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Biochemical and functional characterization of the binding of *Neisseria* Adhesin A (NadA) with Siglec-5 and 14

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Summary

Neisseria adhesin A (NadA) is a proteic recombinant antigen included in Bexsero, a vaccine against serogroup B *Neisseria meningitidis* (*N. meningitidis*). NadA belongs to the trimeric autotransporters family and is composed of a long coiled-coil with three protruding wing-like structures that create an unusual N-terminal head domain. Even though NadA has been extensively described as promoter of both adhesion to and invasion into human epithelial cells, its receptors at the interface with human cells have never been found. To identify the targeted human receptors, we used recombinant NadA (rNadA) as probe on a protein microarray including a expanded collection of surface and secreted human proteins. The screening disclosed three putative interactors, Siglec-5, Siglec-14 and FcγRIIA, expressed on cells of myeloid lineage. We first validated these interactions addressing the biochemical features of the binding, confirming Siglec-5 and -14 as high affinity NadA binding proteins, while FcγRIIA was not. Next, we assessed the membrane properties of NadA binding to Siglecs by using *Escherichia coli* as an heterologous system. Full-lenght Siglecs, expressed on CHO-K1 cells showed an increase of adhesion of *N. meningitidis*, whereas the isogenic strain knock-out NadA did not. Although recombinant Siglec-5 ectodomain was shown to bind to NadA on both capsulated and unencapsulated *N. meningitidis*, we could not find any relevant evidence of NadA/Siglecs interaction on primary and immortalized monocytic cell lines.

In recent years, soluble forms of Siglecs were detected in human serum. In this study, the soluble Siglecs presence was confirmed in human pooled sera and in culture media of monocytes and macrophages. Interestingly, we found that during infection assays *N. meningitids* was able to bind monocytes-released Siglecs in NadA dependent manner. The exogenous addition of the soluble species increased the attachment of bacteria on monocytic cells surface but reduced internalization.

We also demonstrated a novel interaction between Siglecs and complement component 1 (C1q), whose *in vitro* interaction was inhibited by recombinant NadA in a concentration dependent manner. Further, we observed a survival advantage for *N. meningitidis* in presence of bactericidial antibodies anti-NadA by testing sera, as source of complement, depleted of Siglec-5 and -14.

In summary, this work revealed two new human interactors for the neisserial adhesin that could be encountered not only on phagocytes surfaces but also in the bloodstream. On the host side, we characterized a new proteic target for Siglecs on *N. meningitidis* bacterial surface. Moreover, the C1q binding to Siglec-5 and -14 was revealed for the first time, opening questions on Siglecs biology.

In conclusion we found that NadA may not contribute exclusively to the crossing of the epithelial nasopharyngeal barrier but also it may help the bacterium to survive into extracellular host milieu.

Riassunto

Neisseria adhesin A (NadA) è uno degli antigeni proteici ricombinanti inclusi in Bexsero, un vaccino contro il sierotipo B di *Neisseria meningitidis* (*N. meningtidis)* Nonostante NadA sia stata descritta come promotrice dell'adesione e invasione di cellule epiteliali nasofaringee, i recettori responsabili di questa interazione non sono mai stati trovati. Al fine di identificare i recettori umani target, abbiamo utilizzato NadA ricombinante come probe su un microarray di proteine che includevano una collezione di proteine umane di membrana e secrete. Lo screening ha rivelato tre nuovi interattori putativi, Siglec-5, Siglec-14 e FcγRIIA, espressi su cellule della linea mieloide. Abbiamo validato queste interazioni da un punto di vista biochimico, confermando Siglec-5 e 14 come interattori ad affinità elevata, mentre FcγRIIA, non è stato confermato. Successivamente abbiamo investigato il legame NadA/Siglecs utilizzando *Escherichia coli* come sistema di espressione eterologo. I Siglecs full-lengh, espressi in cellule CHO-K1, hanno mostrato un aumento dell'adesione di meningococco non osservata nel ceppo knock-out NadA. Nonostante il dominio extracellulare di Siglec-5 ricombinante lega NadA sia sui ceppi di *N. meningitidis* capsulati che acapsualti, non abbiamo ritrovato evidenze rilevanti sull'interazione Siglecs/NadA su cellule monocitiche primarie e tumorali.

Di recente, forme di Siglec solubili sono state ritrovate nel siero umano, correlabili con condizioni patologiche. A tal proposito, ne abbiamo confermato la presenza nel siero e nei terreni di coltura di linee monocitiche e di macrofagi. È interessante notare che, durante l'infezione, *N. meningitidis* leghi i Siglecs solubili rilasciati dai monociti in maniera dipendente da NadA. Aggiungendo Siglec solubili al batterio in saggi di adesione si osserva una maggiore associazione di batteri sulla superficie monocitaria. Inoltre è emersa una nuova interazione tra Siglec e C1q, il primo componente della via classica della cascata del complemento la cui interazione *in vitro* è inibita da NadA ricombinante. Inoltre, abbiamo osservato un vantaggio sulla sopravvivenza di *N. meningitidis* in presenza di anticorpi battericidi diretti contro NadA, testando sieri umani, come fonti di complemento, depleti di Siglec-5 e 14.

Riassumendo, in questo lavoro abbiamo rivelato una nuova coppia di interattori umani per l'adesina meningococcica presenti non solo sulla superficie dei fagociti ma anche nel flusso sanguigno. Dal punto di vista dell'ospite, abbiamo caratterizzato un nuovo target proteico per i Siglecs sulla superficie *di Neisseria meningitidis*. Inoltre, il legame di C1q con Siglec-5 e -14 è stato rivelato per la prima volta, aprendo nuove domande sulla biologia dei Siglecs.

In conclusione, abbiamo osservato che NadA non partecipa esclusivamente all'attraversamento dell'epitelio nasofaringeo ma potrebbe anche avere un ruolo per la sopravvivenza del batterio nell'ambiente extracellulare dell'ospite.

Introduction

Neisseria meningitidis (*N. meningitidis)* is an encapsulated, gram-negative bacterium, member of the family of Neisseriaceae. *N. meningitidis* is a frequent asymptomatic colonizer of the human nasopharynx tract with carriage rates up to 35%, reported among young-adults (Yazdankhah *et al.* 2004). The carrier status can rarely evolve in invasive meningococcal disease (IMD), a condition characterized by meningitis and septicemia.

Six meningococcal serogroups (A, B, C, W, Y, and X) are commonly associated with disease (Nadine *et al.* 2012). By reverse vaccinology approach, the Bexsero protective vaccine was formulated with three neisserial recombinant antigens (Pizza *et al.* 2000, Giuliani *et al*. 2006) including, Neisseria meningitidis adhesin A (NadA).

