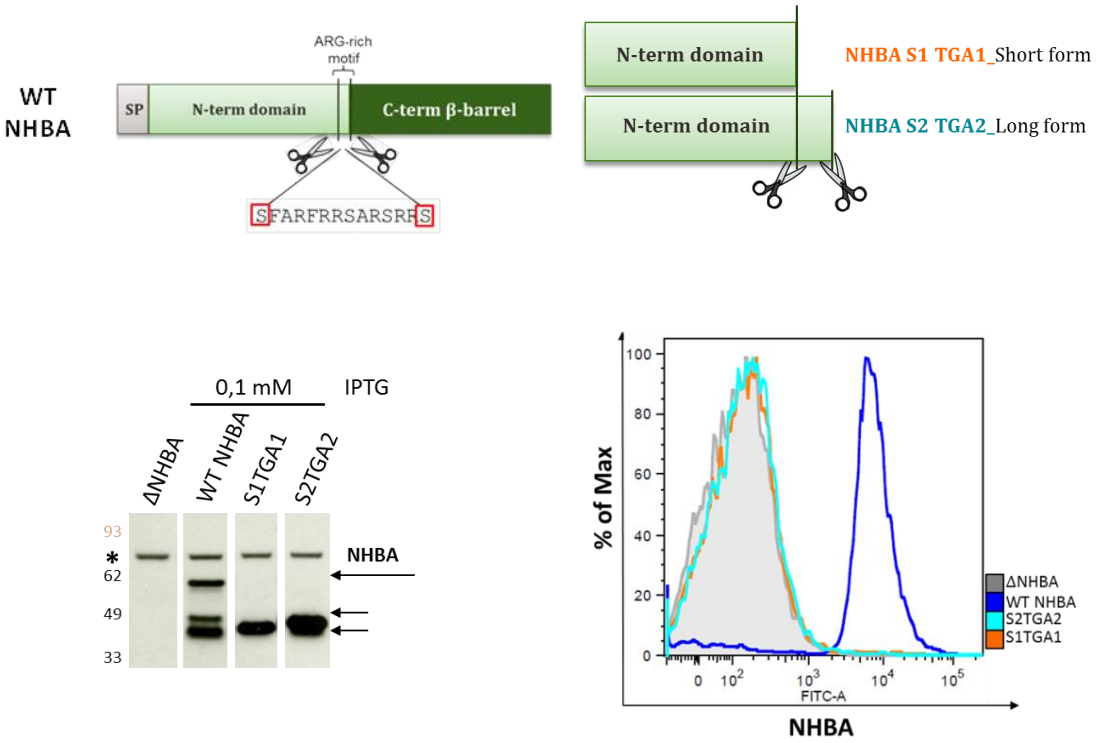


Figure 8. The C-terminal of NHBA is important for surface localization. A) Schematic representation NHBA truncated forms cloned and expressed in *N. meningitidis* MC58 *ΔNHBA* strain. **B)** Western blot analysis. Total lysates collected were loaded on a SDS gel and transferred on nitrocellulose membrane, stained with polyclonal sera α-NHBA (1:2000). Arrows highlighted the different NHBA forms. **C)** FACS analysis on fixed bacteria, they were first stained with α-NHBA polyclonal sera (1:800) and subsequently with FITC-labeled anti-mouse secondary antibodies (1:1000). * aspecific band.

Western blot analysis confirmed the expression of wild-type gene as the full length protein (higher band) and the two cleaved forms (lower bands). Each truncated NHBA gene was stably expressed and detectable (**Figure 8.B**). In the S1TGA1 truncated form only one band, corresponding to the lower N-terminal truncated band in the WT was visible. In the S2TGA2 truncated form the major band corresponds to the longer N-term truncated form and a less abundant band corresponding to the short form were visible.

NHBA surface localization was analyzed by FACS (**Figure 8.C**) using polyclonal α-NHBA sera. No NHBA was detectable on the surface in both strains expressing the truncated forms, while WT NHBA was clearly surface exposed in MC58 WT strain. This suggests that in the absence of the C-terminal β-barrel, the N-terminal domains are expressed but not translocated to the surface.

To further investigate the role of the C-terminal domain in surface translocation; we generated a fusion protein in which the β-barrel portion of NHBA was substituted with the β-barrel of fHbp and we tested if the structure was enough to restore the delivery of N-terminal portion of NHBA onto the bacterial surface. A second fusion protein in which the N-terminal portion of fHbp was fused with the C-terminal portion of NHBA was also generated (**Figure 9.A**). These fusion constructs were cloned under the control of an IPTG inducible promoter and incorporated into the chromosome of MC58 *ΔNHBA ΔfHbp* background by *ex-locus* complementation. Bacterial strains were grown until exponential phase in GC media in the presence of 0,1mM of IPTG, fixed bacteria and total lysates were collected, and protein expression and surface localization were analyzed by Western Blot and FACS respectively.

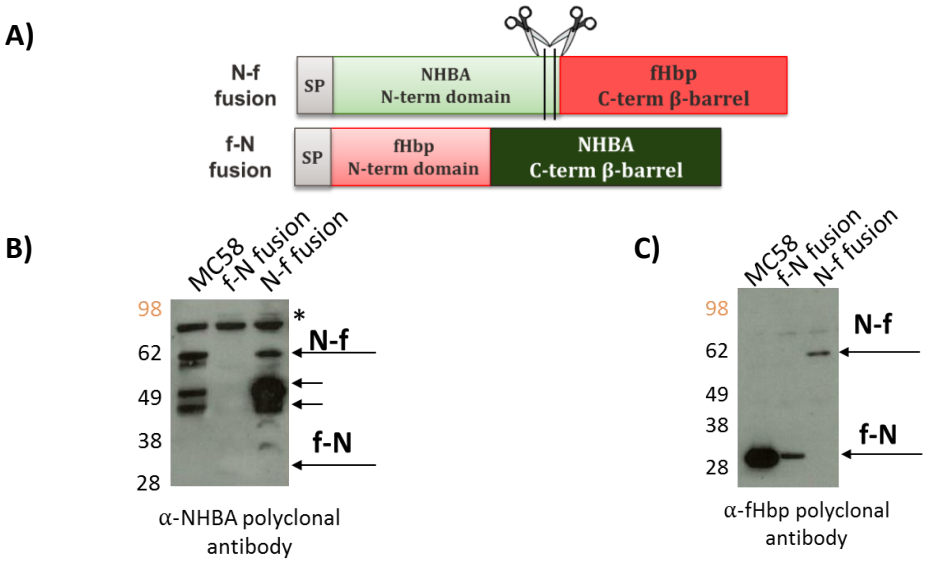


Figure 9. **N-f and f-N fusion proteins were stably expressed**. **A)** Schematic representation of the cloned fusion proteins. N-f fusion (N-term NHBA with the C-term of fHbp), f-N (N-term of fHbp with the C-term of NHBA). Western blot analysis of total lysates collected were loaded on a SDS gel and transferred on nitrocellulose membrane, stain was performed with both **B)** α-NHBA polyclonal sera (1:2000) and **C)** α-fHbp polyclonal sera. Arrows highlighted fusion proteins. * aspecific band

Total cell extracts of MC58 WT and the recombinant strains expressing the f-N and N-f fusion proteins were analyzed by WB and samples were analyzed either with α-fHbp and α-NHBA polyclonal sera (**Figure 9.B** and **C**). Western blot confirmed the expression of both chimeric constructs. Strangely the f-N construct was poorly recognized by Western Blot with anti-NHBA antisera, and reasons for this were not further explored, however, both chimeric proteins were successfully detected by anti-fHbp antisera and it was possible to appreciate the differences in the molecular weight between the WT, the f-N and the N-f protein due to the different N-terminal portions exchanged among fHbp and NHBA.

Surface exposure of these fusion proteins was analyzed by FACS (**Figure 10**).

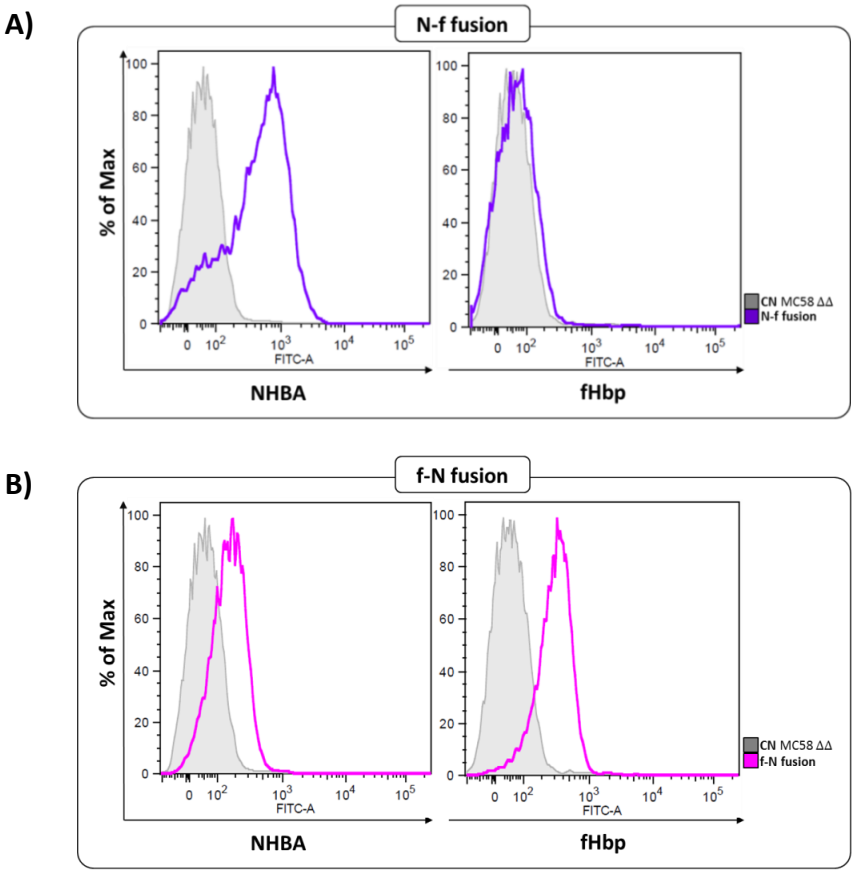


Figure 10 C-terminal β-barrel is required for N-terminal domain surface tranlsocation. FACS analysis on fixed bacteria were performed with both α-fHbp (1:1000) and α-NHBA polyclonal sera (1:800) and subsequently with FITC-labeled anti-mouse secondary antibodies. **A)** N-f fusion was detectable only with α-NHBA sera, because fHbp portion was cleaved out. **B)** f-N was detectable on the surface by both sera.

In the N-f fusion surface expression was confirmed using the α-NHBA polyclonal sera confirming its ability to be translocated onto the surface likely due to the presence of the β-barrel from fHbp. Instead the fHbp C-terminal is not readily recognized on the surface possibly due to its cleavage and release in the supernatant because the two serine cleavage sites are included in the NHBA N-terminal portion (**Figure 10.A**). The f-N fusion was detectable on the bacteria surface either with α-NHBA and α-fHbp polyclonal sera (**Figure 10.B**) confirming the expression and the presence of NHBA β-barrel domain, which was not detectable by Western blot with α-NHBA polyclonal sera (**Figure 8.A**). Expression levels detectable on the bacteria surface of f-N fusion suggest that the reason why f-N fusion was not readily detectable by Western blot using the NHBA antisera might be due to the low levels of the expression reached.

In summary these data suggest that the C-terminal β-barrel domain is necessary for the delivery on the surface of the N-terminal portion of the analyzed lipoproteins and that the β-barrel of NHBA or fHbp is functionally interchangeable in this necessary role.

3.3 Surface lipoproteins assembly modulator 1 (Slam1) expression is conserved among *N. meningitidis* strains

Slam1 was recently identified from Hooda and coworkers as the key component for the surface localization of specific SLPs in group B *N. meningitidis* [59]. The sequence of Slam1 from MC58 was cloned into an expression plasmid and after recombinant expression in *E. coli*, the protein was purified from the inclusion bodies (IB) as described in material and methods (7.4). Purified protein was loaded on SDS gel page (**Figure 11**) and colored with coomassie safe blue staining and its purity was estimated as >90%. The eluted protein was concentrated ten time and then used to immunize mice for the generation of polyclonal sera for use in further experiments.

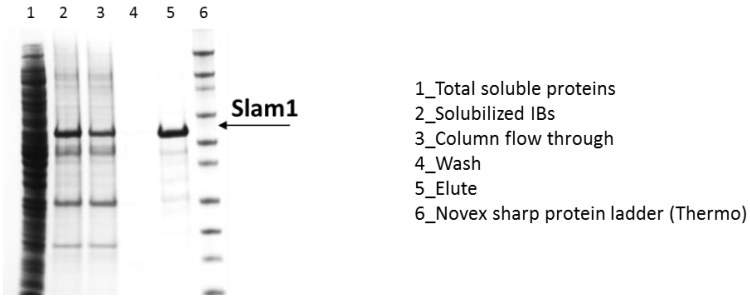


Figure 11 Recombinant Slam1 protein purification. SDS Gel page

In order to characterize better the function of this protein, the *Slam1* gene was deleted in the MC58 strain. The knockout (ΔSlam1) was obtained by replacing the *Slam1* gene with an antibiotic resistance cassette as described in the material and methods section (7.2.1). Western Blot analysis on total lysates of the MC58 (WT) and the ΔSlam1 strains as well as on outer membrane vesicles (OMVs) from MC58 stained with α-Slam1 polyclonal sera (**Figure 12.A**) indicated the correct deletion of the gene in *ΔSlam1* strain, and as expected Slam1 was present in the OMV preparation confirming its presence in the outer membrane. Interestingly while Slam1 was in the OMVs, FACS analysis with α-Slam1 antibodies was negative, suggesting that Slam1 is not exposed enough to be recognized on the surface by the antibodies (**Figure 12.B**).

In order to understand whether Slam1 was conserved among different group B meningococcal strains, we expanded our analysis to a broader panel of clinical isolates from different clonal complexes. Strain characteristics are summarized in **Table 2**. As showed in the Western blot, Slam1 was detectable at comparable levels in all the analyzed strains (**Figure 12.B**)

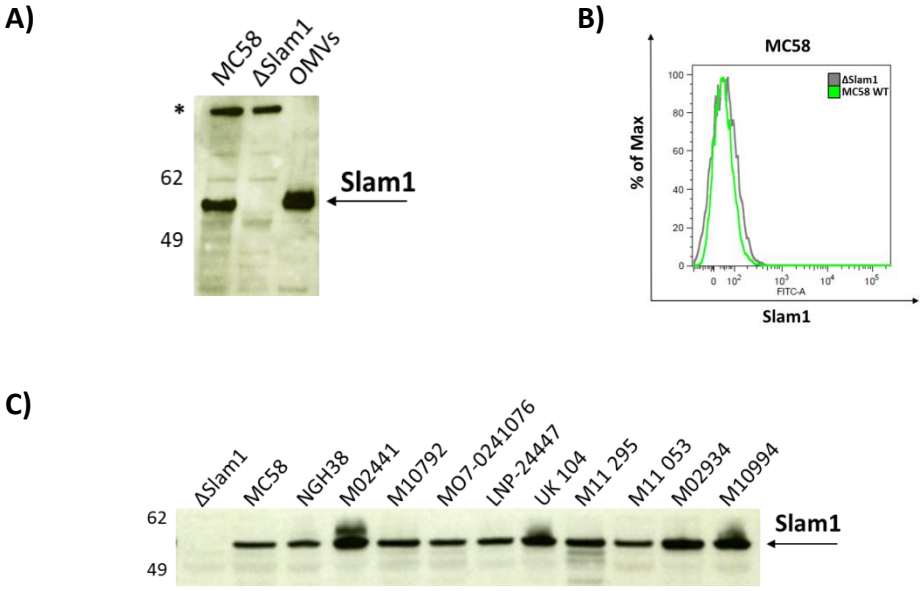


Figure 12 Slam1 expression. A) Western blot anlysis. Total lysate from MC58 WT, ΔSlam1 and 1µg of MC58 OMvs preparation were loaded on SDS gel page and trasferred on a nitrocellulose membrane, stained with polilclonal sera α-Slam1 (1:1000). **B)** FACS analysis on fixed bacteria, they were first stained with α-Slam1 policlonal sera (1:1000) and subsequently with FITC-labeled anti-mouse secondary antibodies (1:1000). **C)** Western blot with α-Slam1 polyclonal sera (1:1000) on a panel of group B *N. meningitidis* strains.