The *nadA* gene is present in ~30% of *N. meningitidis* and in 75% of hypervirulent *N. meningitidis* serogroup B lineages (Bambini *et al.* 2014). Metagenomic analyses revealed that the *NadA* genes can be classified in two distinct groups: group I includes NadA1, NadA2 and NadA3, whereas NadA4, NadA5 and NadA6 are members of group II (Comanducci *et al.* 2004). Less than 50% amino acid identity is found between groups while more than 90% identity is shared among members of each group (Bambini *et al.* 2014). NadA expression levels may vary among isolates by more than 100-fold, predominantly dependent on its transcriptional regulator, NadR, which responds to niche-specific signals during colonization and infection (Fagnocchi *et al.* 2013).

NadA was predicted to be an adhesin via its sequence similarity to *Moraxella* catarrhalis UspA2 (Comanducci *et al*. 2002). NadA is a 43 kDa protein which belongs to the family of trimeric autotransporter adhesins (TAA), obligate homotrimeric proteins. Trimeric autotransporters (TAAs) are outer membrane proteins found in many pathogenic Gram negative bacteria (reviewed in Linke *et al.*, 2006, Łyskowski *et al*., 2011). TAAs mostly mediate adhesion to target cells and extracellular matrix. In addition, TAAs are involved in biofilm formation, autoaggregation, and other important pathogenesis-related processes, such as cytotoxicity, serum resistance, host cell invasion and survival within host cells (Mil-Homens and Fialho 2011). They share a common molecular architecture, owing a multifunctional long passenger domain at the N-terminus (stalk) followed by a highly conserved C-terminal translocation domain (Linke *et al.* 2006). The first solved structure of NadA, performed on variant 5, revealed a novel trimeric autotransporter adhesin that has no close homologs among other TAAs. Indeed, a novel fold predominantly composed of trimeric coiled-coils with three protruding wing-like structures creates an unusual N-terminal head domain (Malito *et al.* 2014). The recently solved x-ray structure of NadA3 (figure 1, Protein Data Bank accession code 6EUN), shows novel crucial residues involved in host interaction. The wingtips at the top of the head emerged as essential for antibody specific recognition (Liguori *et al.* 2018).

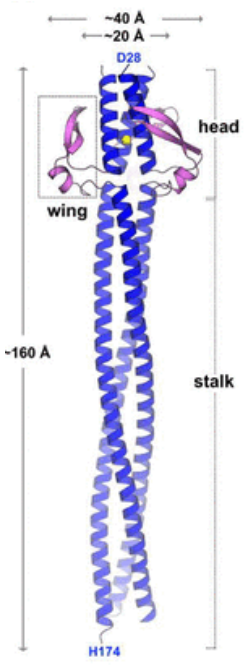


Figure 1. Cartoon representation of NadA3 trimer. N- and C-terminal residues are labeled for one chain. A chloride ion (yellow) is buried within the head domain; wings are depicted in pink. Artwork was prepared using Pymol (Adapted from Liguori *et al.* 2018)

The pathophysiology of *N. meningitidis* is a process that requires several steps: penetration of the nasopharinx mucosal barrier, reaching and surviving in the bloodstream and crossing the blood-brain barrier (BBB). NadA was found to target at least two cell types involved in meningococcal virulence. Earlier reports indicated human epithelial cells as target (Comanducci *et al.* 2002, Capecchi *et al.*2005). Few years later it was found that soluble NadA stimulates monocytes and macrophages to secrete a pattern of cytokines and chemotactic factors but not vasoactive mediators, resulting in a low inflammatory profile but increased recruitment of monocytes (Franzoso *et al.* 2008). NadA on the surface of *Escherichia coli* did not enhance bacterial-monocyte association

or intracellular survival but showed a stimulatory action on these cells (Franzoso *et al.* 2008). Full length NadA in *N. meningitidis* outer membrane vesicles increased their intrinsic efficacy in stimulating human macrophages antigen presentation machinery but not the induction of shock-related cytokines in circulating monocytes (Tavano *et al.* 2009). Recently, an additional role has been suggested at the host peripheral microvasculature and at the BBB through the identification of a NadA putative receptor, whose expression is restricted to endothelial cells (Scietti *et al*. 2016).

Both on human monocytes and epithelial cells, extracellular Heat shock protein 90 (Hsp90) is involved in directing the biological signaling driven by NadA (Montanari *et al*. 2012, Cecchini *et al.* 2011). The chaperone activity is required to allow NadA recycling pathway in epithelial cells and to induce the immunostimulatory activity in human monocytes. Binding of NadA to these cells is independent to the molecular chaperone and could be mediated by another, still unidentified molecule. Accordingly, pre-incubation with anti-Hsp90 antibodies did not compete with rNadA binding to monocytic cells surface (Montanari *et al*. 2012, Cecchini *et al.* 2011). The β1-integrin, a surface protein mainly expressed on epithelial cells, has been reported as NadA receptor on this cell type (Nagele *et al.* 2011). However, this interaction was demonstrated only with heterologous expression system and this evidence could not be confirmed in our laboratories (M. Merola, personal communication). By large-scale proteomic studies, *Scietti et al*. have recently demonstrated a high affinity binding to the scavenger lectin-type oxidized LDL receptor 1 (Lox-1) (Scietti *et al.* 2016), mainly expressed on endothelial cells. However, the biological relevance of this finding has not been elucidated yet. To date, NadA remains orphan of cellular receptors that could indicate a clear mechanism and biological significance.

Sialic acid-binding immunoglobulin-type lectins (Siglecs) are cell-surface single pass transmembrane receptors that are comprised of 2–17 extracellular Immunoglobulin domains, including an amino terminal V set domain which is the sialic acid-binding site (figure 2: orange and green, respectively)(Crocker, Paulson, and Varki 2007).

Siglecs were discovered through converging studies on sialoadhesin (Siglec 1, CD169) and Siglec-2 (CD22) (Crocker *et al.* 1994; Sgroi *et al.* 1993). The human genome contains 14 different Siglecs, which are classified into two groups based on their genetic homology among mammalian species. The first group is present in all mammals and consists of Siglec-1(sialoadhesin), 2, 4 (Myelin Associate Glycoprotein or MAG), and Siglec-15. The second group consists of the CD33-related Siglecs that have evolved rapidly and therefore their repertoire differs between species. The CD33 related Siglecs are Siglec-3 (CD33), -5, -6, -7, -8, -9, -10, -11, 12, -14, and -16. Furthermore, Siglecs may be categorized into three groups based on features of the transmembrane and cytoplasmic tails that likely mirror the major mechanisms by which they mediate their biological functions (figure 2). The first group is made up of Siglec-1 and Siglec-4, lectins that lack inhibitory signaling cytosolic motifs and possess neutral transmembrane domains which may primarily mediate adhesion events (Figure 2A). The second group of Siglecs includes members whose major biological function may be to set the inhibitory tone by cytosolic immunoreceptor tyrosine-based inhibition motifs (ITIMs)(Figure 2B). There is a third category of Siglecs, containing a positively charged residue in the transmembrane anchor region. These Siglecs can in general associate with a disulfide-linked homodimer of DAP12 (DNAX-associated protein of 12 kDa), which owns an aspartate transmembrane residue and a cytosolic immunoreceptor tyrosine-based activation (ITAM) motif (figure 3C) (Pillai *et al*. 2012).

Siglecs are preferentially expressed by specific cell types, resulting in a complex and partially overlapping expression pattern within cells of the innate and adaptive immune systems. Each Siglec has a distinct preference for the binding to sialylated glycans on the surface of mammalian cells (Crocker, Paulson, and Varki 2007). Siglecs binding sites are typically "masked" by *cis* interactions with glycan ligands expressed on the same cell or in *trans* interactions with neighboring cells (Crocker, Paulson, and Varki 2007). Sialic acids (Sias) are a group of acidic sugars, primarily found at the end of glycan chains displayed on cell surfaces. The

distribution is restricted to vertebrates, making them good candidates as one of the "signatures of self" or "self-associated molecular patters (SAMPs)", opposed to "pathogen-associated molecular patterns (PAMPs)". Nevertheless, some pathogenic bacteria developed the ability to acquire sialic acids or sialylated structures from the host to "mimic" structures or even perform *de novo* biosynthesis by convergent evolution. These molecular mimicries have been shown to provide a selective advantage for some bacteria (Pillai *et al.*2012). The Sias expressed by *N. Meningitidis* on its capsule and outer membrane lipooligosaccharides (LOS) contribute to resistance to the bactericidal activity of normal human serum (Vogel *et al.* 1997). Siglec-1 was shown to function either directly or in synergy with other phagocytic receptors to enhance macrophage phagocytosis by binding to sialylated LOS on the surface of *N. meningitidis* (Jones *et al.* 2003). A moderate interaction between *N. meningitidis* and Siglec-5 was further reported, raising the possibility that sialylated LOS on the bacteria may influence macrophage, neutrophil and monocyte functions (Jones *et al.* 2003). Via its sialylated capsule, Group B streptococcus (GBS) was shown to recognize Siglec-9 on neutrophils, blunting oxidative burst and neutrophil extracellular trap (NET) formation (Carlin *et al*. 2009). In addition to the Sias-dependent engagement, some GBS strains can recognize Siglec-5 on macrophages and neutrophils through the surface exposed β-protein. The interaction was demonstrated to promote bacterial attachment to the macrophages surface and influence intracellular signaling (Chang *et al.* 2014). Recently, a novel sialic-acid independent interaction was found for another Neisseriacea, *Neisseria Gonorrhoeae* (Landig *et al.* 2018).

Siglecs that possess identical substrate specificity, such as Siglec-5 and 14, are called paired receptors (Angata *et al.* 2006; Cao *et al.,* 2008). A member of each pair has the cytosolic ITIM, while the other member associates with the ITAM motif, resulting in activating receptor. This implies that paired Siglecs function in a "Yin-Yang" fashion and have evolved in order to provide a balanced signaling to avoid immunosuppression by pathogens (Angata *et al*. 2006; Pillai *et al*. 2012). A particular feature of Siglec-5 and -14 consists on the genetic polymorphism in human population. Indeed, the ancestral SIGLEC gene cluster has SIGLEC14 and SIGLEC5 genes in tandem, whereas a derived (SIGLEC14-null) allele harbors a single SIGLEC14/5 fusion gene. The product of this fusion gene is identical to the SIGLEC5 gene at the amino acid level (Yamanaka *et al.* 2009). Thus, functional implications in modulation of immune response correlated with the loss of a functional Siglec-14 were suggested (Angata, Ishii et al. 2013).

Integral membrane proteins are sometimes released in a soluble form. They not only contribute to the fine tuning of cellular responses, but in some cases, emerge as biomarkers for clinical conditions (Ramasamy *et al.* 2009). Previous studies have reported the presence of alternatively spliced *SIGLEC* mRNA that encodes soluble forms without confirming the presence of a corresponding protein (Connolly *et al*. 2002; Kitzig *et al.* 2002). Recently, soluble form of some Siglecs were found in human blood or cellular culture supernatant (Matsubara *et al.* 2015). Furthermore, soluble Siglecs (sSiglec) are reported as plasma biomarker in different pathologies (Forster *et al*. 2018), such as impaired levels of sSiglec-14 in Chronic Obstructive Pulmonary Disease (COPD) patients' sera (Angata *et. al* 2013). A subsequent study (Huang *et al.* 2018) revealed that sSiglec-14 is derived from an alternatively spliced mRNA that retains an intron encompassing a termination codon truncating the transmembrane domain. Interestingly, sSiglec-14 may exert an anti-inflammatory effect by competing with its membrane bound counterpart. This mechanism implies that the switch of Siglec-14 from membrane bound to soluble secreted isoform may function as a negative feedback mechanism to quell the inflammatory responses triggered by bacterial infections (Huang *et al.* 2018). Altogether, most of the functions ascribed to soluble Siglecs do not have solid experimental validations and their biological function is not yet fully understood.