Table 2 *N. meningitidis* panel of strains tested for Slam1 expression with characteristics

	Clonal Complex	Geography	Year	PorA variant	fHbp variant	NHBA variant	Nad A
MC58	ST-32	GBR	1985	7	1.1	p3	+,v1
NGH38	ND	NOR	1976	18-1	2.24	p2	-
M02441	ST-269	USA	1996	12	3.31	p19	-
M10792	ST-11	USA	2003	22.14	2.22	p139	+, ND
M07-0241076	ST-11	GBR	2007	5-1 10.8	2.23	p20	+, v2
LNP-24447	ST-11	FRA	2008	5.2	3.31	p29	+, ND
UK 104	ST-35	GBR	2008	7.2 4	2.16	p21	-
M11053	ND	USA	2003	22.9	2.19	p17	-
M11 295	ST-32	USA	2003	7.16	1.1	p5	+, v1
M02934	ST-32	USA	1996	7.16	1.1	p5	+, v1
M10994	ND	USA	2003	16.2	2.19	p17	-

3.4 Slam1 has a role in the stable expression as well as in the translocation to the surface of fHbp

The MC58 clinical isolate, normally used as a laboratory strain, is characterized by high expression levels of fHbp and low NHBA amount. The Slam1 knock-out was generated also in NGH38 *N. meningitidis* strain, a representative carrier isolate, which expresses high NHBA levels and low fHbp levels. We grew MC58 WT, ΔSlam1 and NGH38 WT, and ΔSlam1 until exponential phase and fixed bacteria and whole cell lysates were collected. OMVs from stationary phase were also collected as described in the material and methods (7.9.1) and included in the Western Blot analysis. FACS analysis on fixed bacteria showed a marked reduction in fHbp displayed on the surface in the ΔSlam1 strains for both MC58 and NGH38, validating the previous results observed from *Hooda et al*. (**Figure 13**)

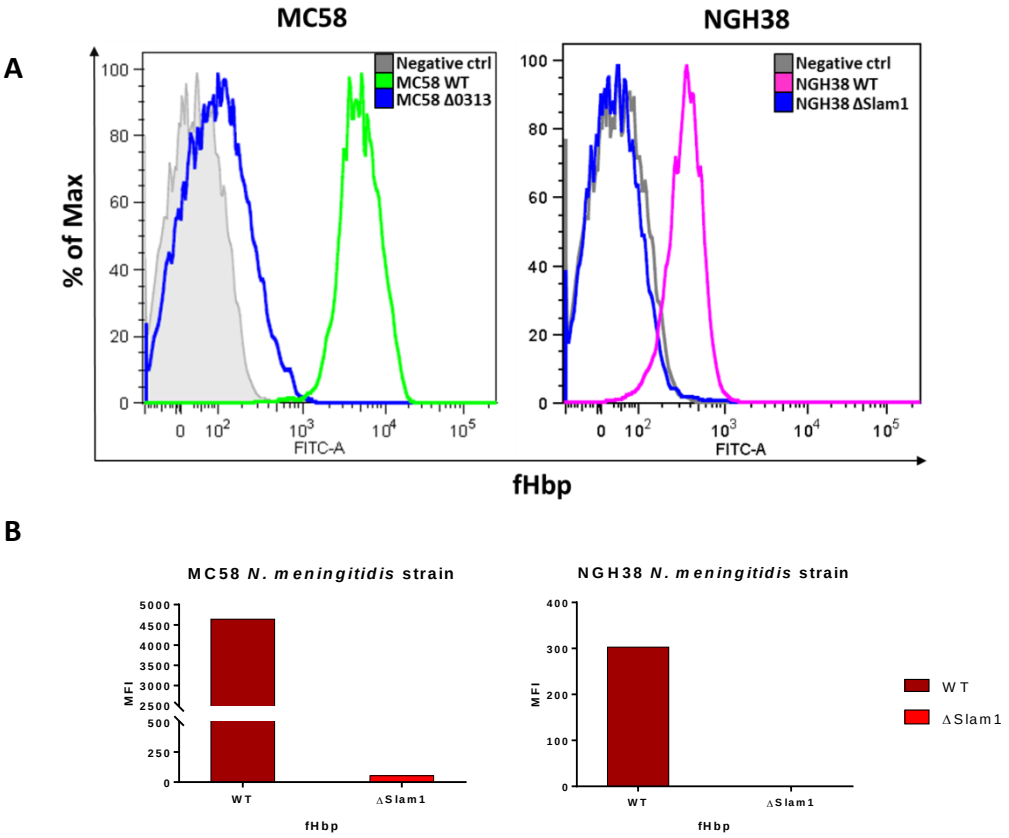


Figure 13 fHbp is not surface exposed in ΔSlam1. FACS analysis on fixed bacteria were first stained with α-fHbp polyclonal sera (1:1000) and subsequently with FITC-labeled anti-mouse secondary antibodies (1:1000). FACS analysis was represented as **A)** histograms and **B)** column graphs with the mean fluorescence intensity. WT strain stained only with 2° Ab was use as a negative control in the analysis

Western blot revealed that the amount of fHbp was strongly decreased in the absence of Slam1 (**Figure 14**). A very slight band was still visible in the MC58 ΔSlam1 total lysate whereas in NGH38 ΔSlam1 fHbp was almost undetectable in the total lysate, probably due to the lower initial fHbp expression levels.

Western blot on OMVs preparation confirmed the differences in fHbp amount between WT and ΔSlam1 was maintained also in the OMVs, furthermore the presence of fHbp in the OM was confirmed. Taken together these data suggest that Slam1 has a critical effect on the amount of fHbp expressed per se and the low amount of fHbp on the surface might be due a general decrease of fHbp amount in the bacteria in the absence of Slam1 and not only to a mislocalization of fHbp.

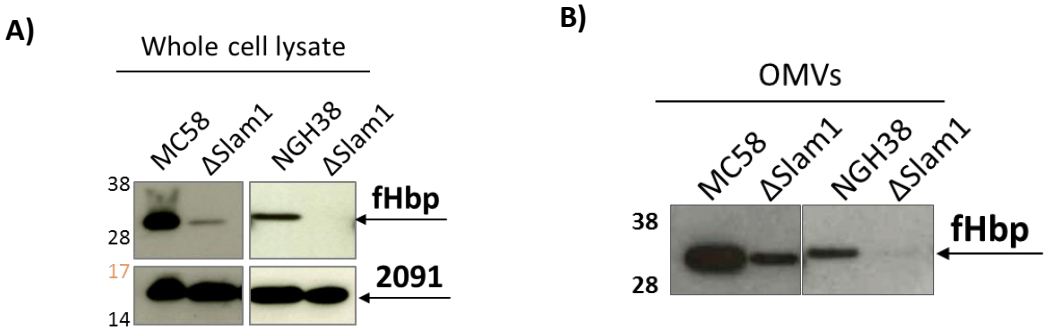


Figure 14 fHbp amount decreased in the ΔSlam1 recombinant strains. Western Blot using α-fHbp polyclonal sera performed WT and ΔSlam1 in MC58 and NGH38 *N. meningitidis* strain on **A)** total lysate derived from bacteria growth in GC liquid media until exphonential phase. **B)** 1μg of OMVs shows the same trend to reduced fHbp amount. Total lysates were stained also with α-2091 used as a loading control.

To further investigate this general reduction of fHbp protein amount in the bacteria, analysis of *fHbp* RNA transcript levels was performed by qRT-PCR. We grew all strains (MC58 and NGH38, WT and ΔSlam1) in GC media until exponential phase and we took samples for RNA extraction and Western blotting.

While there was a slight trend for less RNA in the Slam1 mutants, this reduction was not statistically significant. The differences observed in the RNA transcription levels from WT vs ΔSlam1 were not enough to justify the decrease in fHbp protein amount observed by Western blot analysis (**Figure 15**), this suggests that in the absence of Slam1 there is a greater turnover of the fHbp protein possibly due to an fHbp instability when blocked in the periplasmic space.

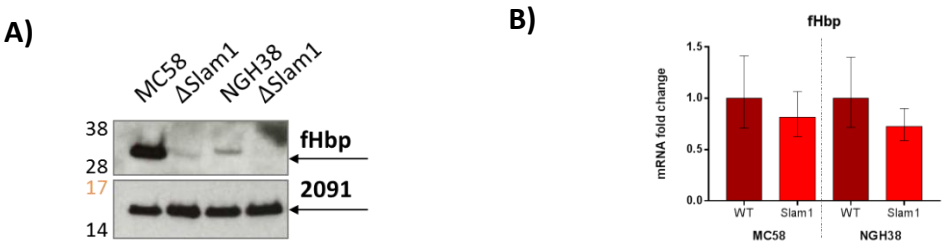


Figure 15 fHbp reduction is not regulated at the transcription level. A) Western Blot using α-fHbp polyclonal sera performed on total lysate derived from bacteria growth in GC liquid media until exphonential phase. Bacteria were stained also with α-2091 as a loading control. **B)** *fHbp* RNA steady state levels were quantified by qRT-PCR and relative expression levels were determined normalizing to *16S-rRNA.* Data set represented is representatives of 2 independent experiments.

3.5 NHBA and Mip are two newly identified substrates for Slam1

In the Neisseria genus, many lipoproteins are known to be surface localized. Some of them were already identified as Slam1 or Slam2 substrates [59], so we decided to check if some of the other identified SLPs were also Slam1 substrates. We focused our attention first of all on NHBA, because it exhibits the same β-barrel structure of the other Slam1 substrates [84], and as shown previously, it was necessary for NHBA surface localization. Secondly we focus our attention on Mip (Macrophage Infectivity potentiator) since it was described to be translocated on the surface in *N. gonorrhoeae* [83], and we checked whether the meningococcal homologue [89] was also surface localized.

We grew MC58 WT and ΔSlam1 in GC medium until 0.5 OD_{600}/ml and samples for Western blotting and FACS analysis were collected. Western blot of MC58 OMVs, WT and ΔSlam1, was also included in the analysis to confirmed the correct delivery in the OM.

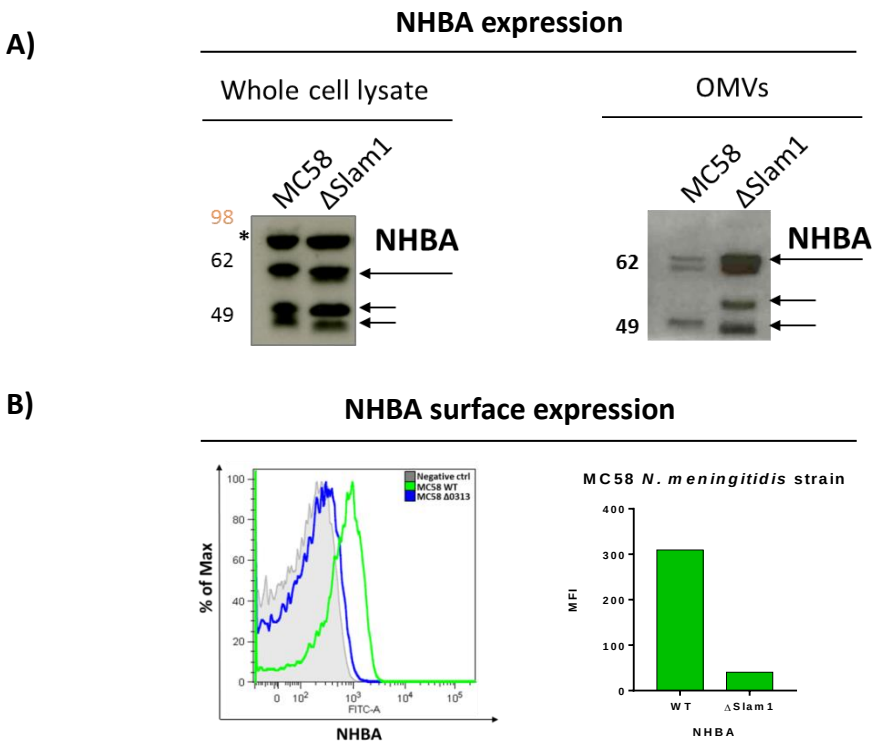


Figure 16 NHBA is translocated on the surface by Slam1 in MC58. **A)** Western blot on the total lysate and 1μg of OMVs were stained using α-NHBA polyclonal sera (1:2000) and confirmed the expression of NHBA and the presence in the OM in both strains. Arrows highligth NHBA full length and the truncated forms. **B)** FACS analyis on fixed bateria bacteria were first stained with α-NHBA polyclonal sera (1:800) and subsequently with FITC-labeled anti-mouse secondary antibodies (1:1000). NHBA was not display on bacteria surface in MC58 ΔSlam1 strain. FACS analysis was visualized both as histogram with the percentage of max and as mean fluorescence. * aspecific band.

Contrary to fHbp, from total lysates there is no difference in the expression of NHBA in the absence of Slam1, and interestingly NHBA is present at higher quantities in OMVs in the Slam1 mutant (**Figure 16.A**). However FACS analysis showed that no NHBA was detectable on the

bacteria surface when Slam1 was not expressed, confirming the role of Slam1 in NHBA surface localization (**Figure 16.B**). This suggests that, in the absence of surface expression in the Slam1 mutant, NHBA appears to accumulate in the OMVs likely due to the absence of turnover from external proteases.

Western blot analysis of the total lysates indicated that Mip was stably expressed either in the WT or in ΔSlam1 and was delivered to the outer membrane as it was present in the OMVs preparation (**Figure 17.A**), but no Mip was detectable on the bacteria surface (**Figure 17.B**), confirming the role of Slam1 also in the translocation of Mip on MC58 bacterial surface.

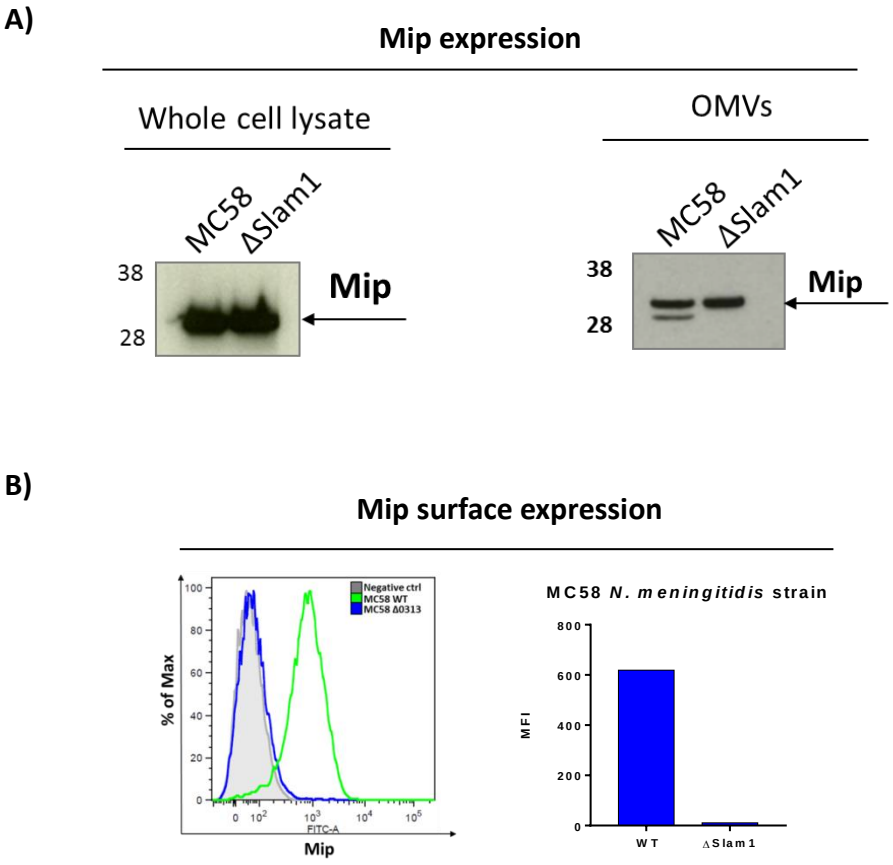


Figure 17 Mip is translocated on the surface by Slam1 in MC58. **A)** Western blot on the total lysate and 1µg of OMVs were stained using α-Mip polyclonal sera (1:1000) confirmed Mip expression in both strains. **B)** FACS analyis on fixed bateria bacteria were first stained with α-Mip polyclonal sera (1:1000) and subsequently with FITC-labeled anti-mouse secondary antibodies (1:1000). Mip was not display on bacteria surface in MC58 ΔSlam1 strain. FACS analysis was visualized both as histogram with the percentage of max and as mean fluorescence.

3.6 Surface exposure of fHbp, NHBA and Mip can be controlled by modulation of Slam1 expression

To further investigate the role of Slam1 in the surface localization of NHBA and Mip we complemented the MC58 Δ*Slam1* strain by inserting a *Slam1* gene under the control of an IPTG-inducible promoter into the genome as described in the material and methods (7.3.1).

MC58 WT, ΔSlam1 and the complemented strain (CiSlam1) were grown in liquid medium in the presence of increasing IPTG concentrations until exponential phase and total lysates for Western blot analysis and fixed bacteria for FACS analysis were collected. We were able to restore Slam1 expression in ΔSlam1 background and to increment Slam1 expression at the high IPTG concentration (0.1 and 1 mM) to higher Slam1 expression compared to WT expression level **(Figure 18**).

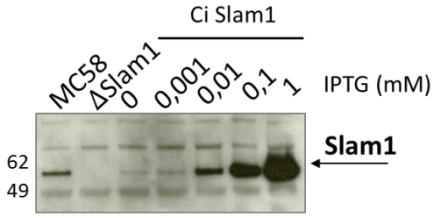


Figure 18 Slam1 can be over expressed in MC58. MC58 WT, ΔSlam1 and CiSlam1 were growth in GC liquid medium in the presence of IPTG (0; 0.001; 0.01; 0.1; 1 mM). Western blot of the total lysate stained with α-Slam1 (1:1000). Slam1 expression was restored in the complemented strain.

From the same samples, Western blot and FACS analysis were performed to observe how fHbp, NHBA and Mip responded to the different Slam1 expression levels (**Figure 19**).

As expected, fHbp expression was not visible in ΔSlam1 mutant strain, whereas its expression was restored at comparable levels to the WT in the complemented strains. From FACS analysis, fHbp surface localization was depending on Slam1 induction and only at the highest IPTG concentration we completely restored fHbp exposure on the surface at comparable levels to the WT (**Figure 19.A**). For Mip, in which no differences in the expression levels were observed in WB, surface localization was depending on Slam1 induction (**Figure 19.B**). Also NHBA surface translocation was depending on Slam1 expression. Furthermore, in contrast with the others SLPs, at the highest IPTG concentration (0.1 and 1 mM of IPTG) we could see higher amounts of NHBA on the surface compared to the WT strain (**Figure 19.C**).

It is worth noting that for NHBA, we observed a different cleavage pattern in the presence of different Slam1 amounts. In the absence of Slam1, or at low levels of induction, we observed a greater cleavage of NHBA, while in the presence of Slam1 (WT or high induction levels) more NHBA full length was present. Interestingly this suggests that we get highly processed when it is not exposed on the surface.

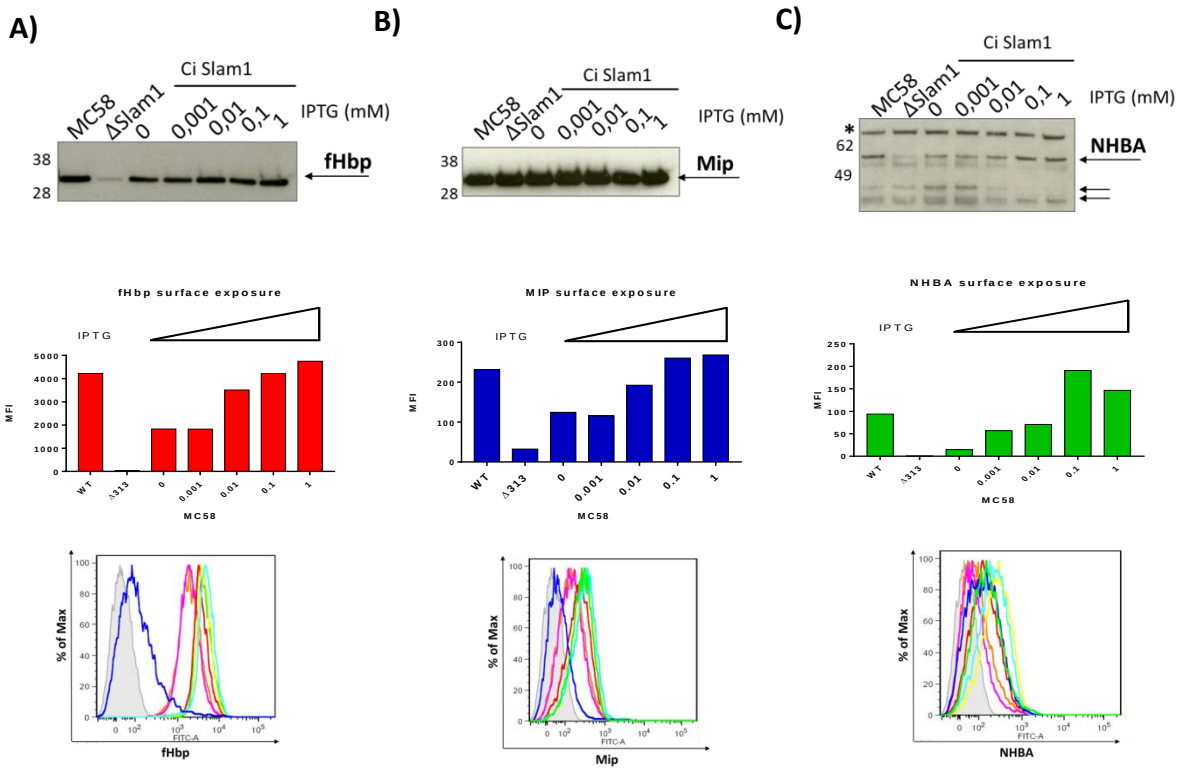


Figure 19 SLPs surface exposure can be modulated by Slam1 expression in MC58. MC58 WT, ΔSlam1 and CiSLam1 strain were analyzed for **A)** fHbp, **B)** Mip and **C)** NHBA expression by WB and surface localization by FACS using FITC α-mouse secondary antibody. FACS analysis were reported as mean fluorescence and as histogram with the percentage of max. Histogram colour key: Negative control (grey,); WT (pink) ΔSlam1 (blue), Ci0313 with IPTG 0 (orange); 0.001 (green); 0.01 (red); 0.1 (yellow); 1(light blue). *aspecific band

We then analyzed how NGH38 *N. meningitidis* strain was responding to Slam1 inductions by restoring expression with complementation in the NGH38 Slam1 mutant (**Figure 20**). NGH38 strains were grown as previously, with different IPTG concentrations in GC liquid media, and samples for Western Blot and FACS analysis were collected.

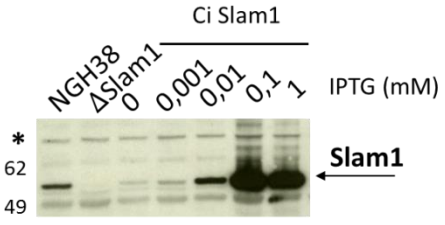


Figure 20 Slam1 can be over expressed in NGH38. NGH38 WT, ΔSlam1 and CiSlam1 were grown in GC liquid medium in the presence of IPTG (0; 0.001; 0,01; 0,1; 1mM). Western blot of the total lysate stained with α-Slam1 (1:1000). Slam1 expression was restored in the complemented strain. * aspecific band

Previous results were confirmed in the NGH38 strain, where fHbp and NHBA surface localization can be modulated by Slam1 induction (**Figure 21**). Furthermore in NGH38 at the highest Slam1 induction the translocation of SLPs on the surface was higher for both fHbp and NHBA as compared to WT.

Interestingly also in NHG38 by Western blot, an inverted trend in the accumulation of the different NHBA forms was visible: decreased amount of NHBA truncated forms was followed by an accumulation of NHBA full length in the presence of Slam1 and the restoration of surface localization.

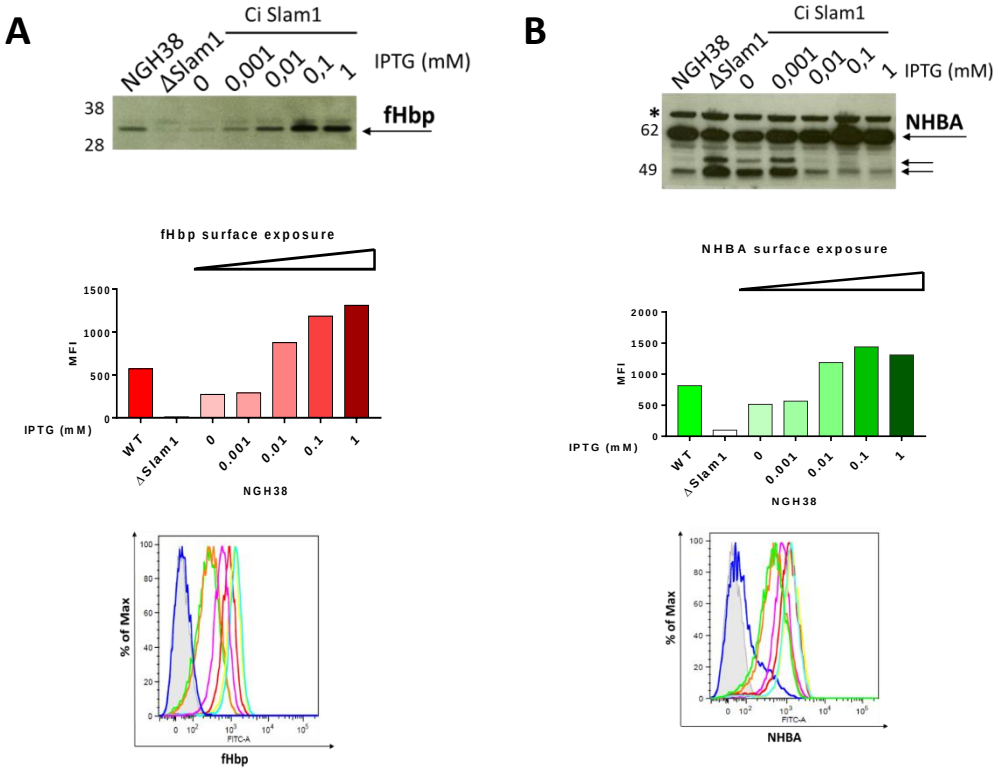


Figure 21 SLPs surface exposure can be modulated by Slam1 expression in NGH38. NGH38 WT, ΔSlam1 and CiSlam1 strain were analyzed for **A)** fHbp and **B)** NHBA expression by WB and surface localization by FACS. FITC α-mouse was used as secondary antibody. FACS analysis were reported as mean fluorescence and as histogram with the percentage of max. Histogram colour key: Negative control (grey,); WT (pink) ΔSlam1 (blue), Ci0313 with IPTG 0 (orange); 0.001 (green); 0.01 (red); 0.1 (yellow); 1(light blue). *aspecific band

3.7 Mip is not translocated to the Outer membrane in NHG38

When we analyzed Mip surface localization in NGH38 strains, surprisingly we noticed that Mip was not detectable on the surface even in the NGH38 WT strain (**Figure 22.A**).

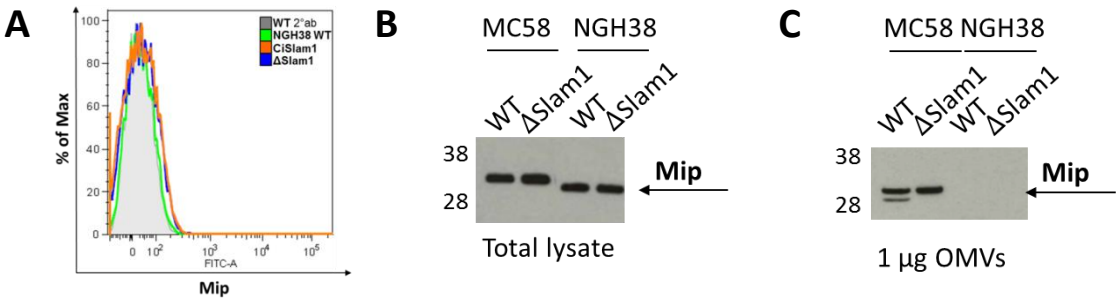


Figure 22 Mip is not translocated to the outer membrane in NGH38. NGH38 strains were grown in GC liquid media until exponential phase, total lysate and fixed bacteria were collected. **A)** Facs analysis of NGH38 with α-Mip polyclonal sera and subsequently with FITC α-mouse secondary antibody. Wester Blot of **B)** total lysate **and C)** 1µg of OMVs stained with α- Mip polyclonal sera.

Western Blot analysis with total lysate from MC58 WT, ΔSlam1 and NGH38 WT, ΔSlam1 stained with α-Mip polyclonal sera, confirmed Mip expression in all strains (**Figure 22.B**). However, from western blot analysis it was also possible to notice that Mip from NGH38 strains showed lower molecular weight compared to Mip expressed in MC58.

Western blot on OMVs from both WT and ΔSlam1 showed that Mip was not present in NGH38 OMVs preparation, suggesting that, in this strain Mip is not translocated to the OM (**Figure 22.C**).

The Mip gene sequence from NGH38 were sequenced and the results indicated that NGH38 (**Figure 23**) has a deletion of four amino acids close to the lipobox motif as well as two point mutations within the coding sequence. The first one was in proximity to the deletion, a Serine (S) was exchanged with an Alanine (A); and the second in the middle of the sequence, in which an Aspartic acid (D) was replaced by a Glutamic acid (E) (**Figure 23**).

Interestingly this suggests that these sequences may be important signals for lipoproteins localization in this bacterium.

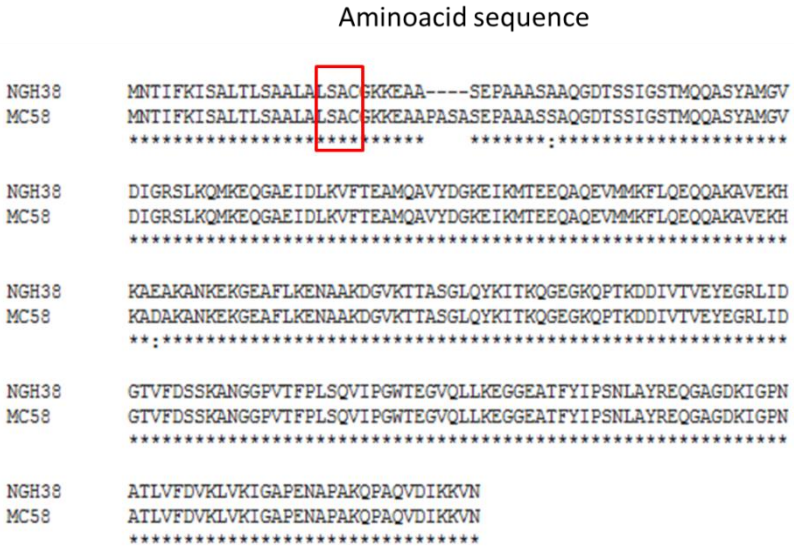


Figure 23 Mip amino acid sequence aligment. NMB1567 aminoacid sequence from NGH38 was aligned with the aminoacid sequence of MC58. Red box shows conserved lipobox motif.

3.8 Slam1 has a role in bactericidal activity by influencing SLPs localization in *N. meningitidis*

In order to test how Slam1 expression and the resultant different exposure of SLPs on the surface may alter the immunogenicity of OMVs, OMV preparations from the NGH38 panel of strains (WT, ΔSlam1 and CiSlam1) were purified and included in an immunization scheme in mice. NGH38 strains were grown as described in the material and methods (paragraph 7.9.1). Complemented strain was grown in the presence of 0.1mM of IPTG. OMVs were purified, and analyzed by SDS gel page (**Figure 24**).

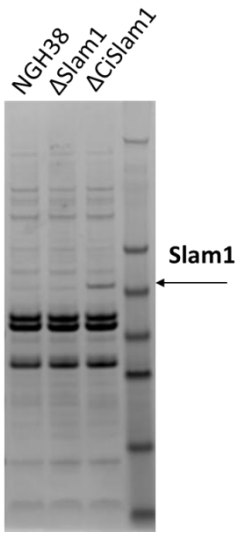
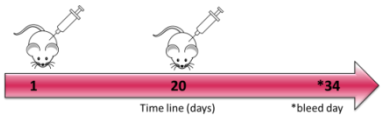


Figure 24 SDS gel page of NG38 OMVs preparation. 7µg of OMVs were loaded on SDS gel page and stained with comassie safe blue. Marker used was SeeBLue Nu page.

OMVs showed comparable pattern of protein bands among preparations. Slam1 overexpression was visible in the complemented strain even in a SDS Gel page stained with coomassie safe blue, but other than that there were no significant differences in proteins profile among OMVs preparations.

These OMV preparations were used to immunize mice according to the scheme summarized in **Figure 25**. In short, CD1 mice were immunized with two doses of 2.5 µg of OMVs intraperitoneally. The sera collected 14 days post the second immunization were tested for bactericidal activity in the presence of rabbit complement (rSBA) (paragraph 7.14) and by ELISA (paragraph7.13) for the presence of fHbp and NHBA antibodies.



Antigen	Dose	Adiuvant	Route
NGH38 wt OMVs	2 µg	3 mg/mL Al (OH)3	IP
NGH38 ΔSlam1 OMVs	2 µg	3 mg/mL Al (OH)3	IP
NGH38 CiSlam1 OMVs	2 µg	3 mg/mL Al (OH)3	IP

Figure 25 Immunization scheme. Upper panel: Eight CD-1 mice were immunized intra-peritoneally (IP) two times, as indicated. Bottom panel: composition of OMVs formulations.

rSBA analysis was performed on the pooled sera against a panel of strains, selected to be representative as divergent for the major antigens and clonal complex. Strains characteristics are summarized in **Table 3**.

Both the homologous NGH38 strain, and the 4 other heterologous strains from different clonal complexes and expressing diverse PorA variants were tested in serum bactericidal assay with rabbit complement.

Table 3

TABLE OF STRAINS TESTED

	Clonal Complex	PorA variant	fHbp variant	NHBA variant	Nad A variant
MC58	ST-32	7	1.1	p3	+,v1
NGH38	NA	18-1	2.24	p2	-
NZ 98/254	ST-41/44	7-2	1.14	p2	-
DE8221	ST-231	5	2.24	p2	-
5/99	ST-8	5.2	2.23	p20	+, v2

As was shown in **Figure 26** no bactericidal activity was detected against three different strains (MC58 NZ98/254 and DE8221) probably due to high differences from NGH38 and the tested strains. However, high bactericidal titers were observed against the homologous strain NGH38 and the heterologous 5/99 strain which expresses a different PorA subtype. Interestingly, pooled sera from mice immunized with ΔSlam1 showed slightly lower titers against the homologous strain, and no bactericidal activity was measurable against the 5/99. Complementation of the mutant restores high titers against the homologous (NGH38) and heterologous (5/99) strain.

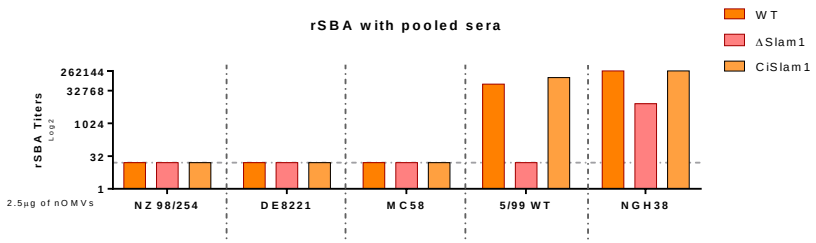


Figure 26 Serum bactericidal activity (SBA) performed on a panel of strains.

rSBA assay performed with single mice from the three immunization groups against NGH38 and 5/99 strains shows the same trend observed with pooled mice sera. Again a slight but significant decrease in the bactericidal activity was observed against the homologous strain (NHG38) (**Figure 27. A**); while no bactericidal titers were elicited against the heterologous strain (5/99) in the absence of PorA mediated killing (**Figure 27.B**).

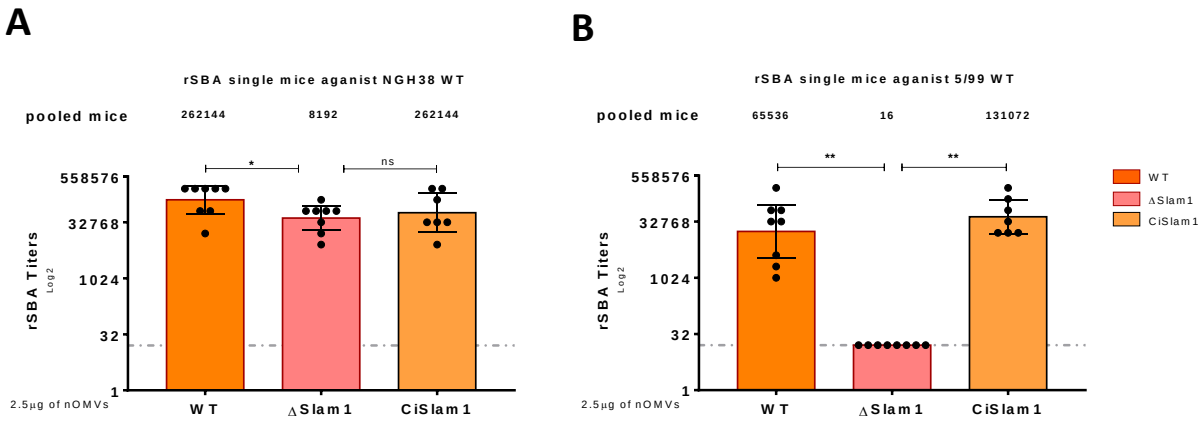


Figure 27 Serum bactericidal activity (SBA) with single mice performed against 5/99 and NGH38 WT Neisserial strains. A) rSBA against NGH38 reference strains. Statistical analysis performed with multiple comparison was not significant. While analyzing each component with t test student non parametric, (Mann-Whitney test) WT and ΔSlam1 was significant (* p value 0.0193); ΔSlam1 and CiSlam1 was not significant (ns). **B)** rSBA against 5/99 reference strains. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test **p: 0.0033 (WT vs ΔSlam1) and p: 0.0010 (ΔSlam1 vs CiSlam1)

To further investigate whether the Slam1-dependent bactericidal activity could depend on different NHBA and/or fHbp surface localization we performed rSBA on a panel of 5/99 strain with the over-expressing NHBA variant p2 or lacking fHbp and NHBA (**Figure 28**).