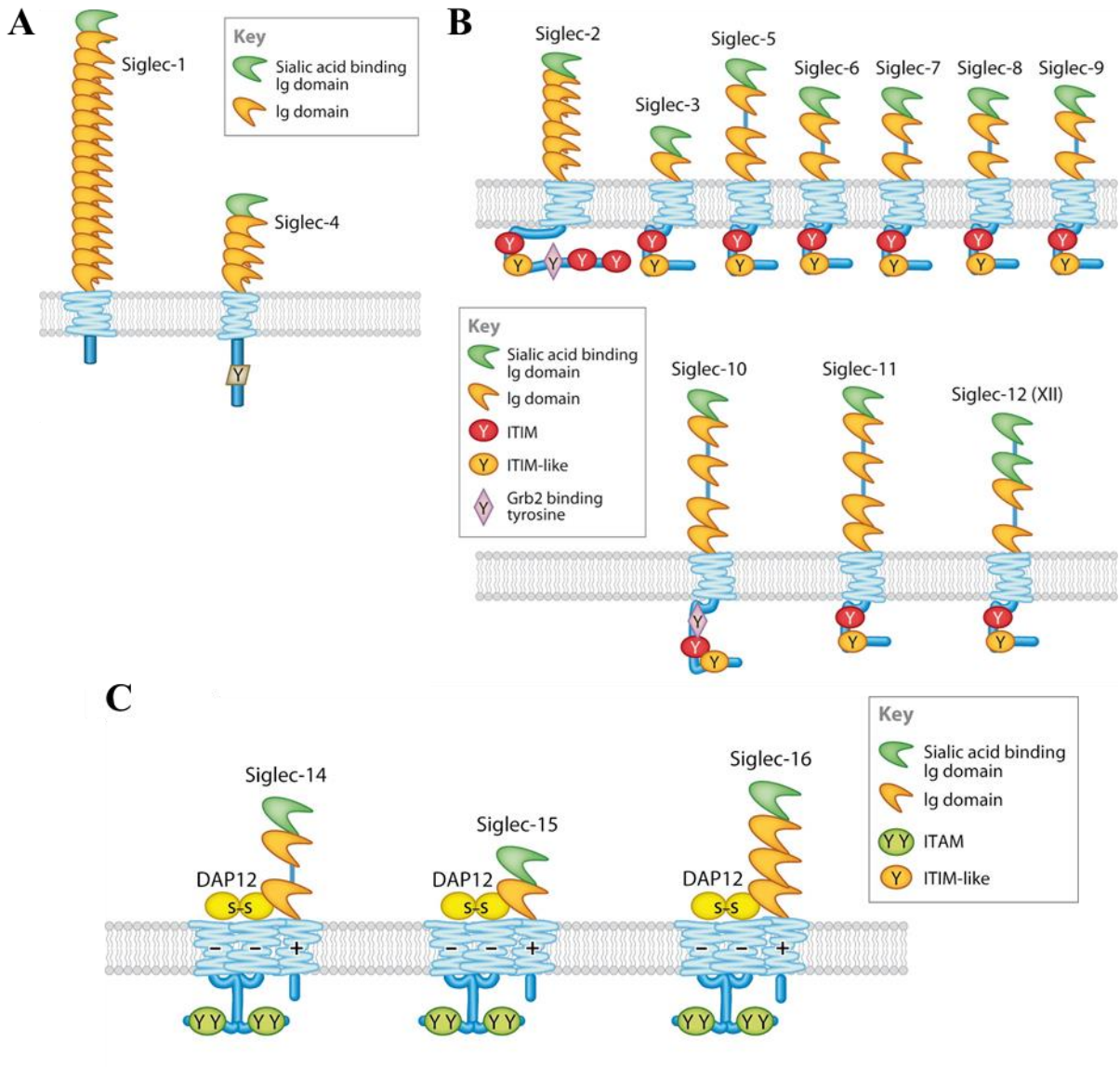


Figure 2. Schematic illustration of human Siglecs family (Adapted from Pillai *et al.* 2012)

A) Siglecs that lack defined cytoplasmic tail signaling motifs or a charged transmembrane residue;

B) Siglecs that contain ITIM motifs in their cytoplasmic tails;

C) Siglecs that contain a transmembrane lysine (+) and associate with DAP12.

Aim of the work

NadA was discovered as protective antigen by the reverse vaccinology approach and the recombinant protein was included as antigen in 4CMenB vaccine against *N. meningitidis*, serogroup B (Pizza *et al.* 2000; Comanducci *et al.* 2001). Subsequent characterization indicated NadA as member of the trimeric autotransporter family and revealed its feature as adhesin and invasin on human epithelial cells (Capecchi *et al.* 2004). Recombinant NadA was found to bind to human monocytes and macrophages. However, NadA on the surface *E. coli* did not enhance bacterial-monocyte association or intracellular survival (Tavano *et al.* 2008).

Although integrin β1 was reported as NadA receptor (Nagele *et al.* 2011), this evidence was never confirmed.. As interactors, the molecular chaperone Hsp90 and Lox-1 (Montanari *et al*. 2012, Cecchini *et al.* 2011, Scietti *et al.* 2016) were shown to bind to the adhesin but no conclusive studies have been performed to assess any receptorial feature. Remarkably, Lox-1 is mainly express by endothelial cells, a cell type not described to be targeted by the adhesin. To date, NadA remains still orphan of cellular receptors, an information required to define the biological outcome of the NadA-dependent interaction with human cells.

The present work aims to look for NadA interactors/receptors by a large-scale screening of a human surface protein library (*B.Benucci* and *Z.Spinello*, manuscript in preparation). The putative NadA interactor(s) disclosed were deeply investigated with different approaches. The workflow may be divided into four sections:

1: Biochemical validation of the binding. This includes expression and purification of the interactors as soluble proteins. Recombinant NadA and putative interactors were used to determine the affinity constant of the binding by label-free technologies (such as BioLayer interferometry and surface plasmon resonance). The precise region of interaction was mapped by Hydrogen deuterium exchange (HDX) and mass spectrometry analysis;

2: Characterization of the binding in a membrane context. Heterologous expression systems were employed to express both NadA in *E. coli* and human putative interactors on the surface of non-permissive cells (such as Chinese Amster Ovary Cells, CHO-K1), in order to mimic the membrane environment;

3: NadA contribution in the context of whole bacterium. *Neisseria Meningitidis* strains (serotype B) wild type and knock-out NadA were employed to further explore the role of the adhesin at the interface with host cells. Outer membrane vesicles obtained from the abovementioned strains were used as stimulants to investigate the effects on human target cells;

4: biological consequences of NadA-host interactors were analyzed by mean of Colony forming Unit Assay, flow cytometry and confocal microscopy.

Results

1.Identification and biochemical characterization of NadA interactors

1.1 Siglec-5 and -14 are NadA interactors

In order to identify NadA putative receptors, a large-scale protein microarray screening was performed spotting about 7000 recombinant soluble proteins (6500 human and 500 mouse) from an expanded GNF library, available *in house* (Scietti *et al.* 2016). 1 µM of recombinant NadA was used and interactions were detected using rabbit anti-NadA polyclonal antibody (B.Benucci, Z.Spinello *et. al, manuscript in preparation*).