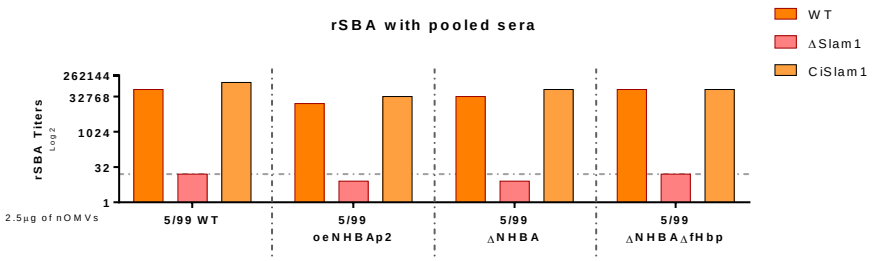


Figure 28 Serum bactericidal activity (SBA) performed on a panel of strains. Except for 5/99 WT all other tested strains were all deleted for NadA antigen. WT sequence of NHBA (5/99 ΔNHBA) and fHbp (5/99ΔNHBAΔfHbp) was also removed. In 5/99ΔoeNHBAp2 NHBA variant p2 was complemented ex-locus under the control of an IPTG inducible promoter in a 5/99 ΔNadAΔNHBA background.

No differences in bactericidal activity of the pooled sera were observed among the tested WT or recombinant 5/99 strains, suggesting that neither α-NHBA nor α-fHbp antibodies were mediating the killing activity of the OMVs immune-sera.

ELISAs were performed on the individual sera on plates coated with NHBA and fHbp proteins, however, no anti-fHbp nor anti-NHBA titers were measurable from any of the sera of the study (data not shown).

In summary, we conclude that Slam1 has a drastic effect on the immunogenicity of OMVs. In NGH38, Slam-dependent responses are independent of PorA-driven responses and may be through the surface translocation of bactericidal antigens, likely Slam1-dependent lipoproteins other than NHBA and fHbp.

4 Discussion and conclusion

The main aim of this work was the characterization of the lipoproteins translocation mechanisms in *N. meningitidis*. Several aspects were investigate: we investigated sorting signals for Neisserial lipoproteins localization; we better characterized the role of Slam1 for lipoproteins surface exposure and identify two new targets; and we test whether Slam1 influences the immunogenicity of OMVs by modulating SLPs on the surface.

E. coli have been largely used as a model to investigate translocation signals and mechanisms of lipoproteins localization system [90]. However not all Gram negative bacteria, share the same features: sometimes they have conserved the general pathway and differ in signals [56] or translocator components [46]. From the evidence presented here this would seem to be the case also for *N. meningitidis*. While homologues of the *E. coli* Lol components can be identified in *N. meningitidis* [58], from our systematic lipoprotein analysis we predict that lipoproteins are over-represented in *N. meningitidis* (at over 3% of the total number of ORFs) compared to *E. coli*. Furthermore from consolidating known proteomic data we show that the +2 rule does not hold true as a retention signal for the IM. Through the unexpected result of the absence of Mip on the bacteria surface in the NGH38 strain, we serendipitously identify that the area +8-and +11 may be important for IM retention. Indeed analyzing whole cell lysate and OM preparation (OMVs) from MC58 and NGH38, Mip was stably expressed in both strains, but was not present in the OMVs preparation from NGH38 indicating that a 4 amino acid deletion (+8;+11) alters significantly the translocation of this lipoprotein and abrogates translocation to the OM in this strain. The deletion in this area may result in the generation of a retention signal or compromise the recognition by LolCE and blocked the surface lipoprotein at the level of the inner membrane. This hypothesis should be further tested in order to elucidate the precise signals for lipoproteins localization in *N. meningitidis*.

The presence of surface lipoproteins and the recent Slam OMP family identified by Hooda and co-workers, pointed out the presence of additional mechanisms for surface localization in *Neisseria*. Here we show that Slam1 was stably expressed in different *N. meningitidis* clinical isolates either invasive or carriage meningococcal strains. We also identify two new substrates for Slam1, NHBA and Mip. Indeed both NHBA and Mip were stably expressed in the Slam1 mutant but not translocated on bacteria surface when Slam1 was missing.

Since some Slam1 substrates show similar β-barrel domain structures [49] we hypothesize that the signal for surface localization was present in the common domain. The C-terminal domain structure of NHBA has been reported [84] and, as the other Slam1 substrate, is folded with a TbpB-like structure. Our data show that in the absence of the β-barrel C-terminus, truncated forms of NHBA were not able to be exposed on bacterial surface indicating the β-barrel is

essential for surface localization. To further investigate this aspect, we generated different SLPs mutants in which the C-terminal portion was exchanged, among surface lipoproteins. The importance of the C-terminal domain was confirmed, indeed when C-terminal domains were replaced between NHBA and fHbp, both fusion proteins were able to reach bacteria surface. All these results were support the hypothesis that the C-terminal portion and the β-barrel domain play a major role in the interaction whit Slam1 for SLP translocations across the OM.

Additionally we proved that meningococcal Mip lipoprotein [89], which was described to be surface exposed in *N. gonorrhoeae* [83], was depend on Slam1 for its surface exposure in *N. meningitidis*. No structure was available for Neisserial Mip, but a model based on the structure from *L. pneumophila* homologous has been described [89]. Meningococcal Mip consist in a globular C-terminal with the functional domain, and a long α-helix domain involved in Mip dimerization. From this model Mip was not folded with a TbpB-Like shape, unlike the other identified Slam1 substrates. This suggested that not all of the β-barrel domain may be necessary for recognition and delivery of SLPs to bacteria surface and possibly conserved elements may be present in Mip and the β-barrel and it is possible that only few amino acids, with similar characteristic or specific folding, can be necessary for the recognition and/or requirement from Slam1.

As mentioned before we were able to identify two new Slam1 substrates NHBA and Mip that were stably expressed and only their surface localization were affected depending on Slam1 expression. On the contrary for fHbp results demonstrated a general decrease in the fHbp total amount in the ΔSlam1, and not only in its surface exposure. This observed reduction was conserved among tested strains and was not driven from feedback mechanisms at transcriptional levels, as no significative differences in the transcription level was observed in the analyzed strains between WT and ΔSlam1. This suggests that Slam1 directly or indirectly has an effect on the turnover or stability of fHbp protein post-transcriptionally.

While the precise reason for this is unknown, that the stability of fHbp is affected positively by Slam1 and this phenomenon is specific only for fHbp, of the three SLPs tested here. This could be related either to a direct mechanism: it is possible that Slam1 directly interacts with fHbp thereby stabilizing the protein, or due to an indirect mechanism: fHbp degradation from proteases present in the periplasm when not localized on the surface.

Lipoproteins are emerging as key targets for protective immunity [91]. We have identified 2 other Slam1 substrates and probably other SLPs remain not yet identified. Our data suggests also the possibility to modulate lipoprotein exposure by regulating Slam1 expression. Moreover the over-expressing of Slam1 gave evidence of a concomitant increase of lipoproteins on bacteria surface compared to the WT condition. To test whether different levels of SLPs on the surface might affect immunogenicity we tested the immunogenicity of OMVs purified from

strains with and without Slam1. Rabbit- serum bactericidal activity (rSBA) performed on a panel of strains confirmed the role of Slam1 in driving bactericidal activity of the OMVs. There was a small but significant reduction in bactericidal activity against the homologous strain in sera raised against OMVs lacking Slam1. PorA is recognized as an immunodominant antigen of meningococcus and generally OMVs will be protective towards strains with similar PorA subtypes. From our data, we can conclude however that Slam1 contributes to full bactericidal activity against the NHG38 strain. We tested 4 heterologous strains all with different subtypes to investigate the immunogenicity of non-PorA antigens in the OMVs. Some strains (MC58, NZ 98/254 and DE8211) were not killed by any of the OMV antisera, probably due the high differences within NGH38 strain. On the contrary, other 5/99 strain was killed with high titers from sera derived from NGH38 WT OMVs, indicating that NGH38 OMVs contain non-PorA bactericidal antigens which cross-react with 5/99. Surprisingly, all bactericidal activity against 5/99 was dependent on Slam1, and OMVs prepared from Slam1 mutant were not bactericidal. Further analyses revealed also that immunogenicity was not driven by NHBA or fHbp, as no NHBA and fHbp antibodies were detected by ELISA and the 5/99 lacking NHBA and fHbp expression was still killed by OMV sera.

Since all strains expressed Slam1 to similar extent with conserved sequence, it is unlikely that bactericidal activity was driven by Slam1. Furthermore, FACS analysis indicated that Slam1 is not surface exposed and accessible to polyclonal antisera, suggesting that Slam1 maybe not act directly as a target for bactericidal antibodies. These results together suggest the presence of bactericidal SLPs, which are Slam1 substrates distinct from NHBA and fHbp, that can have a role in OMVs immunogenicity.

Many aspects have to be further investigated and characterized. How Slam1 interact with SLPs is still an open question. No Slam1 homologous were identified so far [63] in *B. burgdorferi* and *Selverda et al*. have published that the N-terminal part of fHbp was enough for the successful surface display of Borellia OspA on meningococcal OMVs [91], in contrast to what we have discovered about the importance of the C-terminal domain. In our hands truncated forms of NHBA where stably expressed but not delivered on bacterial surface. On the other hand this could be in agreement with the ability of Slam1 to translocate Mip on the surface even if is not folded with TbpB-like structure. These results might suggest an additional role of the N-terminal portion for recognition and surface translocation for some SLPs.

In summary, signals for inner membrane retention or outer membrane translocation are distinct in *N. meningitidis* with respect to the *E. coli* model and more research is needed to fully elucidate these. The identification of Mip as a Slam1 substrate and its mutant allele in NGH38 could help in the future to characterize meningococcal SLPs as well as the signal for IM retention. Here we have widened our knowledge on Slam1 substrates, and the immunogenicity studies suggest that

other Slam-dependent bactericidal SLPs might be present in *N. meningitidis*. Lipoproteins present on bacteria surface represent a key target for protective immunity so the new Slam1 translocator component identified in many gram negative bacteria might represent a new powerful strategy for engineer bacteria for over-expressed immunogenic lipoproteins on bacteria surface.

Chapter Two

Exploiting Slam1 for heterologous Over-Expression of Neisserial Surface exposed lipoproteins

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Outer Membrane vesicles (OMVs) are small particles spontaneously released from bacterial membranes, in particular from Gram-negative bacteria. They are often considered a non-replicative representation of the parent bacterium as they contain Outer Membrane Proteins (OMPs) and oligosaccharides, present on the bacterial surface. OMVs contain innate immunostimolatory properties able to facilitate the immune system activation; hence OMVs purified from several pathogens are able to induce protective immune responses against the pathogen from which they are purified from [80, 92, 93]. In the table1 are reported some examples of OMVs from different bacteria able to elicitates a functional immune response.

Table 4 Overview OMVs vaccine tested in mice or other rodents. Spontaneous released (s-OMVs), detergent extracted (d-OMVs) and native (n-OMVs) extracted with detergent free methods.

Pathogen	type of OMVs	reference
Acinetobacter baumannii	s-OMVs	[94]
Bordetella pertussis	d-OMVs	[95]
Borrelia burgdorferi	n-OMVs	[96]
Brucella melitensis	s-OMVs	[97]
Burkholderia pseudomallei	s-OMVs	[98]
Francisella	s-OMVs	[99]
Helicobacter pylori and H. felis	s-OMVs	[100]
Klebsiella pneumoniae	s-OMVs	[78]
Neissieria meningitidis	s-OMVs	[101]
Salmonella typhimurium	s-OMVs	[74]
Shigella	s-OMVs	[102, 103]
Vibrio cholerae	s-OMVs	[104]

Thanks to the ability to genetically engineer the OMVs-producing bacteria, this system proves to be versatile to be exploited as a vaccine platform [80, 81]. In the past, many approaches were investigated for the expression of heterologous antigens using OMVs as a delivery system within their lumen or their surface [105]. Surface localization seems to be preferred for the generation of functional antibodies against the carried antigens [106-108] and for this purpose different strategies were developed. In general, antigens or proteins normally founded on bacterial outer membrane, totally or partially exposed on the surface, might be used for the surface delivery of foreign antigens in heterologous or homologous systems. For example Kim and co-workers were

able to delivery GFP molecules on OMVs surface taking advantage of *E. coli* ClyA (vesicles associated toxins) [109] and the same system was use to delivery Influenza A antigen on vesicles surface [110]. Other approaches involved autotrasporter proteins. As the name suggests, this class of protein contains a translocator domain, which span the OM, and is responsible for self-transport of the passenger domain on bacterial surface. In this case, the antigen could be fused at the level of the passenger domain and displayed on bacteria surface thanks to the translocator domain [111, 112]. A similar approach was described by Salverda and co-workers that were able to display OspA, *B. burgdoferi* surface lipoprotein, on meningococcal surface by fusing it to different portion of Neisserial fHbp surface lipoprotein [91]. Interstingly different unrelated studies also find antibody-mediated immune responses against luminal heterologous antigens [113-115].

Lipoproteins are molecules characterized by an acyl moiety, either di- or tri- acylated, at the N-terminal portion of the proteins, which allows their anchoring to the membrane. The mechanisms by which lipidated lipoproteins can reach the Outer Membrane (OM) is conserved among bacteria and involves the Lipoprotein outer membrane localization (Lol) system; composed by an ABC transporter (Lol CDE) at the levels of IM, a chaperonine (LolA) and a lipoprotein (LolB) able to transfers lipoproteins from LolA and anchors them to the inner leaflet of the OM [30]. Lipoproteins are usually found to be attached to the Outer Membrane (OM) or the Inner Membrane (IM) facing the periplasm, but the number of reports regarding lipoproteins anchored to the outer leaflet of the OM has increased in the last years, highlighting the importance of this emerging class of lipoproteins named Surface Lipoproteins (SPLs)[39, 57].

The SLPs identified up to date [49, 116-124] are highly heterogeneous, lack common features and the mechanism of OM translocation seems to be different among bacteria [40]. SLPs can be partially surface exposed, as for *E. coli* RcsF [48], or transiently display on surface like *E. coli* Lpp and Pal [125, 126] or nontypeable *H. influenza* P6 lipoproteins [127]. SLPs from Neisseria genus are the most studied group of this lipoprotein family. Eight different SLPs have been experimentally localized on *Neisserial* surface [49, 53, 83, 128], among them factor H binding protein (fHbp) [19] and Neisseria Heparin binding antigen (NHBA)[50]. Both fHbp and NHBA are antigens against *N. meningitidis* serogroup B [128, 129], demonstrating the importance of SLPs as vaccine antigen.

Recently a family of outer membrane proteins called Slam (Surface-lipoprotein assembly modulator), involved in surface exposure of specific *N. meningitidis* lipoproteins was described [59]. *N. meningitidis* contains two different Slam proteins: Slam1 which is necessary for the translocation of different meningococcal SLPs and Slam2 which is specific for HpuA (hemoglobin-haptoglobin utilization protein A) translocation. Hooda and colleagues showed the ability of Slam to potentiate the functional display of selected SLPs on *E. coli* laboratory strain,

which does not possess any Slam or SLPs homologous [63]. Hooda shows the capacity of Slam1 to localize fHbp and other *N. meningitidis* SLPs on the surface of non-pathogenic *E. coli* [59]. On the contrary Fantappiè and co-workers have recently reported the ability of *N. meningitidis* fHbp and NHBA to reach *E. coli* surface even when Slam1 was not expressed, suggesting the presence of a sub-class of SLPs which can always cross outer membrane, even when transplanted from one organism to another [130].

In this work, taking advantage of the recently identified Slam1 translocator component, and using strategies of co-expression with SLPs in the heterologous background of *E. coli*, we were able to generate OMVs differentially enriched in immunogenic SLPs and potentially with different localization (surface exposed or in the lumen of OMVs) of these SLPs.
The hypothesis investigated is therefore whether the co-expression of meningococcal SLPs with Slam1 generates more immunogenic *E. coli* OMVs. We clearly demonstrate the importance of the co-expression of SLPs with Slam1 for the correct display of antigens and we demonstrate that, depending on the presence of Slam1, heterologous *E. coli* OMVs enriched with *N. meningitidis* SLPs elicit different antibody responses.

5 Results

5.1 Co-expression of Neisserial SLPs with Slam1 in *E. coli* results in high expression and their translocation to bacterial surface

5.1.1 Slam1 has a role in the stable expression as well as in the translocation to the surface of fHbp

The ultimate goal of this study was to generate *E. coli* OMVs able to display SLPs such as fHbp or NHBA on their surface, and to investigate the role of Slam1 in promote the heterologous expression of SLPs.

In order to co-express Neisserial Slam1 and SLPs in *E. coli*, two different strategies were tested. Firstly we generate *E. coli* strains where fHbp and Slam1 were co-expressed on different plasmids as described in material and methods (7.2.2). *E. coli* BL21 (DE3) strain was co-transformed with 2 distinct compatible plasmids carrying *fHbp* or *Slam1,* respectively. Schematic representation of the plasmid used in this study, is shown in **Figure 29.A**.

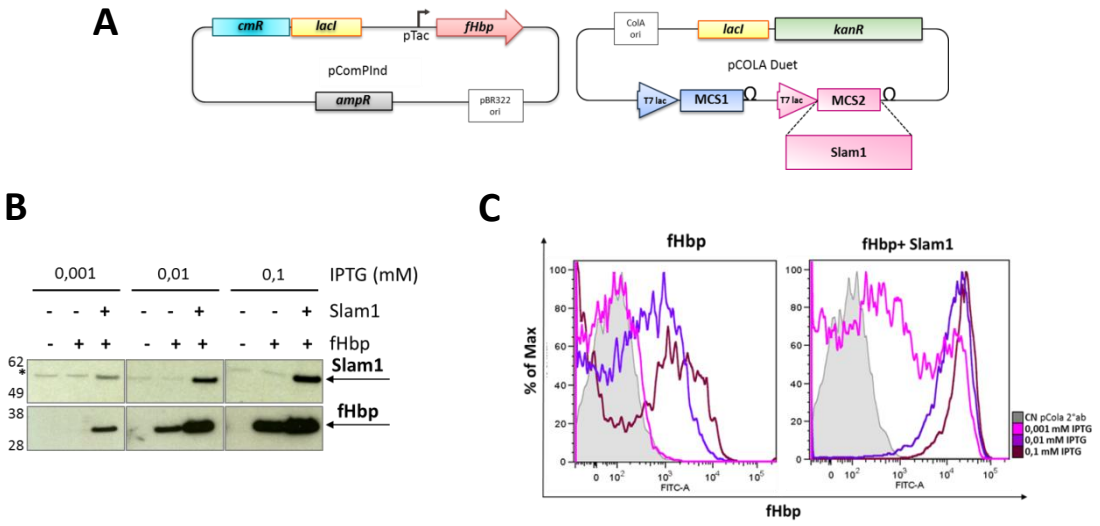


Figure 29 Heterologous expression of Slam1 and fHbp in *E. coli*. A) Schematic representation of plasmids used for fHbp and Slam1 co-expression in *E. coli.* Plasmids were both carrying LacI repressor gene and have compatible origin of replication with different promoter and resistance cassettes. Bacteria transformed with both plasmids were growth on LB AGAR plate with different IPTG concentration (0.001 mM, 0.01mM or 0.1mM IPTG). After O/N growth at 37°C, total lysate bacteria or fixed bacteria were collected. **B**) Western blot analysis with α-fHbp polyclonal mouse (diluted 1:5000) and α-Slam1 (1:1000) antisera on empty bacteria, bacteria expressing only fHbp or co-expressing fHbp and Slam1 were show. **C**) FACS analysis performed using polyclonal α-fHbp mouse (1:1000) polyclonal antisera against the same samples collected analyzed by Western blot to check fHbp surface localization. *aspecific band

The resulting strains expressing fHbp in the presence or absence of Slam1 were grown on LB agar plates with different IPTG concentrations (final concentration 0.001; 0.01; 0.1 mM IPTG). Expression levels of both proteins responded to IPTG induction and the expression of both proteins were confirmed by WB analysis of total lysate (**Figure 29**.**B**) translocation on the surface was examined with FACS analysis (**Figure 29**.**C**).

In the presence of Slam1 the amount of fHbp in the total extracts strongly increased compared to the strain expressing fHbp alone, and this higher fHbp amount was reflected by higher detectable fHbp on *E. coli* surface. fHbp expressed alone was visible by WB in all samples but FACS analysis reveals that fHbp was detectable on bacteria surface only at higher IPTG concentrations (0.01mM and 0.1mM). However when co-expressed of Slam1, fHbp was appreciable on the surface already at the lower IPTG concentration (0.001mM). Furthermore, observing fHbp amount on bacteria total lysate, even if fHbp amount is comparable (fHbp+Slam1 induced with 0,01mM IPTG and fHbp alone induce with 0,1mM IPTG) the presence of Slam1 results in greater exposure of fHbp on bacteria surface.

Despite these promising results, the co-expression strategy, in our hands was not applicable to liquid culture as bacteria tend to lose plasmid carrying fHbp (data not shown).

In order to avoid plasmid loss, in a second strategy, we cloned fHbp and Slam1 on the same plasmid as described in the material and methods (7.2.2). *E. coli* BL21 (DE3) was transformed with this new construct, and as controls *E. coli* carrying plasmid expressing fHbp or Slam1 alone and the empty pCOLA plasmids (Ø) were also used. (**Figure 30**.**A**). BL21 transformed with this set of plasmids were plated on LB agar plates with different IPTG concentration (0.001 or 0.1mM) and after O/N growth at 37°C bacterial total lysate and fixed bacteria were collected from all *E. coli* strains.

As previously described, fHbp and Slam1 expression was modulated by adding different IPTG concentration, and also in this case the presence of Slam1 positively affected fHbp expression and surface localization.

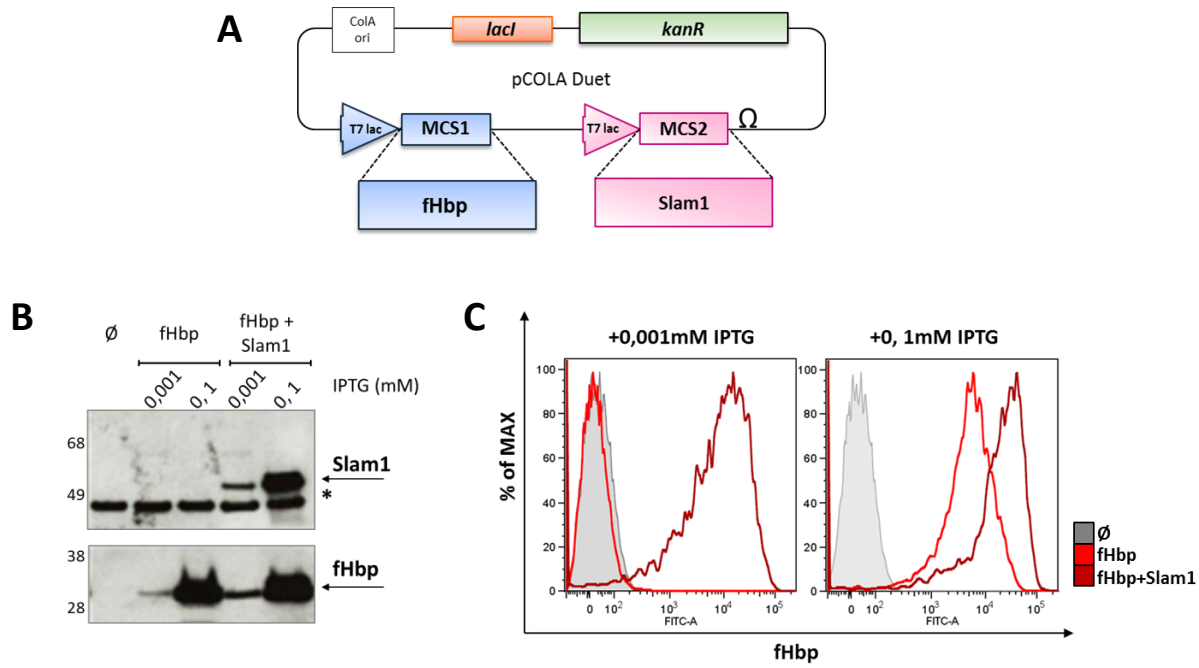


Figure 30. Heterologous expression of Slam1 and fHbp in *E. coli,* with new set of constructs. A) Schematic representation of the new set of plasmids used for co-expression. fHbp was cloned in the first multi-cloning site, while Slam1 was cloned in to the second multi cloning site. Expression of both proteins was IPTG regulated. **B**) Western blot of bacteria total lysate collected after O/N incubation at 37°C on LB agar plates in the presence of different IPTG concentrations (0.01mM and 0.1mM IPTG). Proteins expression was checked by Western Blotting using α-fHbp (1:5000) or α-Slam1 (1:1000) mouse polyclonal sera. **C)** FACS analysis performed using polyclonal anti-fHbp mouse polyclonal antisera (1:1000) against the same samples collected analyzed by Western blot, was done to control fHbp surface localization. *aspecific band, empty vector (Ø) control.

Western Blot analysis of lysates from bacteria grown on plates indicated only minor differences in fHbp amount in cultures in the presences or absence of Slam1 at either IPTG concentration (0.001 or 0.1mM), (**Figure 30.B**). FACS analysis, on the other hand, revealed that fHbp was detectable on the surface of both *E. coli* strains under conditions of 0.1mM IPTG, but when Slam1 was also present, fHbp surface exposure was more compared to the one expressing fHbp alone (**Figure 30.C**), At the lower IPTG concentration (0.001 mM) fHbp was only detectable on the surface in the presence of Slam1. This suggests that when expression levels are lower the role of Slam1 in the stable surface localization of the fHbp is more critical.

Liquid cultures were set up in order to produce OMVs. Since OMVs are usually released during stationary phase; specific growth protocol was set up (7.9.2). Briefly bacteria strains were grown in defined liquid media (HTMC) overnight in the presence of IPTG from the beginning of the growth inoculating from a pre-culture which had been grown at 37 °C for several hours as described in materials and methods.

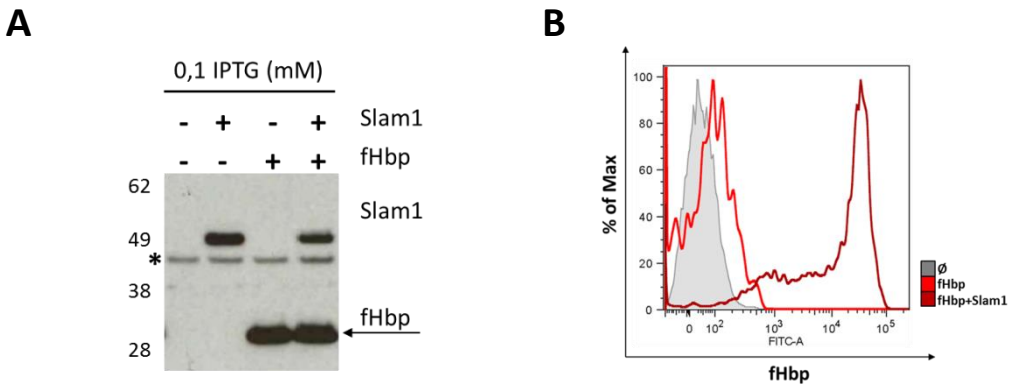


Figure 31. Slam1 is necessary for fHbp surface localization in certain growth condition. Bacteria were growth on plate O/N. Liquid growth on HTMC media was performed after O/Day pre-inoculum. 0.1mM IPTG was added in the media at the beginning of the O/N incubation. After 12/14 hours bacteria total lysate for WB analysis and fixed bacteria for FACS analysis were collected. **A)** Western blot analysis stained with α-Slam1 (1:1000) or α-fHbp (1:5000) polyclonal sera confirmed the expression of both proteins. **B)** FACS analysis performed using polyclonal α-fHbp mouse polyclonal antisera (1:1000) against the same samples collected analyzed by Western blot, was done to control fHbp surface localization. *aspecific band.

Under these conditions we were able to observed clear differences in fHbp surface exposure depending on the presence of Slam1. Western blot analysis confirms fHbp and Slam1 expression in all constructs (**Figure 31.A**) and FACS analysis confirm the presence on the surface of fHbp only when Slam1 was expressed (**Figure 31.B**). Cell free supernatants were collected from these preparations and OMVs were purified as described in material and methods (7.9.2).

These findings are someway in contrast to the findings of *Fantappiè et al.*, where expression of fHbp and NHBA in *E. coli* results with their surface localization. In order to understand if we could also repeat the results of *Fantappie et al.*, we performed the culturing of our *E. coli* strains as described in the reported article. As such, initially, bacteria were grown until exponential phase without IPTG and then protein expression was induced for 3hours. After induction total lysate and fixed bacteria were collected (**Figure 32**).

Proteins induction was confirmed by WB. Slam1 and fHbp in both conditions (with or without Slam1) were visible as reported on WB (**Figure 32.A**). FACS analysis show only a slight decrease in fluoresce intensity when fHbp was expressed alone (**Figure 32.B**). Therefore under conditions of optimal and high protein expression, fHbp can be detected on the surface by FACS in the absence of Slam1 co-expression in agreement with the results obtained with plate cultures.

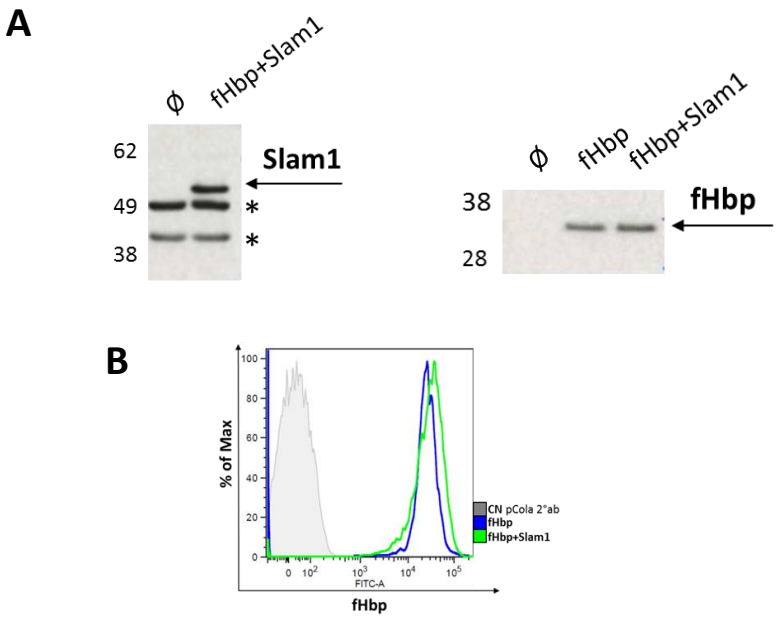


Figure 32. **After three hours induction in liquid culture Slam1 is not required for surface exposure**. Bacteria were growth on plate O/N. Liquid growth on LB media was then performed. 0.1mM IPTG was added for 3 hours to the media when bacteria have reached exponential phase, after that bacteria total lysate for WB analysis and fixed bacteria for FACS analysis were collected. **A)** Western blot analysis stained with α-Slam1 or α-fHbp confirmed the expression of both proteins. *aspecific band, empty vector (Ø) control. **B)** FACS analysis performed using polyclonal α-fHbp mouse polyclonal antisera against the same samples collected analyzed by Western blot, was done to control fHbp surface localization.

5.1.2 Slam1 is necessary for NHBA surface translocation in *E. coli*

N. meningitidis NHBA was identified as a lipoprotein translocated on bacterial surface by Slam1 as described before. As for fHbp, NHBA was cloned with or without Slam1 in pCOLA Duet plasmid, as described in the material and methods (7.2.2).

BL21 (DE3) *E. coli* strain was transformed with the plasmids set. These bacteria strains were grown (described in paragraph 7.9.2) in defined liquid media (HTMC) in the presence of IPTG from the beginning of the growth, as was done for fHbp. Bacteria total lysate and fixed bacteria for FACS analysis were collected.

Western blot analysis confirmed NHBA expression in bacteria total lysate from both *E. coli* strains, when NHBA was expressed alone and when was co-expressed with Slam1 (**Figure 33.A**); interestingly as for fHbp NHBA amount seems to be higher when Slam1 was also expressed.

On the other hand FACS analysis reveal that no NHBA was detectable on the bacteria surface when Slam1 was not expressed (**Figure 33.B**). Curiously bacteria co-expressing Slam1 and NHBA show two different populations expressing different levels of NHBA on the surface.

Cell free supernatants were collected from these preparations and OMVs were purified as described in material and methods.

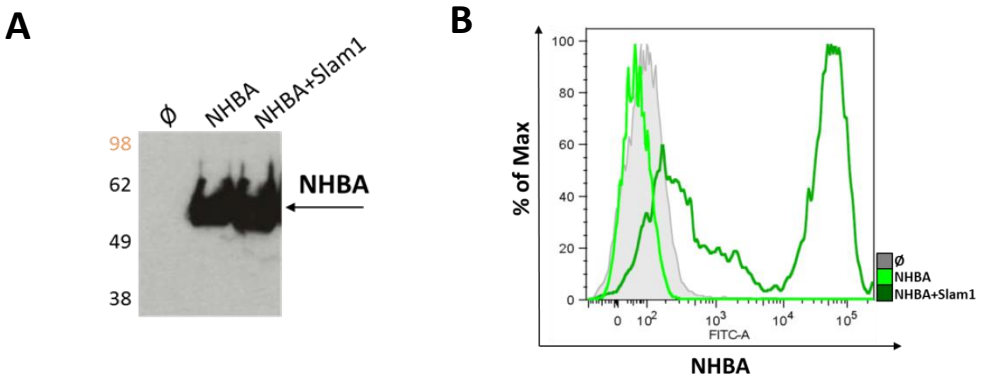


Figure 33 NHBA required Slam1 for surface exposure. Bacteria were growth on plate O/N. Liquid growth on HTMC media was performed after O/Day pre-inoculum. 0.1mM IPTG was added in the media at the beginning of the O/N incubation. After 12/14 hours bacteria total lysate for WB analysis and fixed bacteria for FACS analysis were collected. **A)** Western blot analysis stained with α- N term NHBA monoclonal antibodies (1:4000) confirmed NHBA expression. **B)** FACS analysis performed using α- N term NHBA monoclonal antibodies (1:800) on fixed bacteria, was done to control NHBA surface localization. Empty vector (Ø) control

5.2 *E. coli* OMVs are enriched on Neisserial proteins

5.2.1 Neisserial fHbp lipoprotein is present in heterologous OMVs preparations at high level

OMV were purified using a detergent-free method from cell-free culture supernatants through ultracentrifugation. At electron microscopy analysis by negative staining (**Figure 34**) indicated the resulted vesicles appear round and with comparable size. OMVs integrity was also confirmed.

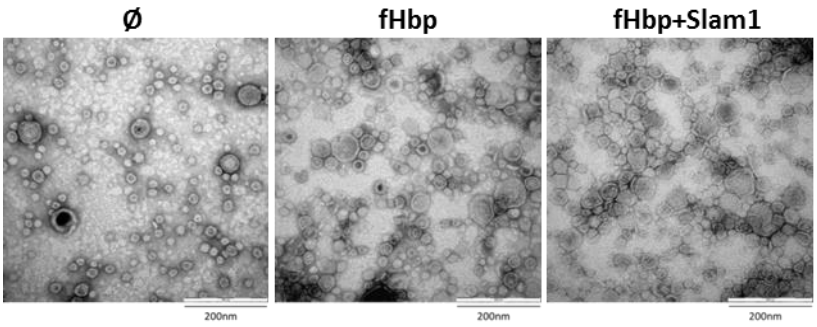


Figure 34 Electron microscopy on *E. coli* OMVs. Negative staining of purified *E. coli* OMVs expressing fHbp alone or with Slam1. OMVs purified from empty control bacteria were also show (Ø).

Equal amounts of OMVs with respect to total protein quantities from all preparations were loaded onto SDS-PAGE gel and either visualized by Simply BlueTMSafe staining or blotted onto nitrocellulose membrane and then the proteins of interest detected using polyclonal mouse sera. *E. coli* OMVs showed similar pattern bands among preparations, furthermore bands at the expected molecular weight of fHbp and Slam1 are visible in the SDS-PAGE .

SDS-Gel page and WB analysis (F**igure 35.A .B)** both confirmed differences in fHbp amount in OMVs depending on Slam1 expression. It is worth noting that OMVs from *E. coli* co-expressing fHbp and Slam1 contain really high amounts of fHbp where it appears as the most abundant protein in the OMVs as is shown in SDS Gel page. In order to quantify better the differences in the total amount of fHbp between OMVs preparation expressing fHbp alone or co-expressed with Slam1, Western Blot with OMVs serial dilutions was performed (F**igure 35.C**). OMVs with fHbp co-expressed with Slam1, results in over 10 times more fHbp than in OMV where fHbp is present alone.