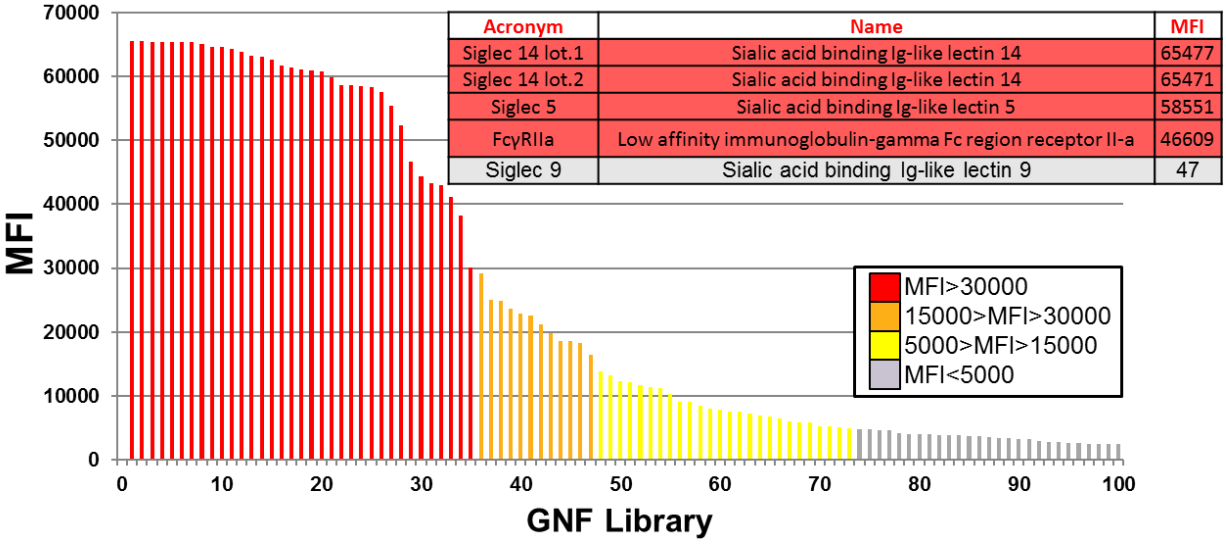


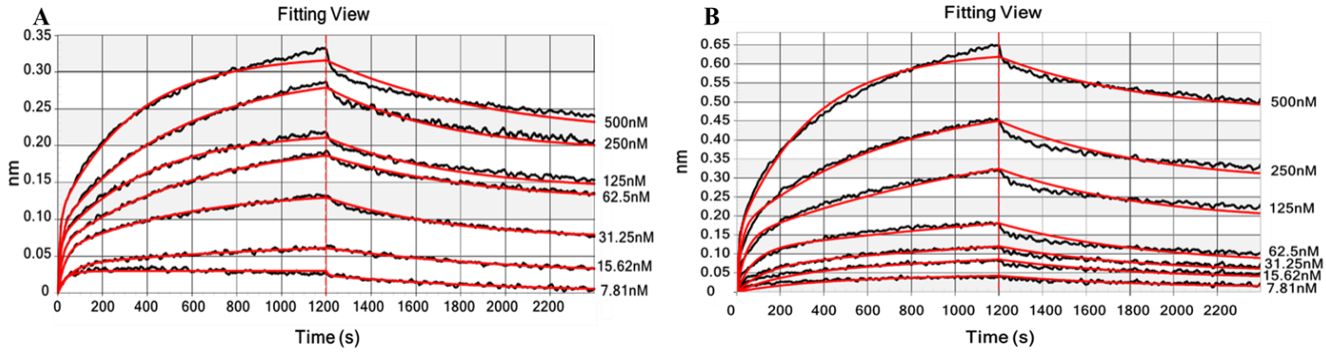
Figure 3. Large scale screening for NadA interactors. Mean Fluorescence Intensity (MFI) of spotted human and mouse proteins tested against NadA (1 µM). Red bars represent high reactivity proteins (MFI>30000), orange bars medium reactivity proteins (15000>MFI>30000), yellow bars represent low reactivity proteins (5000>MFI>15000) and grey bars no reactivity proteins (MFI<5000). Table shows protein acronym, name and the respective MFI values of the relevant interactions.

The interactions with the highest scores were obtained with three proteins expressed on cells of myeloid origins, such as monocytes and macrophages. Two replicates of the Sialic acid-binding immunoglobulin-type lectins receptor 14 (Siglec-14) with a Mean Fluorescence Intensity (MFI) score of 65477 and 65471 respectively, the Sialic acid-binding immunoglobulin-type lectins receptor 5 (Siglec-5) with a MFI score of 58551 and the Low affinity immunoglobulin gamma Fc region receptor II-a (FCγRIIA) with a MFI score of 46609 were detected. Since the Sialic acid-binding immunoglobulin-type lectins receptor 9 (Siglec-9) revealed MFI score of 47, we decided to use this Siglec as negative control in our experiments. Moreover, the LOX-1 interactor found by Scietti *et al.* was confirmed (not shown) owning a lower signal with respect to the abovementioned species.

1.2 Siglecs are high affinity NadA interactors

To assess the binding kinetics between recombinant NadA and recombinant Siglec-5 or 14 receptors, Bio Layer Interferometry (BLI) technology was used. BLI is a label-free technology for measuring biomolecular interactions. It is a technique based on the optical phenomenon of wave interference. It analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on a biosensor tip and an internal reference layer. The binding between a ligand immobilized on the biosensor tip surface and an analyte in solution produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift (Δλ in nm), which is a direct measure of the change in thickness of the biological layer.

rNadA was first immobilized onto the BLI biosensor and each receptor used individually as analyte. To calculate affinity of rSiglec5 and rSiglec14 on immobilized rNadA, serial dilutions (from 500 nM to 7.81nM) were tested. By adding increasing concentration of the single analytes, the constant of dissociation (K_d) was calculated as $6.2E^{-8}$ M for Siglec 5 and $2.6E^{-7}$ M for Siglec 14 (figure 4 C). These values indicate a very strong interaction between NadA and monocytic putative receptors.



C

Ligand	Analyte	Kd
NadA	Siglec-5	$6.2E^{-8}$ M
NadA	Siglec-14	$2.6E^{-7}$ M

Figure 4. Binding kinetics of NadA and Siglec-5 or 14 interaction
A) Blank subtracted sensograms of Siglec 5 (500 - 7.8 nM) tested on biotinylated NadA immobilized on SA biosensors. Association and dissociation curves were fitted in a 2:1 model
B) Blank subtracted sensograms of Siglec 14 (500 - 7.8 nM) tested on biotinylated NadA immobilized on SA biosensors. Association and dissociation curves were fitted in a 2:1 model
C) Calculated Kd from the A and B graphs.

For FcγRIIA putative ligand the Surface Plasmon resonance tool was used to deeply study the biophysical interaction (Supplementary results). However, the interaction found on protein microarray was not confirmed.

1.3 Siglec-5 binds NadA through the head domain

For a precise mapping of the NadA region implicated in the Siglec-5 binding, a hydrogen-deuterium exchange experiment was performed. Epitope mapping by the HDX approach is based on the differential rate of deuterium incorporation by an antigen when it is free or bound to the partner protein following incubation in deuterated solvents. The rate at which backbone amide hydrogens exchange in solution is directly dependent on the dynamics and structure of the protein. When a protein complex forms, such as antigen–receptor complex, the interface between the binding partners may occlude solvent accessibility, reducing the exchange rate (Hager-Braun and Tomer 2005).

Mapping by HDX-MS was performed in two parallel steps. Deuterium incorporation was performed on rNadA alone (which represents the reference sample) and on the complex of rNadA and recombinant Siglec-5. Both samples were digested by pepsin, and deuterium incorporation was monitored for 95 peptides covering 96.3% of NadA sequence (Malito et al. 2014) and compared. In figure 5A, HDX results are simplified by reporting the extend of deuterium uptake for 5 peptide fragments covering the head domain of NadA. The interaction involves the segment peptide Val^{36}-Gly^{50} and Ile^{60}-Asp^{75}.

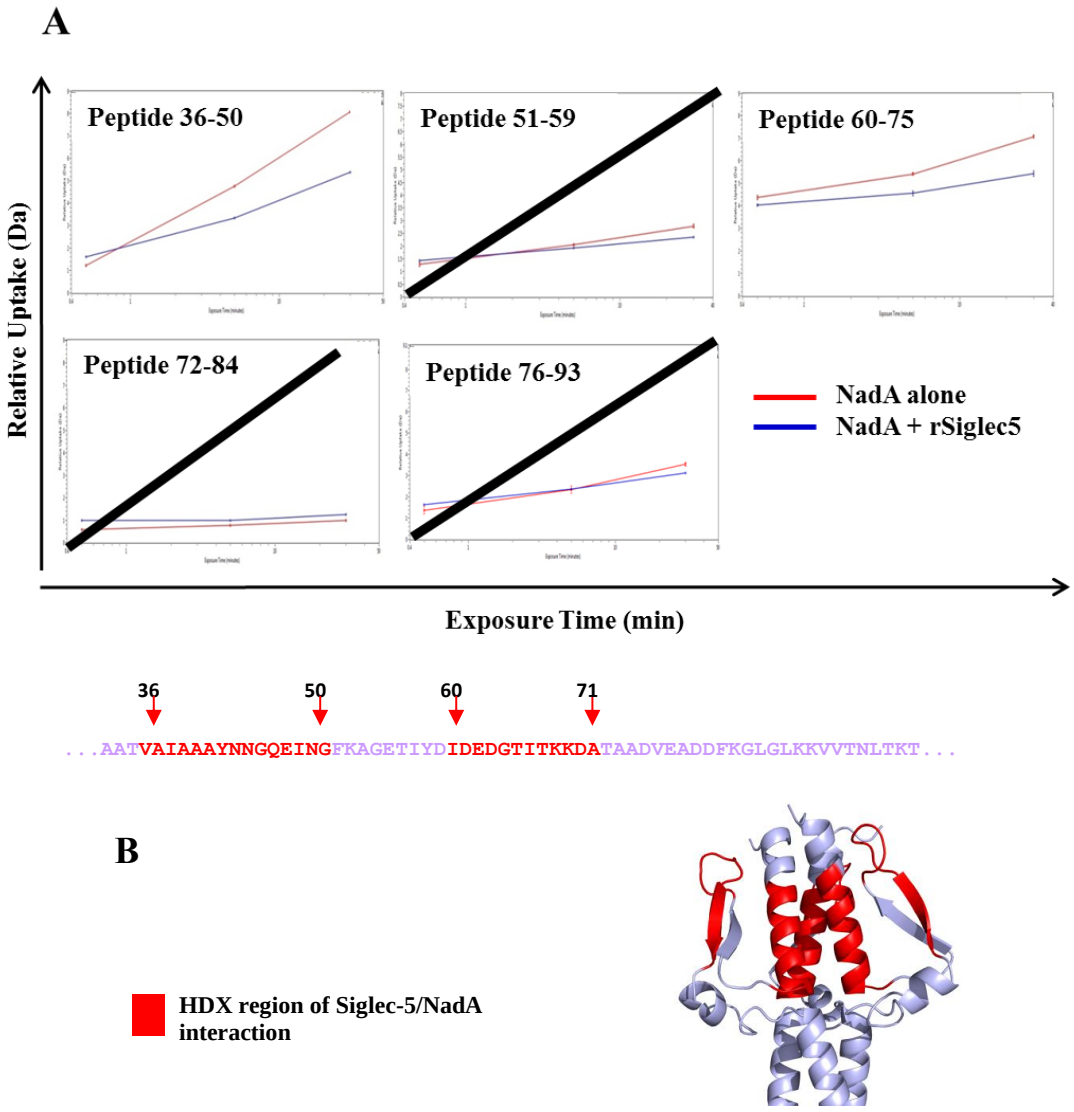


Figure 5. Time course of deuterium incorporation of the peptides covering the NadA head domain. A) Deuterium uptake of each peptide is calculated as the difference between extracted centroid mass of each undeuterated peptic peptide and respective deuterium-labeling time point peptide using DynamX software. The blue curves are derived from complex NadA–Siglec 5, whereas the red curves are derived from NadA alone.There are 2 peptides showing a significant difference in deuterium uptake between the free and bound NadA forms (peptide Val^{36}-Gly^{50} and Ile^{60}-Asp^{75}) while the 3 peptides not affected by deuterium protection are highlighted with a black bar (peptide Phe^{51}-Asp^{59}, Thr^{72}-Leu^{84} and Val^{76}-Leu^{93}).**B)** The figure reports the *in silico* model of NadA variant 3 built from the X-ray structure of NadA variant 5 (Malito et al. 2014). The NadA/Siglec 5 interacting region (peptide Val^{36}-Gly^{50} and Ile^{60}-Ala^{71}) is colored in red.

C *-Surface view* *-Top head view*

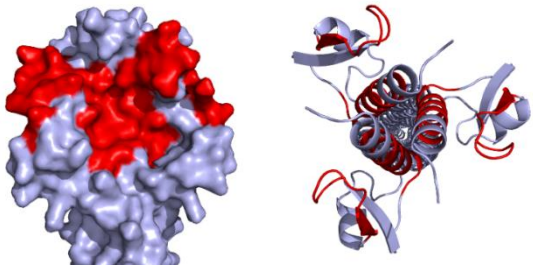


Figure 5 C) NadA/Siglec 5 interaction region highlighted on the *in silico* model of NadA variant 3 head domain. NadA/Siglec 5 interaction region is depicted in red on the top and surface views of the *in silico* model of NadA variant 3 head built from the X-ray structure of NadA variant 5. Dashes show regions with low sequence homology or unknown secondary structure (Malito et al. 2014).

In figure 5B and 5C, the region of interaction is depicted on the *in silico* model of NadA. The 3D view highlights the conformational nature of Siglec-5 and NadA interaction.

2. NadA interaction with Siglecs in heterologous systems

Biochemical studies carried out with the recombinant proteins confirmed a strong binding between NadA and Siglecs-5 and -14 in solution. To endorse the interaction in a membrane context, we proceeded by employing heterologous expression systems. Furthermore, this approach allows the exclusion of other membrane interactors or co-interactors both on the host and pathogen.

2.1 Siglecs recognize NadA expressed on E. coli

To this end, we expressed full length NadA on *E. coli*. NadA processed and assembled in oligomers on *E. coli* surface, as described by Capecchi *et al.* 2005. To test whether Siglecs recognized NadA inserted on a membrane, the recombinant extracellular domains (ectodomain) of Siglec-5 and 14, comprising the Immunoglobulin domain and the sialic acid binding sites, were employed. Wild-type *E. coli* and *E. coli* expressing NadA were incubated for 1h at 37 C° with recombinant Siglec-5 or 14. Then, bacteria were washed and double stained with primary antibodies against Siglec-5 or -14 and NadA. Next, the appropriate secondary antibodies were used. The fluorescence was detected by confocal microscopy. As illustrated in figure 6, NadA expressed by *E. coli* recognizes and co-localizes with Siglec-5 and -14. The NadA dependence of Siglecs binding was confirmed by the absence of fluorescence in *E. coli* wt samples, as shown in the bottom panel of figure 6.