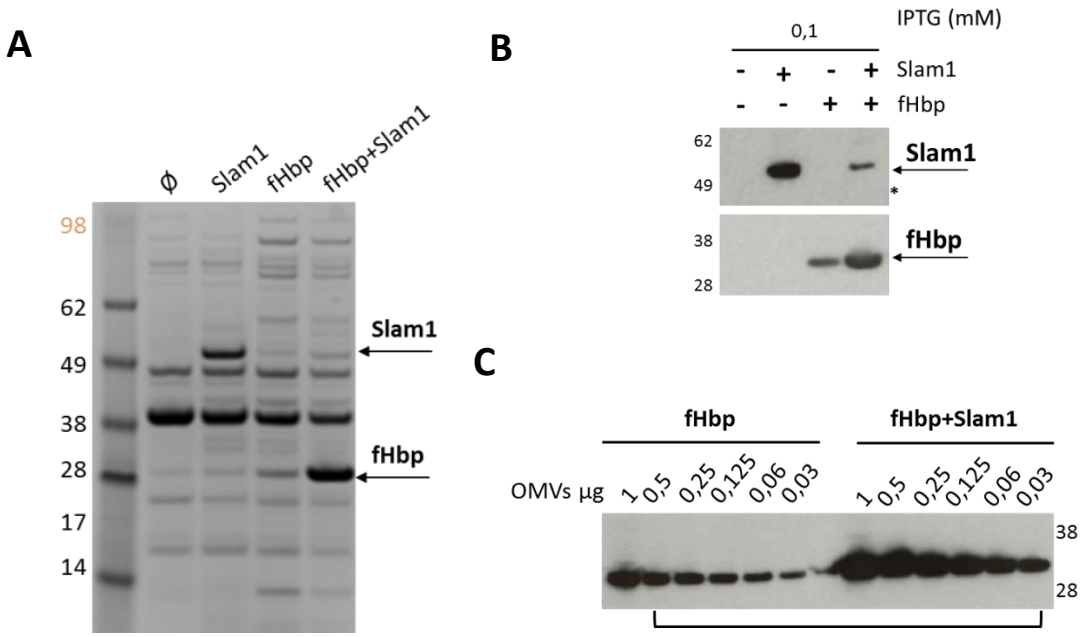


Figure 35 Characterization of *E. coli* OMVs preparations. A) SDS gel page with 7.5 µg of OMVs loaded stained with Simply BlueTMSafe; **B)** Western blot performed on 1.5 µg (protein content) of OMVs stained with α-fHbp polyclonal sera (1:5000) and Slam1 policlonal sera (1:1000); **C)** Western blot performed on OMVs serial dilutions with α-fHbp polyclonal sera (1:5000).

5.2.2 NHBA Neisserial lipoprotein is present in *E. coli* OMVs

As for fHbp preparations OMV were purified using a detergent-free method from cell-free culture supernatants, collected after O/N growth, through ultracentrifugation

Equal amounts of OMVs with respect to total protein quantities from all preparations were loaded onto SDS-PAGE gel and visualized by Simply BlueTMSafe staining. OMVs preparations showed similar pattern bands, and in preparations where proteins were induced, bands at the expected molecular weight of NHBA and Slam1 were visible (**Figure 36.A**). SDS gel Page of OMVs confirmed the difference in NHBA amount observed by western blot of bacterial total lysate. Even if, expression level of NHBA were not comparable with expression reached by fHbp expression with or without Slam1, slight difference between NHBA expressions among preparations was observed.

Western blot on serial dilution of OMVs carrying NHBA alone or with Slam1 was performed (**Figure 36.B**) and shows high number of NHBA dependent bands, probably due to intrinsic predisposition of NHBA cleavage [50] (Ø: 1 µg of empty OMVs as control for antibody specificity). A part of that, different amount of NHBA among the two preparations was appreciable.

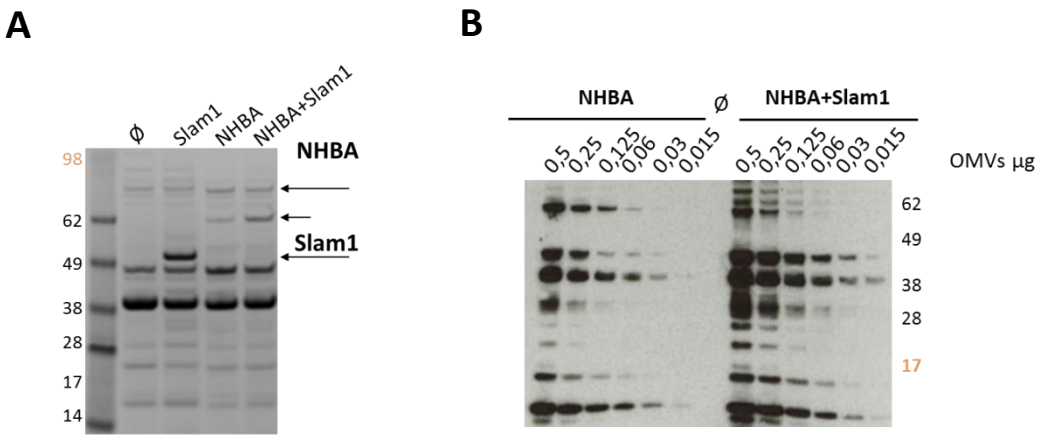
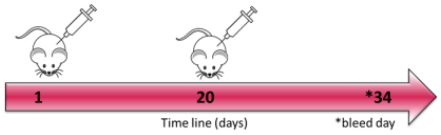


Figure 36 Characterization of E coli OMVs preparations. A) SDS gel page with 7.5 µg of OMVs loaded stained with Simply BlueTMSafe; **B)** Western blot performed on serial OMVs serial dilutions with α N-term monoclonal antibody (1:4000).

5.3 Neisserial SLPs delivered by heterologous OMVs are able to induce coverage against *N. meningitidis*

5.3.1 fHbp delivered on heterologous OMVs elicited high functional antibody titers

CD-1 mice were immunized intra-peritoneally two times with: 2µg or 0.2 µg native *E. coli* OMVs and 1 µg of recombinant fHbp protein variant 1.1 (rfHbp) (**Figure 37**) to evaluate whether the co-expression of Slam1 with meningococcal SLP in the heterologous *E. coli* background has an effect on the immunogenicity of the resulting OMVs.



Antigen	Dose	Adiuvant	Route
Ø OMVs	2 µg	3 mg/mL Al (OH)3	IP
Slam1 OMVs	2 µg	3 mg/mL Al (OH)3	IP
fHbp OMVs	2 µg	3 mg/mL Al (OH)3	IP
fHbp OMVs	0,2 µg	3 mg/mL Al (OH)3	IP
fHbp+ Slam1 OMVs	2 µg	3 mg/mL Al (OH)3	IP
fHbp+Slam1 OMVs	0,2 µg	3 mg/mL Al (OH)3	IP
rfHbp	1 µg	3 mg/mL Al (OH)3	IP

Figure 37 Immunization scheme. Upper panel: eight CD-1 mice were immunized intra-peritoneally (IP) two times, as indicated. Bottom panel: Bottom panel: representation of the immunization scheme. Amount of native *E. coli* OMVs, adjuvant dose and the site of injection were indicated. (Ø: Empty native OMVs)

Elicited antibody titers were evaluated by ELISA analysis using immunized sera of individual mice from each group and rfHbp as a coating antigen as described in the material and methods (paragraph 7.13) (**Figure 38**).

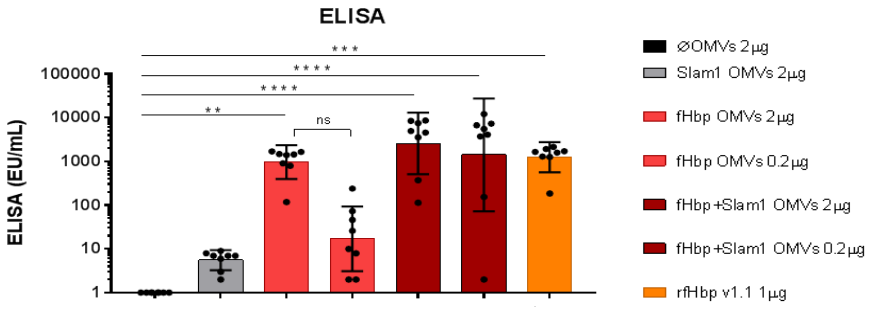


Figure 38 Analysis of sera from mice immunized with *E. coli* OMVs preparation or recombinant fHbp protein. IgG α-fHbp antibody titers elicited measured by ELISA. Each dot represents an individual mouse serum while bars indicates the median value within each immunization group. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test (ns: not significant; **p<0.0065; ***p<0.0009, ****p<0.0001).

Formulation of OMVs carrying fHbp, with the exception of 0.2 µg of OMVs carrying fHbp alone, elicitates antibody titers which were significantly higher than the negative controls (Ø OMVs and Slam1 OMVs) and comparable to the recombinant protein (rfHbp). A trend dose-dependent anti-fHbp titers from sera immunized with fHbp OMVs preparation was visible, while apparently no difference between the two doses of fHbp+Slam1 OMVs preparation were detected.

Antibody fuctionality was evaluated by serum bactericidal assays with rabbit complement (rSBA) as describe in the material and methods (paragraph7.14). Mice sera were tested against *N. meningitidis* H44/76 meningococcal natural strain, normally used as a reference strain for fHbp variant 1.1. (**Figure 39**).

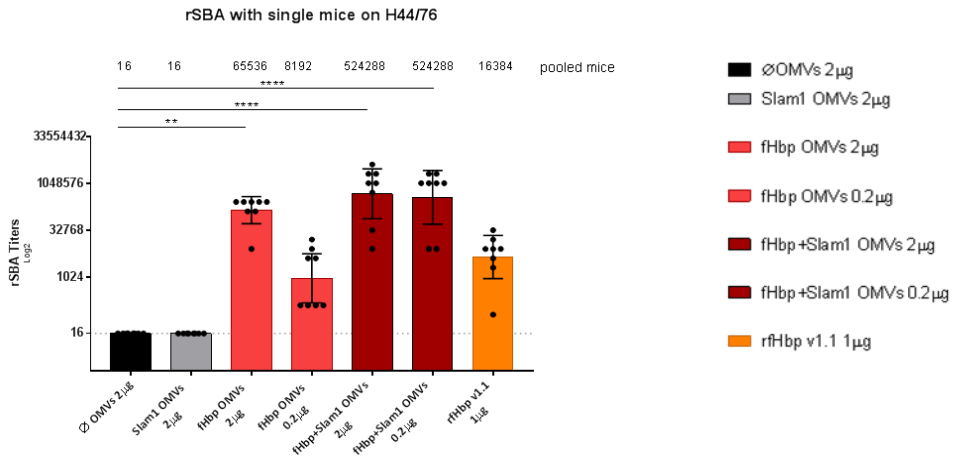


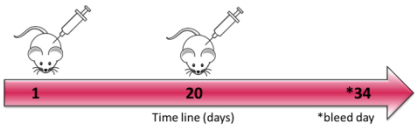
Figure 39 Serum bactericidal antibody (SBA) titers against H44/76 meningococcal strain. Dots represent SBA titers of individual mouse sera against the defined strain. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test (**p<0,0024, ****p<0.0001)

Accordingly with ELISA titers, no killing was achieved with sera derived from controls (Ø OMVs and Slam1 OMVs); while good functional response was achieved with sera derived from mice immunized with the preparation expressing fHbp.

Titers from 6 out of 8 mice from the immunizations (2 µg and 0.2 µg) of the fHbp+Slam1 OMVs preparation are really high, but again no differences were detected between the two doses of OMVs. In addition, differently from what emerged from ELISA titers, 2 µg of fHbp OMVs, and both doses of fHbp+Slam1 OMVs preparation gave higher bactericidal titers compared to 1µg rfHbp v1.1, indicating that fHbp expressed in its natural conformation on OMVs improves the functional immune response as compare to the recombinant protein.

5.3.2 NHBA delivered in the lumen or on the surface of *E. coli* OMVs elicitates different functional antibody

OMVs preparations expressing NHBA variant p3 with or without Slam1 and relative controls preparations were included into the immunization scheme as summarized in **Figure 40**. CD-1 mice were immunized intra-peritoneally two times with 2 µg OMVs or 1 µg of recombinant NHBA (rNHBA) variant p2, formulated with aluminum hydroxide.



Antigen	Dose	Adiuvant	Route
Ø OMVs	2 µg	3 mg/mL Al (OH)3	IP
Slam1 OMVs	2 µg	3 mg/mL Al (OH)3	IP
NHBA OMVs	2 µg	3 mg/mL Al (OH)3	IP
NHBA+ Slam1 OMVs	2 µg	3 mg/mL Al (OH)3	IP
rNHBA	1 µg	3 mg/mL Al (OH)3	IP

Figure 40 Immunization scheme. Upper panel: five CD-1 mice were immunized intra-peritoneally (IP) two times, as indicated. Bottom panel: representation of the immunization scheme. Amount of native *E. coli* OMVs, adjuvant dose and the site of injection were indicated**.**

Elicited antibody titers were evaluated by ELISA assay using rNHBA protein as a coating antigen (**Figure 41**). ELISA revealed that all preparations, including NHBA OMVs, elicited α-NHBA antibodies which were specific and significantly higher than negative controls (Ø OMVs and Slam1 OMVs). Sera from mice immunized with NHBA+Slam1 OMVs show higher antibodies titers in comparison to sera of mice immunized with OMVs with only NHBA expression, and show a trend to be higher than mice immunized with 1µg of rNHBA protein.

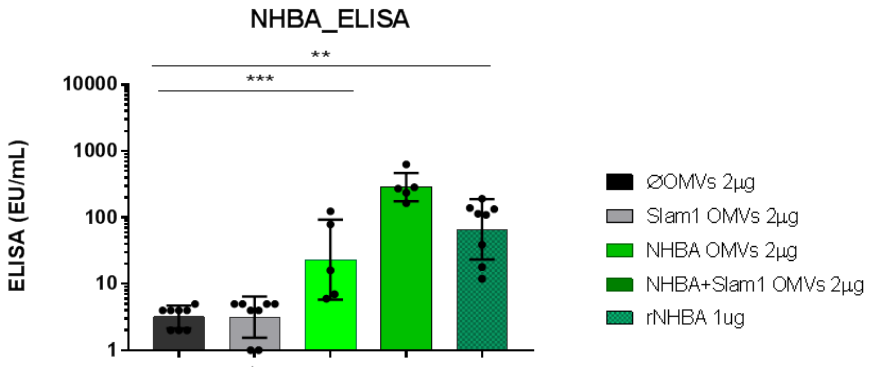
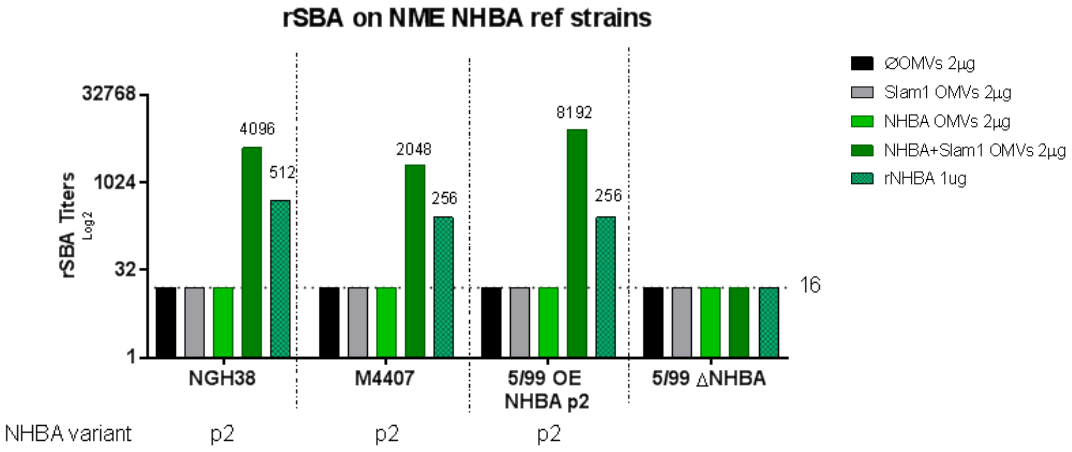


Figure 41 Analysis of sera from mice immunized with E. coli OMVs preparation or recombinant NHBA protein. IgG α-NHBA antibody titers elicited measured by ELISA. Each dot represents an individual mouse serum while bars indicates the median value within each immunization group. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test (**p<0.0060; ***p<0.0002)

Antibodies efficacy were evaluated with rSBA assay, performed with pooled mice sera, against a set of *N. meningitidis* reference strains, expressing differently NHBA variant p2 (**Figure 42.A**) or single mice sera (**Figure 42.B)**

A



B

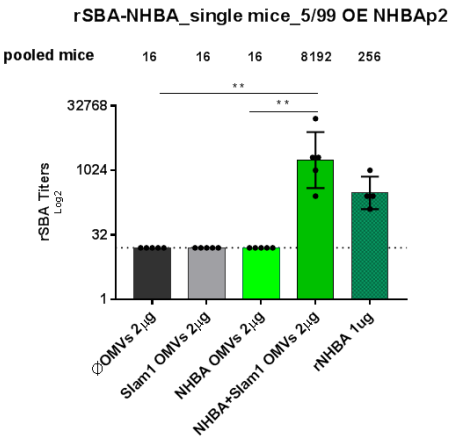


Figure 42 Serum bactericidal activity (SBA) performed on a panel of strains. **A)** Pooled mice sera were tested against strains naturally expressing NHBA p2 variant (NGH38 and M4497); Over expressing NHBAp2 (5/99 OE NHBA p2) or not expressing NHBA (5/99 ΔNHBA). rNHBA variant p2 immunized mice sera were also tested as a control. **B)** Single mice sera were tested against 5/99 OE NHBA p2 reference strain**.** Dots represent SBA titers of individual mouse sera against the defined strain. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test (test: **p<0,0051).