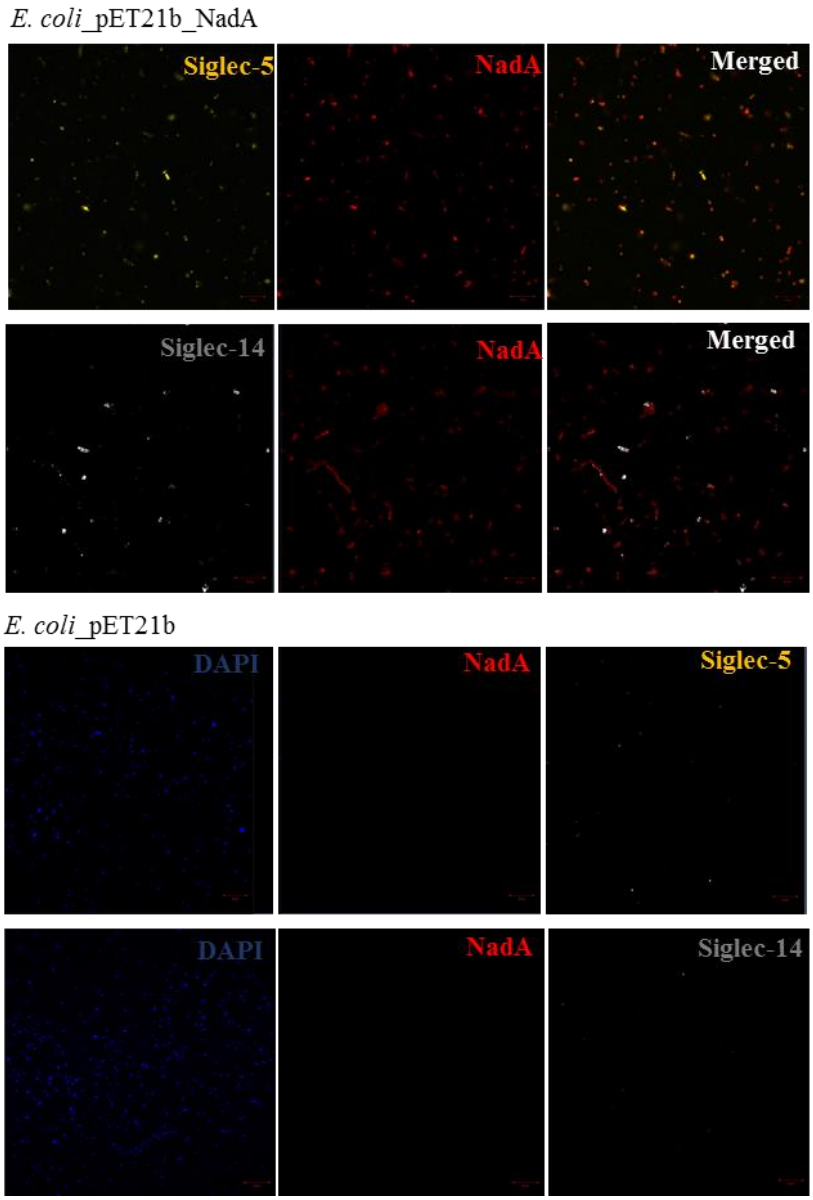


Figure 6. Recombinant Siglec 5 and rSiglec 14 specifically bind to *E. coli* surface exposed NadA: *E. coli*–NadA and *E. coli*–pET were incubated with 200 µg/mL of rSiglec 5 or -14 for 1 hour at RT. After extensive washings, bacteria were stained with rabbit polyclonal anti Siglecs antibodies and mouse polyclonal anti-NadA antibody followed by Alexa Fluor 488 and 647-conjugated antibodies and DAPI mounting medium to stain bacterial chromosome. Immunofluorescence allowed to visualize the rSiglec-5 and -14 bound to bacterial surface (yellow and grey, respectively) and the bacterial chromosome (DAPI) only with *E. coli*–NadA. NadA expression was verified (red).

2.3 rNadA and N. meningitidis strains specifically binds Siglecs in a eukaryotic heterologous system

To determine whether the recombinant NadA binds to Siglecs anchored on eukaryotic cell membrane, we transiently transfected Chinese hamster ovary (CHO-K1) cells, a suitable transfection host, with Siglec-5, Siglec-14 or Siglec-9 expression plasmids. CHO-K1 cells did not naturally express Siglecs receptors (figure 7 A) neither are permissive to NadA binding.

Figure 7A. Expression of Siglecs on CHO-K1 cells transfected or not. Cell lysates of CHO-K1 cells transfected individually with pEZ-M14 FLAG-tag plasmids carrying Siglec-5, Siglec-14 and Siglec-9 respectively were analyzed by immunoblotting. The transfected proteins were revealed by using anti-3x flag antibody.

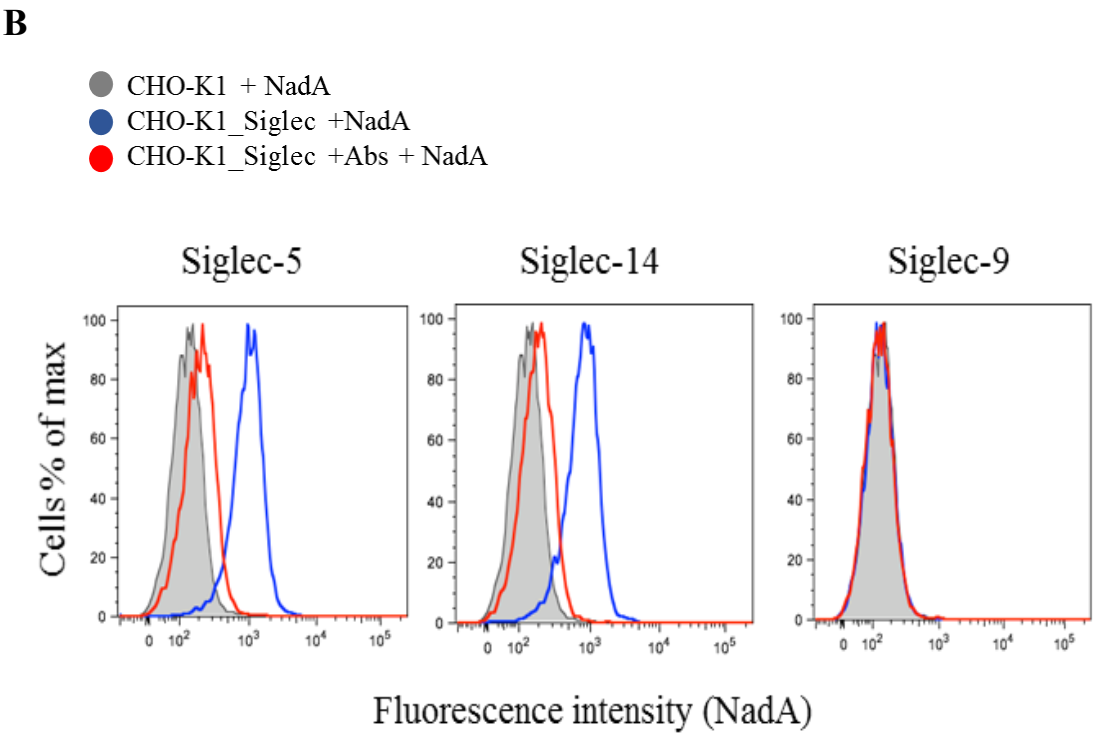


Figure 7 B. rNadA binding to CHO-K1 cells expressing Siglec-5, 14 or 9. Flow cytometry histograms representative of rNadA binding on transfected CHO-K1. Grey curves represent the negative controls (Mock transfected CHO-K1 cells incubated with rNadA), blue curves represent the rNadA binding on transfected cells and the red curves the inhibition of the binding by antibodies against each Siglec. Histograms are representative of 3 experiments performed in duplicate.