Conversely from what we observed with ELISA assay, no bactericidal activity was achieved by the sera derived from mice immunized with NHBA OMVs, whereas pooled sera from OMVs expressing both NHBA and Slam1 (NHBA+Slam1 OMVs) exhibited higher responses than the pooled sera from the group immunizes with 1 µg of recombinant protein. To be notice also that *E. coli* OMVs expressing NHBA variant p3 are able to kill the selected panel of *N. meningitidis* strains expressing the same variant of the rNHBA used in the immunization as control (NHBA

variant p2) and the bactericidal activity is specifically mediate by NHBA since the strain lacking NHBA (5/99ΔNHBA) in not killed with none of the sera.

SBA was performed using single mice sera against selected *N. meningitidis* strain: 5/99 over expressing NHBA strain (5/99 OENHBAp2) (**Figure 42**. B) confirmed results obtained with pooled mice sera.

6 Discussion and conclusions

OMVs have an intrinsic adjuvant effect on the antigens which are delivered. Immunization with OMVs purified from a pathogen can result in protection against the homologous bacteria [74, 95, 131]; but also against heterologous antigens that can be incorporated on the OMVs or combined in the same formulation [81, 91, 110]. Antigen localization in OMVs is thought to be important for the type of immune response induced [132], and surface localization is generally preferred because antigens can be easily engaged and presented by immune cells and stimulate high antibody levels [106]. Never the less, previous studies have shown the ability of *E. coli* OMVs to stimulate good immune responses when delivering foreign antigens, in their lumen [107, 108, 114, 115]. In this work we wanted generally to ask if Slam1 has an impact on immunogenicity of SLPs heterologously expressed in *E. coli* OMVs; and more specifically to exploit the role of Slam1 in surface localization of meningococcal SLPs in order to compare the immune response elicited by OMV delivered heterologous antigens with different localization.

Two of the main antigens of the multicomponent Bexsero vaccine against group B meningococcus included in the formulation as recombinant proteins [128, 129] are surface lipoproteins, namely Neisserial Heparin Binding Antigen (NHBA) and factor H binding protein (fHbp). And we chose to heterologously express these proteins with and without Slam1 in *E. coli*. Two different groups have recently investigated the heterologous surface localization of *N. meningitidis* fHbp (Hooda *et al*. and Fantappiè et al.) and NHBA (Fantappiè et al.) and two different outcomes emerged. Hooda et al. demonstrated the importance of Slam1 for correct fHbp display [59]. On the other hand, Fantappiè and co-workers, proposed the presence of a new sub-class of Surface Lipoproteins which is able to reach the bacterial surface even when transplanted in a different Gram-negative bacterium, suggesting that no additional translocator components are necessary for heterologous lipoproteins surface delivery [130].

In this study, we generated *E. coli* strain expressing Nm SLPs with or without Slam1 by using expression plasmids, we checked surface translocation of selected SLPs in different growth conditions and investigate the differences in the immunogenicity due to the presence of Slam1.

Our results indicate that Slam1-dependency in surface exposure of the analyzed SLPs depends on the growth conditions, and possibly on the extent of the over-expression of the heterologous expression levels. In particular when SLPs expression was induced from the beginning of the growth, Slam1 was necessary for the surface localization of fHbp and NHBA as *Hooda et al*. described. On the contrary, when protein expression was induced maximally, for three hours, only when bacteria have reached exponential phase, no differences were observed in fHbp surface localization, similarly to what *Fantappiè et al.* showed.

Different hypothesis could be made to explain this phenomenon. *E. coli* could activate unknown mechanisms which allow *N. meningitidis* SLPs surface display. For example it is possible that the

same mechanism involved in the anchoring of RcsF to *E. coli* outer leaflet of the OM, could have a role in this [48, 133, 134]. Also, expression levels reached could, in some way, saturate the periplasm and impact on the outer membrane stability/integrity, therefore making fHbp detectable from antibodies even if it is attached to the OM but facing the periplasm. Or possibly under maximal over-expression conditions, a subset of the cells die and lyse and SLPs re-associate with the surface of the *E. coli* cells.

Nevertheless, from our results it is clear that we can generate *E. coli* expressing fHbp and NHBA with different localization depending on the presence of Slam1 and, assuming that OMVs are a representation of the bacterial outer membrane, we can obtain OMVs preparations enriched with heterologous lipoproteins either within the lumen or surface exposed. In particular, we generated OMVs from *E. coli* expressing the fHbp and NHBA SLPs either on the surface or not depending on the co-expression of Slam1. SLPs expression increases in the presence of Slam1, this is particularly evident for fHbp which was 10-fold more and becomes the most abundant protein within *E. coli* OMVs when co-expressed with Slam1. Instead the quantity of NHBA in OMVs when co-expressed with Slam1 was at most 2-fold more in the presence of Slam1. It is possible that the correct localization of fHbp and to a much lesser extent NHBA, or an additional stabilizing function of Slam1 could result in the accumulation of SLPs in the heterologous outer membrane. These data are in alignment with the impact of Slam1 on expression of fHbp in meningococcus that we described in the previous chapter. Sera from mice immunized with OMV preparations expressing SLPs at different levels and with different localization due to Slam1 presence were analyzed in comparison to sera from mice immunized with the respective recombinant proteins. Both OMVs co-expressing fHbp and Slam1 or NHBA and Slam1 were able to elicit higher bactericidal titers compared to the preparations expressing SLPs alone or the control recombinant proteins. Even if ELISA titers show induction of similar antibody levels, the functionality of such antibodies were significantly different. This clearly demonstrates the advantage of co-expression of Slam1 on the immunogenicity of heterologously expressed SLPs. Importantly no killing was achieved by the sera derived from mice immunized with NHBA OMVs preparation. In both cases the presence of Slam1 positively stimulates the production of functional α-fHbp or α-NHBA antibodies confirming the necessity of the translocator component for the correct localization of the selected SLPs on *E. coli* surface and the reinforcing the concept that surface display is preferred for the generation of functional immune system responses.

7 Materials and methods

7.1 Bioinformatics analysis

Alignment of the first 50 aminoacid of the predicted list of lipoproteins was performed using the Clustal W algorithm incorporated within the Geneious software (Biomatters).

7.2 Generation *N. meningitidis* recombinant strains and plasmids for *E. coli* proteins expression

7.2.1 *Construction of N. meningitidis* mutants and complemented strains

All recombinant strains and plasmids used in this study are listed in **Table 9**, while all the oligonucleotides used are listed in **Table 8**. DNA manipulations were carried out routinely as described for standard laboratory methods [135]. Generation of 5/99 recombinant strains (5/99 ΔNadA ΔNHBA; 5/99 ΔNadA ΔNHBA oeNHBA p2 and 5/99 ΔNadA ΔNHBA ΔfHbp) are described in *Serruto et al.* [50].

The plasmid pGEMT-UD*slam1*Kan was constructed to generate the *nmb0313* isogenic mutant (ΔSlam1 strains). This plasmid contains the Kanamycin resistance gene (KanR) within the *nmb0313* upstream and downstream flanking regions. Briefly flanking regions of *nmb0313* were amplified from the MC58 chromosome by PCR using KAPA Hi-Fi polymerase (KAPA Biosystem). Upstream (0313UP_F/NMB0313RV UP) and downstream (NMB0313RV DO/0313DO_R) flanking were amplified, purified and digested with appropriate restriction enzyme sites and cloned into pGEMT plasmid. Kanamycin cassette was cloned as 1,4 kb XbaI fragment into the XbaI site between the two flanking regions. This plasmid was used to transform NGH38 and MC58 *N. meningitidis* strains using Kanamycin resistance cassette for selection. Complementation of *nmb0313* was achieved by insertion of a copy of the *nmb0313* in the noncoding region between the converging ORF NMB1428 and NMB1429 of Δ0313 strains chromosome (CiSlam1). To do this, plasmid pInd0313 was generate by amplifying *nmb0313* gene (primers 0313 pC_F/R) from chromosome and cloned as AseI/NsiI fragment under the control of the inducible promoter pTac and the LacI repressor into pComPInd plasmid [136]. Both NGH38ΔSlam1 and MC58ΔSlam1 were complemented *ex-locus* with this construct and Chloramphenicol resistance cassette present in the plasmids was used for selection.

Fusion proteins, with N-terminal portion exchanged between fHbp and NHBA, (N-f and f-N fusions) sequences were ordered from Invitrogen and cloned in pCompIND plasmid. Nucleotide sequences of N-f fusion (NHBA pC_F/fHbp pC_R) and f-N fusion (fHbp pC_F/NHBA pC_R) were amplified by PCR using KAPA Hi-Fi polymerase (KAPA Biosystem). Inserts were cloned as NdeI/NsiI in pCompIND vector. This plasmid was used for *ex-locus* complementation, as described above, in MC58 *ΔNHBA ΔfHbp* background. Truncated NHBA forms were generated by

mutagenize each serines (2132mS1 F/R and 2132mS2F/R) in the cleavage site of NHBA already cloned in pCompIND vector. This plasmid was used for *ex-locus* complementation in MC58 *ΔNHBA* background.

The correct nucleotide sequence of each plasmid was confirmed by DNA sequencing. Plasmids were linearized and used for the transformation of the *N. meningitidis* strains. All meningococcal transformants were verified both by PCR analysis and Western blot.

All primers (**Table 8**) and generated plasmids (**Table 9**) and transformants (**Table 10**) are reported in the appendix.

7.2.2 *E. coli* expression plasmids cloning

DNA manipulations were carried out routinely as described for standard laboratory methods [137].

For fHbp and Slam1 co-expression two different plasmids, with compatibles origin of replications (ORI) and antibiotics resistance cassettes, were used for express singularly Slam1 and fHbp. *Slam1* sequences were amplified (i0313_F/R) from *N. meningitidis* MC58 genome by PCR using KAPA Hi-FI polymerase (KAPA Biosystem) and cloned as MfeI-XhoI fragment into MCS2 pCOLA Duet empty vector. *fHbp* v1.1 from *N. meningitidis* MC58 was cloned into pCompIND inducible plasmids [136, 138] and used for fHbp expression. BL21 (DE3) *E. coli* strain was transformed with both plasmids together and as negative controls *E. coli* were transformed with pCOMpIND *fHbp* and pCOLA empty; pCOMpIND empty and pCOLA *Slam1* or pCOMpIND empty and pCOLA empty.

To construct *E. coli* co-expressed fHbp or NHBA with Slam1 on the same vector pet Duet plasmid family were used. Sequences were amplified from *N. meningitidis* MC58 genome by PCR using KAPA Hi-FI polymerase (KAPA Biosystem). *fHbp* (ifHbp_MCS1_F/R) and *NHBA* (iNHBA_MCS1_F/R) were amplified and inserts were cloned as NcoI/NotI in the Multi Cloning site (MCS1) of pCOLA empty vector, for single expression; or Slam1 pCOLA plasmid for proteins co-expression. Correct nucleotide sequence of each plasmid was confirmed by DNA sequencing.

All plasmids generated and primers used in this study were summarized in the appendix section in the **Table 9** and **Table 8** respectively.

7.3 Bacterial strains and culture conditions

7.3.1 *N. meningitidis* growth conditions

Neisseria meningitidis (Nm) serogroup B strains (MC58, NHGH38 and its isogenic derivatives) and Escherichia coli (Ec) (DH5α) strains used in this study are listed in **Table 10**.

N. meningitidis strains were routinely grown on Gonococcus (GC) agar (Difco) plates supplemented with Kellogg's supplement I at 37°C, 5% CO2 overnight. When required,

Kanamycin or Chloramphenicol was added on the plates (final concentration of 150µg/mL and 5µg/mL respectively). Liquid cultures were grown under the same conditions in GC with Kellogg's supplement I. Colonies from overnight growth were used to inoculate 7 ml cultures at ~0.05 OD_{600}/mL. The cultures were incubated at 37°C with shaking until exponential phase (~0.5 OD_{600}/mL). When required isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) was added to culture medium at the indicated final concentrations. Strains were stocked in GC medium with 15% glycerol and were stored at −80°C.

7.3.2 *E. coli* growth conditions

The list of bacteria strains used in this work is reported in the table of strains (Table 10). *E. coli* DH5α [139] and BL21 (DE3) were used for cloning and proteins expression respectively.

Plasmids cloning were performed with DH5α *E. coli* strain; bacteria were cultured on Luria Bertani (LB) medium in the presence of Kanamycin (final concentration 50µg/mL) or Ampicillin (final concentration 100µg/mL) for selection.

BL21 (DE3) *E. coli* strains were cultured on LB agar plate or LB broth at 37 °C, in the presence of Kanamycin and/or Ampicillin to achieved final concentration of 50 µg/mL or 100 µg/mL respectively. When required, isopropylβ-D-1-thiogalactopyranoside (IPTG) (Sigma) at different final concentrations (0.001; 0.01; 0.1 mM) was also added.

7.4 Expression of Slam1 recombinant protein for Slam1 anti-sera production

The gene fragment encoding Slam1 (*nmb0313*), corresponding to residues 32-488, was amplified by PCR from *N. meningitidis* MC58 genomic DNA using primers (i313His_F/R) listed in the table of primers (**Table 8**). PCR fragment was cloned into the pET21 vector using the PIPE method [140] to obtain a protein lacking the signal peptide and carrying a 6X His-tag at the C-terminus. Protein expression was performed in *E. coli* BL21(DE3) cells, by using EnPresso B growth systems (BioSilta, Cambridgeshire, United Kingdom) supplemented with 100 µg/ml ampicillin. Bacteria were grown at 30°C for 12 h, and recombinant protein expression was then induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25°C for other 24 hours. Bacteria were harvested by centrifugation at 4000rpm for 20 minutes at 4°C and lysated by sonication. Protein was extracted from the insoluble fraction with Tris HCl 20mM, NaCl 300mM, Urea 8M, pH8 and then purified by immobilized metal ion affinity chromatography (IMAC) using HiTRAP (GE Healthcare Life Sciences) and eluted in PBS dilutions in Tris HCl 20mM, pH8.

5 CDI Female 6-8 weeks old received 20 µg of purified recombinant Slam1 protein adjuvanted with 3 mg/ml aluminum hydroxide, for three immunizations intra-peritoneal, with a 3 weeks interval. Sera were collected after 49 days at bleed out.

7.5 Bacteria total lysate preparation.

N. meningitidis or *E. coli* colonies from overnight plate cultures were re-suspended in proper media to ~0.5 OD_{600}/mL. One milliliter of the suspension was centrifuged for 5 min at 15000 × *g* and the pellet re-suspended in 100 µl in 2X SDS-PAGE loading buffer (50 mM Tris Cl [pH 6.8], 2.5% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 50 mM dithiothreitol [DTT]) [141]. For liquid culture, 1 ml of each sample was collected and the concentration normalized in 2x SDS-PAGE loading buffer respect to the relative optical density at 600 nm.

7.6 Polyacrylamide gel electrophoresis and Western blotting

Protein extracts were separated by SDS-PAGE on NuPAGE® Novex® 4-12% Bis-Tris Protein Gels in MES 1X (Life Technologies) and transferred onto nitrocellulose membrane using an iBlot Dry Blotting System (Invitrogen). Membranes were blocked for 1 hour at room temperature with PBS with 0.05% (v/v) Tween 20 (Sigma) and 10% (w/v) powdered milk (Sigma) and then incubated for 60 min at room temperature with the specific primary antibodies diluted in PBS + 0.05% (v/v) Tween 20 (Sigma) and 3% (w/v) powdered milk (Sigma). A horseradish peroxidase-conjugated anti-mouse IgG antibody were diluted (1:5000) in PBS + 0.05% (v/v) Tween 20 (Sigma) and 3% (w/v) powdered milk (Sigma) and the Western Lightning ECL (Perkin Elmer) were used according to the manufacturer's instructions. Antibodies dilutions used were specified in the **Table 7**.

7.7 Fluorescence Activate Cell Sorting (FACS) analysis

Strains were growth (plate or liquid) as described in paragraph 7.3 and OD_{600} 0.05 bacteria, for each antibodies staining, were collected by centrifugation (10000 × *g* for 5 min). Pelleted bacteria were fixed by 1h incubation at room temperature with PBS containing 0.5% (v/v) formaldehyde (Sigma). Fixed bacteria are then suspended in PBS 1%BSA and 10 µl of preparation is plated on GC agar plate and incubated O/N at 37°C to check inactivation (for menigococcal bacteria) or LB (for *E. coli*). Inactivated bacteria are then incubated for 1 h at room temperature with secondary antibody alone, mouse monoclonal antibodies or mouse polyclonal sera diluted to specific concentrations indicated in the **Table 7** in PBS containing 1% (w/v) BSA. Subsequently cells were incubated for 1 h at room temperature with a secondary rabbit anti-mouse immunoglobulin G (whole molecule) FITC-conjugated (Sigma). After a final washing step,

cells were re-suspended in 200 µl of PBS. All data were collected using a BD FACS CANTO II (BD Bioscience) by acquiring 10,000 events, and data were analyzed using the Flow-Jo software (v.8.6, TreeStar Inc.).

7.8 Quantitative real-time PCR (qRT-PCR) experiments

Bacterial cultures were grown in 7 ml of liquid medium to until exponential phase. Three ml of the culture were then poured onto 3 ml of frozen medium to immediately chill the culture and stop transcriptional changes. Cells were then harvested by centrifugation at 3400 × *g* for 10 min. Total RNA was isolated using an RNeasy Mini kit (Qiagen) as described by the manufacturer. A second step of DNase treatment was performed using RQ1 RNase-free DNase (Promega), for one hour at 37°C and purified with the RNeasy Mini kit. RNA was quantified using a Nanodrop 1000 spectrophotometer and its overall quality was assessed by running samples on a 1% agarose gel. 2 µg of total RNAs were reverse-transcribed using random hexamer primers and SuperScript® II RT (ThermoFisher), following manufacturer's instructions. As controls, all RNA samples were also incubated without reverse transcriptase.

Quantitative real time-PCR was performed in triplicate per sample in a 25 µl reaction volumes using Platinum® SYBR® Green qPCR SuperMix-UDG with Rox (ThermoFisher) according to the manufacturer's instructions and containing 2.5 ng of cDNA, and 0.4 µM of gene-specific primers (See **Table 8**). Amplification and detection of specific products were performed with a Mx3000P Real-Time PCR system (Stratagene) using the following procedure: 95ºC for 10 min, followed by 40 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s then ending with a dissociation curve analysis. The *16S RNA* gene was used as the endogenous reference control and the relative transcript change was determined using the $2^{-\Delta\Delta Ct}$ relative quantification method [142].

7.9 OMVs purification

7.9.1 Isolation of native *N. meningitidis* Outer Membrane Vesicles (OMVs)

The nOMV vaccines were prepared growing NGH38 and derivative strains in MCDMI - Meningitidis Chemical Define Medium I- medium. The *N. meningitidis* strains were first pre-inoculated into 7 mL of MCDMI at an OD_{600} ranging from 0.15-0.2 and incubated at 37°C at 180 rpm until mid-exponential phase (=0.8-0.9 OD). Due to the stable nature of the chromosomal integration, no antibiotics were added to the growth medium. The mid-exponential pre- cultures were inoculated into 50 mL medium in 250 mL baffled flasks and grown over night (16-18 h) until late stationary phase at 37°C and 180 rpm. After Overnight growth total lysate for Western Blot analysis and fixed bacteria for FACS analysis were collected. Bacterial cells were pelleted by centrifugation for 30 min at 5000 × *g* and 4°C and supernatants filtered (pore size 0.22 µm, millipore). nOMVs were collected from cell free supernatants by high speed centrifugation, 2 hours at 175,000 × *g*, 4°C. An additional washing step with 1X PBS was performed. OMVs pellets were re-suspended in 50-200 µl of 1X PBS and filtered with 0,22µm filter (millipore). OMV were quantified through the Lowry assay (DC Protein Assay, BioRad) for total protein content following the manufacturer's instructions.

Table 5 Growth defined media MCDMI recipe

MCDMI		
Component	**Final concentration**	
	(g/L)	**(mMol)**
Soy peptone (BBL Phytone)*	15	NA
Sodium chloride	5.8	99.2
Potassium sulphate	1	5.7
Potassium phosphate dibasic	4	23.0
L-glutamic acid	5	34.0
L-arginine	0.3	1.7
L-serine	0.5	4.8
L-cysteine	0.25	1.9
Magnesium chloride hexahydrate	0.41	2.0
Calcium chloride **	0.021	0.189
Ferric citrate ***	0.002	0.008
Sodium DL-lactate	7.5	66.9
Glycerol ****	5	NA
pH 7.2		

7.9.2 Isolation of native *E. coli* Outer Membrane Vesicles (OMVs)

BL21(DE3) strains were pre-cultured over-day in 7 ml of LB medium supplemented with of Kanamycin final concentration 50µg/ml. Cultures were diluted 1:100 in 50 ml of HTMC liquid medium with Kanamycin 50µg/mL and IPTG at the properly final concentrations (specified for each experiment) and incubated overnight (14-16 h) at 30°C, 5% CO_2, 160 rpm. After Overnight growth total lysate for Western Blot analysis and fixed bacteria for FACS analysis were collected. Bacterial cells were pelleted by centrifugation for 30 min at 5000 × *g* and 4°C and supernatants filtered (pore size 0.22 µm, millipore). nOMVs were collected from cell free supernatants by high speed centrifugation, 2 hours at 175,000 × *g*, 4°C. An additional washing step with 1X PBS was performed. nOMV pellets were re-suspended in 50-200 µl of 1X PBS and filtered with 0,22µm filter (millipore). OMV were quantified through the Lowry assay (DC Protein Assay, BioRad) for total protein content following the manufacturer's instructions.

Table 6 Growth defined media HTMC recipe

HTMC	
Component	**g/L**
Glicerol	15
Yeast Extract(polvere)	30
$MgSO_4 \circ 7H_2O$	0,5
K_2HPO_4	20
KH_2PO_4	5

*pH 7.35 with KOH 1M

7.10 Negative Stain Electron microscopy

OMVs were fixed using 4% PFA in PBS. Droplets of sample suspensions (10 µl) were placed on formvar-carbon coated grids and allowed to adsorb for 60 sec. Excess liquid was removed gently touching the filter paper. The adsorbed specimen was then processed for negative-staining, by first washing the specimen grid on a drop of negative stain (2% uranyl acetate in distilled water), blotting and repeating this step once more, this time leaving the specimen grid for 60 sec on a new drop of negative stain solution. Samples were observed at a JEOL 1200 EX II electron microscope. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software.

7.11 Ethical statement

Animals were housed in the GSK Vaccines Animal Facility and experiments were conducted in compliance with the ARRIVE guidelines, the current Italian legislation (Legislative Decree 116/92), and with the GSK Animal Welfare Policy and Standards.
All animal experiments were performed in compliance with the approval of the local Animal Welfare Body (AWB2015-01).

7.12 Mice immunization

Groups of 8 or 5 mice between 6- to 8-week old female CD-1 mice (Charles River) were immunized intra-peritoneally (IP).
For each injection, the mice received a total dose of 2.5, 2 or 0.2 μg of OMV or 1 μg of recombinant proteins as described for each specific experiment in the result paragraphs. The OMV or the recombinant protein vaccines were absorbed with aluminum hydroxide (Alum, 3 mg/ml) and administered in two doses at day 1, 21. Blood was taken at day 0; for further analysis and bleed out was performed on day 34. The experiment complied with the relevant guidelines of Italy and the institutional policies of GSK Vaccines

7.13 ELISA

Serum antibody titers against fHbp or NHBA were measured by ELISA. Microtitter plates were coated overnight at 4°C with 0.015 μM of purified recombinant proteins (rfHbp v1.1 or rNHBA p2) and incubated overnight at 4°C. Afterwards saturation buffer (2.7% polyvinylpyrrolidone 10 in water) was added to each well and plates incubated for 2 hours at 37°C. Each wells were, then, incubated with the single mice sera followed by alkaline phosphatase-conjugated anti-mouse antibodies diluted 1:2000 in dilution buffer (1% BSA, 0.05% Tween-20, in PBS 0.074M). Each steps were followed by three washing steps with the washing buffer (0.05%Tween20,in PBS 0.074M). In the end p-nitrophenyl phosphate was added and optical density was analyzed using a plate reader at a dual wavelength of 405/620-630 nm. Antibody titers were quantified via interpolation against a reference standard curve.

7.14 Serum Bactericidal Activity (SBA) analysis

Serum bactericidal activity against *N. meningitidis* strains was evaluated as previously described [143] with pooled baby rabbit serum (Cedarlane) used as the complement source (rSBA). Bacteria were grown at 37°C with shaking until early exponential phase (OD600 of ~0.25) in Mueller Hinton broth (MH), plus 0.25% (w/v) glucose, when required 1mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) was added. Bacteria are then diluted in Dulbecco's saline phosphate buffer (Sigma) with 0.1% (w/v) glucose and 1% (w/v) BSA (Bovine Serum Albumin)

to approximately 10^5 CFU/ml. The incubation with 25% of baby rabbit complement with or without polyclonal mouse sera at different dilutions was performed at 37°C with shaking, for 60 min.

Serum bactericidal titers were defined as the serum dilution resulting in a at least 50% decrease in the CFU/ml of bacteria after 60 min of incubation with the reaction mixture, compared to the control CFU/ml at time zero.

8 Appendix

Table 7 List of the antibodies and respective working dilutions

Table of antibodies				
	N. meningitidis		E.coli	
Antibodies	**WB dilution**	**FACS dilution**	**WB dilution**	**FACS dilution**
α-NHBA polyclonal serum	1: 2000	1: 800	/	/
α-Mip polyclonal serum	1: 1000	1: 100	/	/
α-fHbp polyclonal serum	1: 5000	1: 1000	1: 5000	1: 1000
α-NHBA Nterm monoclonal Ab	/	/	1: 4000	1: 800
α-Slam1 polyclonal serum	1: 1000	/	1: 1000	/
α-mouse-FITC	/	1:1000	/	1:1000
α-mouse-HRP	1:2000	/	1:5000	/

Table 8: Table of primers.

Restriction sites are underlined when present

Table of primers			
Primer Name	**Restriction site**	**Sequence**	**Application**
		N. menigitidis	
0313UP_F	XmaI	GagatctagaGCCGGcattcgggcaaaaacc	Slam1 KO_Up flanking
0313DO_R	XhoI	gtgtctcgagCTTTTCGCCGATACGGTTTG	Slam1 KO_Do flanking
NMB0313_RV_UP	XbaI	AACAGCAACCCGGGTATCAATCGGCGGAT	Slam1 KO_Up flanking
NMB0313_FW_DO	XbaI	CCGATTGATACCCGGGTTGCTGTTCCTTTTCG	Slam1 KO_Do flanking
0313 pC_F	NdeI	Gtgtattaatatggttatttttattttgtg	Amplification Slam1
0313 pC_R	NsI	Gtgtatgcattcagaacgttttattaaactc	Amplification Slam1
NHBA pC_F	NdeI	atatcatatgTTTAAACGCAGCGTAATCG	Amplification NHBA
NHBA pC_R	NsI	ATATATGCATTCAATCCTGCTCTTTTTTGC	Amplification NHBA
fHbp pC_F	NdeI	ATATCATATGAATCGAACTGCCTTCTGC	Amplification fHbp
fHbp pC_R	NsI	ATATATGCATTTATTGCTTGGCGGCAAG	Amplification fHbp
2132 mS1 R		TTATAAACCTAAACCCACTT**G**ATTTGCGCGATTTAGGCGTT	Mutagenesis of serine1 NHBA in vector for NHBA p3 variant *ex-locus* complelmentation
2132 mS1 F		AACGCCTAAATCGCGAAAT**C**AAGTGGGTTTAGGTTTATAA	Mutagenesis of serine1 NHBA in vector for NHBA p3 variant *ex-locus* complelmentation
2132mS2 R		TTCTGCACGGTCGAGGCGGT**G**ACTTCCGGCCGAGATGCCGCT	Mutagenesis of serine2 NHBA in vector for NHBA p3 variant *ex-locus* complelmentation
2132mS2F		AGCGGCATCTCGGCCGGAAGT**C**ACCGCCTCGACCGTGCAGAA	Mutagenesis of serine2 NHBA in vector for NHBA p3 variant *ex-locus* complelmentation
S3_0313F		GCAAATTTCCAATTCGCTGG	Slam1 Sequencing check
S5_0313R		CGCGGGACAACAATTCG	Slam1 Sequencing check
S_0313F		GCCTGCCGTCATATCGTTG	Slam1 Sequencing check
S_0313R		CAACGATATGACGGCAGGC	Slam1 Sequencing check
pKX-US-F		CCGGCTCGTATGTTGTGTGG	sequencing check pGEMt insertion
pKX-DS-R		CGAAAGGGGGATGTGCTGC	sequencing check pGEMt insertion
Tac2_fw		GCATAATTCGTGTCGCTCAAGG	pCOM pInd insertion and sequencing check

Primer Name	Restriction site	Sequence	Application
COM-C-REV		ACCGGCATCGGCAACTACAC	*Ex-locus* complementation check
CM-C-Fwd		CCTCGAGCCGCTGACCGAAGG	*Ex-locus* complementation check
ComSQ		tttgaaaatgagattgagc	pCOM pInd insertion and sequencing check
kan_int_fwd		ATTATCGAGCTGTATGCGGAGTG	Kanamicine insertion check
kan_int_rev		GCAATCCACATCGGCCAGAT	Kanamicine insertion check
pRTNM16sII.F2		CACACTGGGACTGAGACACG	qRT-PCR *16S* gene
pRTNM16sII.R2		CAGCCTTTTCTTCCCTGACA	qRT-PCR *16S* gene
pRTfHbpU.F		GGCTTGCCGATGCACTAAC	qRT-PCR *fhbp* gene
pRTfHbpU.R		GTTTTTTCCGCACCTTGTGC	qRT-PCR *fhbp* gene
		E. coli	
i0313F	Mfe1	GCAGATCTCAATTGatggttatttttttattttgtg	Cloning of Slam1 in pCOLA vector MCS2
i0313R	XhoI	TTACCAGActcgagtcagaacgttttattaaactc	Cloning of Slam1 in pCOLA vector MCS2
ifHbp_MCS1_F	NcoI	GGAGATATAccatggTGAATCGAACTGCCTTCTG	Cloning of fHbp v1.1 in pCOLA vector MCS1
ifhbp_MCS1_R	NotI	AGCATTATgcggccgcTTATTGCTTGGCGGCAAG	Cloning of fHbp v1.1 in pCOLA vector MCS1
iNHBA_MCS1_F	NcoI	GGAGATATAccatggTCTTTAAACGCAGCGTAATC	Cloning of NHBA p3 in pCOLA vector MCS1
iNHBA_MCS1_R	NotI	AGCATTATgcggccgcTCAATCCTGCTCTTTTTTGC	Cloning of NHBA p3 in pCOLA vector MCS1
ACYCDuetUP1_FW		GGATCTCGACGCTCTCCCTT	universal primer FW MCS1 pCOLADuet-1
DuetDOWN1_RV		GATTATGCGGCCGTGTACAA	universal primer RV MCS1 pCOLADuet-1
DuetUP2_FW		TTGTACACGGCCGCATAATC	universal primer FW MCS2 pCOLADuet-1
T7 Terminator_RV		GCTAGTTATTGCTCAGCGG	universal primer RV MCS2 pCOLADuet-1

Table 9 Table of plasmids used in this study

KanR: Kanamycine resistance; AmpR: Ampicillin resistance; CmR: Chloramphenicol resistance

Table of plasmids

NAME	Description	Antibiotic resitance cassette	Reference of source
	N. meningitidis		
pGEM-T	*E. coli* cloning vector	AmpR	Promega
pET21	*E. Coli* expression vector	AmpR	
pET_0313	*E. Coli* expression vector with 0313 (aa 32-488) fro protein purifiaction	AmpR	This study
pGEMT-UDnmb0313Kan	pGEM-T containing the flanking region of nmb0313 with Kan resistance cassette cloned as XmaI fragment between flanking regions	AmpR, KanR	This study
pComPIND CmR	Plasmid for allelic replacement at a chromosomal location between ORFs NMB1428 and NMB1429 and inducible expression under the control of the PTAC promoter and the lacI repressor. Upstream of the cloning site is a Cm resistance cassette	AmpR, CmR	REF [60]
Pind_0313	Plasmid for the complementation of *nmb0313* in the Com region with an IPTG-inducible tac prmoter. In *Δnmb0313* background	AmpR, CmR	this study
Pind_N-f	Plasmid for the complementation of NHBA-fHbp fusion protein in the Com region with an IPTG-inducible tac prmoter. In MC58 *ΔNHBA ΔfHbp* background	AmpR, CmR	this study
Pind_f-N	Plasmid for the complementation of fHbp-NHBA fusion protein in the Com region with an IPTG-inducible tac prmoter. In MC58 *ΔNHBA ΔfHbp* background	AmpR, CmR	this study
pIND_NHBA S1TGA1	Plasmid for the complementation of short NHBA truncated form in the Com region with an IPTG-inducible tac prmoter in MC58 *ΔNHBA* background	AmpR, CmR	this study
pIND_NHBA S2TGA2	Plasmid for the complementation of long NHBA truncated form in the Com region with an IPTG-inducible tac prmoter. In MC58 *ΔNHBA* background	AmpR, CmR	this study

Table of plasmids

NAME	Description	Antibiotic resitance cassette	Reference of source
	E. Coli		
Empty (Ø)	pCOLA DUET vector: encodes two multiple cloning sites(MCS). With T7 promoter, COLA replicon from ColA, lacI repressor and KanR	Kanamycin	Novagen
Slam1	Construct to express recombinant *N.meningitidis* Slam1 protein in MCS2 of pCOLA Duet in *E. coli.*	Kanamycin	This study
NHBA	Construct to express recombinant *N.meningitidis* NHBA p3 protein in MCS1 of pCOLA Duet in *E. coli*	Kanamycin	This study
fHbp	Construct to express recombinant *N.meningitidis* fHbp v1.1 protein in MCS2 of pCOLA Duet in *E. coli.*	Kanamycin	This study
NHBA_Slam1	Construct to co-express recombinant *N.meningitidis* NHBA p3 and Slam1 proteins in MCS1 and MCS2 respectively of pCOLA Duet in *E. coli.*	Kanamycin	This study
fHbp_Slam1	Construct to co-express recombinant *N. meningitidis* fHbp v1.1 and Slam1 proteins in MCS1 and MCS2 respectively of pCOLA Duet in *E. coli.*	Kanamycin	This study
pIND fHbp	Plasmid fHbp expression with an IPTG-inducible tac prmoter.	Ampicillin, Cloramphenicol	Biagini et al 2016

Table 10 Table of strains

KanR: Kanamicine resistance, AmpR: Ampicillin resistance, CmR Chloramphenicol resistance

Table of Strains

NAME	Description	Antibiotic resitance cassette	Reference of source
	***N. meningitidis* strains**		
MC58	Laboratory-adapted *N.meningitidis* reference strain		
MC58 ΔSlam1	*Slam1* null mutant of MC58	KanR	this study
MC58 CiSlam1	Complemented *Slam1* in Δ*Slam1* MC58 background	KanR, CmR	this study
MC58ΔNHBA	*Nhba* null mutant of MC58	EryR	Ref
MC58ΔNHBAΔfHbp	*Nhba* and *fHbp* null mutants in MC58	EryR	
MC58 N-f	Complemented mutant expressing NHBA-fHbp fusion protein under the control of IPTG inducible promoter.	EryR, CmR	this study
MC58 f-N	Complemented mutant expressing fHbp-NHBA fusion protein under the control of IPTG inducible promoter.	EryR, CmR	this study
MC58 NHBA S1TGA1	Complemented mutant expressing short NHBA truncated form in the Com region with an IPTG-inducible Tac prmoter in MC58 ΔNHBA background	EryR, CmR	this study
MC58 NHBA S2TGA2	Complemented mutant expressing long NHBA truncated form in the Com region with an IPTG-inducible Tac prmoter in MC58 ΔNHBA background	EryR, CmR	this study
NGH38	Clinical Isolate		
NGH38 ΔSlam1	*Slam1* null mutant of NGH38	KanR	this study
NAME	**Description**	**Antibiotic resitance cassette**	**Reference of source**
NGH38 CiSlam1	Complemented *Slam1* in Δ*Slam1* NGH38 background	KanR, CmR	this study
5/99	Clinical Isolate		

5/99 ΔNadA ΔNHBA	*NadA* and *NHBA* null mutants in 5/99 strain	
5/99 ΔNadA ΔNHBA oeNHBA p2	Complemented *NHBA* variant p2 under the control of IPTG inducible promoter	KanR, EryR, CmR
5/99 ΔNadA ΔNHBA ΔfHbp	*NadA,NHBA* and *fHbp* null mutants in 5/99 strain	KanR, EryR, CmR

***E. coli* strains**		
Escherichia coli DH5-α	supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Life Technologies
Escherichia coli BL21-DE3	hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)	Life Technologies

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