Firstly, we used the recombinant NadA to investigate binding on CHO-K1 expressing Siglecs. The incubation was conducted by adding 200 µg/ml of rNadA for 30 minutes at 37°C. NadA binding was revealed by mouse anti-NadA antibody and the proper secondary fluorescent antibody. Results are shown in figure 7B. Mock transfected CHO cells are not permissive to NadA binding (gray shaded area). When CHO-K1 cells express either Siglec-5 or -14, the binding could be detected by flow cytometry. Instead, expression of Siglec-9

receptor did not restore NadA binding. Furthermore, when CHO cells are pre-incubated with antibodies against Siglec-5 or 14, the binding was abolished (figure 7B, red curves).

To investigate whether NadA expressed on *N. meningitidis* could bind Siglec-5 and 14 expressed on the membrane, we used transfected CHO-K1 cells and determine the interactions with the pathogen. *N. meningitidis* serogroup B strain 5/99 was selected. Since it was reported that the capsule has anti-adherent properties on host cells (Stephens *et al.* 1993, Virji *et al.* 1993), the 5/99 strain was deprived of the capsule by performing the Knock-Out (KO) of *synX* gene (Fisseha *et al.*2005). Synx is a polysialic acid capsule biosynthesis protein expressed by *N. meningitidis*. In addition, the isogenic strain depleted of NadA (*ΔnadA*) was used.

To check NadA Siglecs interaction, an adhesion assay was performed by incubating *N. meningitidis* strains with CHO-K1 cells (multiplicity of infection, MOI 200:1) at 37 C°. After 3 hours of infection, non-adherent bacteria were washed out and cells were permeabilized with 1% saponin, as described in Jonas *et. al.* 2003. Colony Forming Unit counting revealed bacteria bound on CHO- K1 cells transfected with Siglec-5 or -14 expression plasmids or empty vector. Higher number of 5/99 *N. meningitidis* were found to adhere on Siglec-5 and -14 expressing cells compared to non transfected cells. In contrast, a significant lower number of *ΔnadA* meningococci adhered to Siglec-5 and -14 expressing CHO-K1 cells. CHO-K1 expressing Siglec-9, used as negative control in the adhesion assay, did not show any NadA dependent *N. meningitidis* adhesion (figure 8). This experiment suggests that binding of *N. meningitidis* to the cell surface could be a direct result of NadA -mediated binding to Siglec-5 and 14.

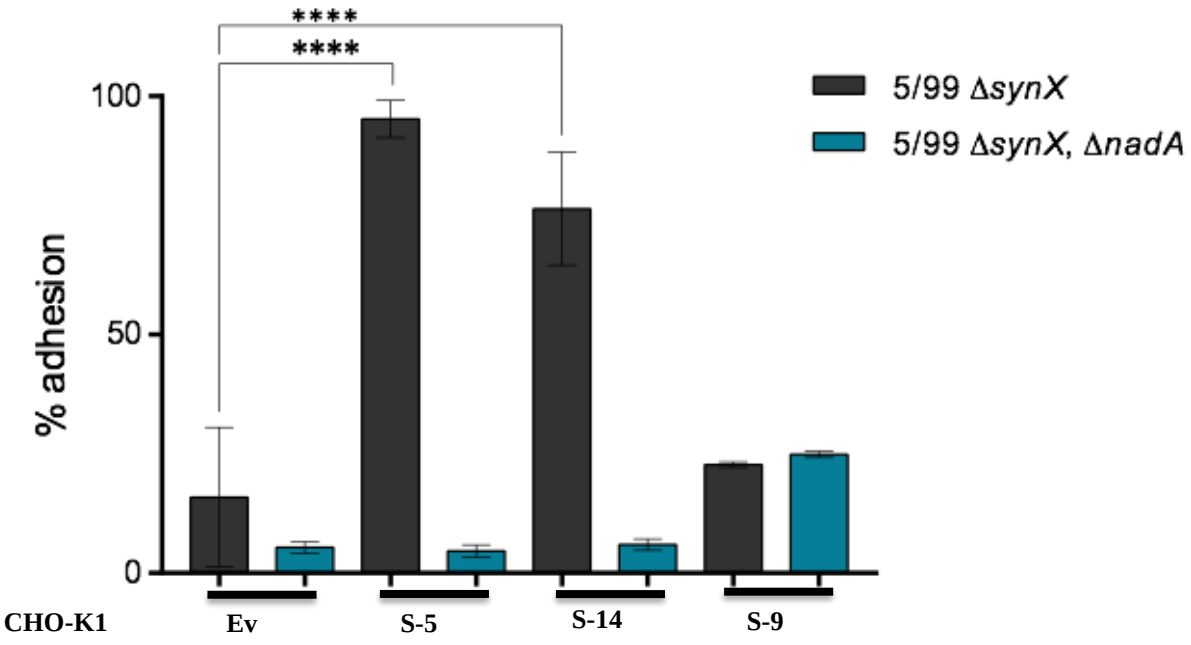


Figure 8. Adhesion assay of *N. meningitidis* to CHO-K1 cells either expressing Siglec-5, 14 or S9. CHO-K1 cells incubated for 3hrs with of 5/99 *ΔsynX* or *ΔnadA*.
The results are reported as percentage of CFU counts for each strain with respect to 5/99 on CHO-K1_S-5 (grey bar, set as 100%). Data represents the mean and standard deviation of n=3 experiments each performed in duplicate. Reported p-value **** < 0.0001 (one -way ANOVA test).

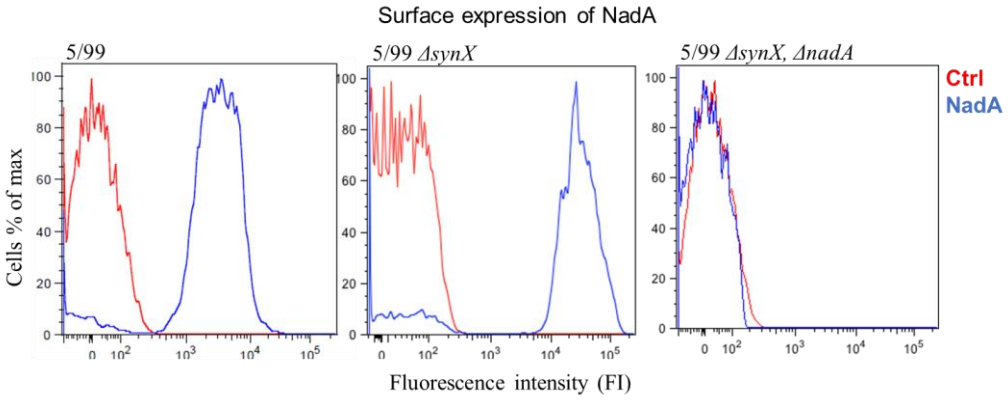
3.NadA interaction with Siglecs in a physiological membrane context

3.1 NadA is required for N. meningitidis interaction with rSiglec-5 ectodomain

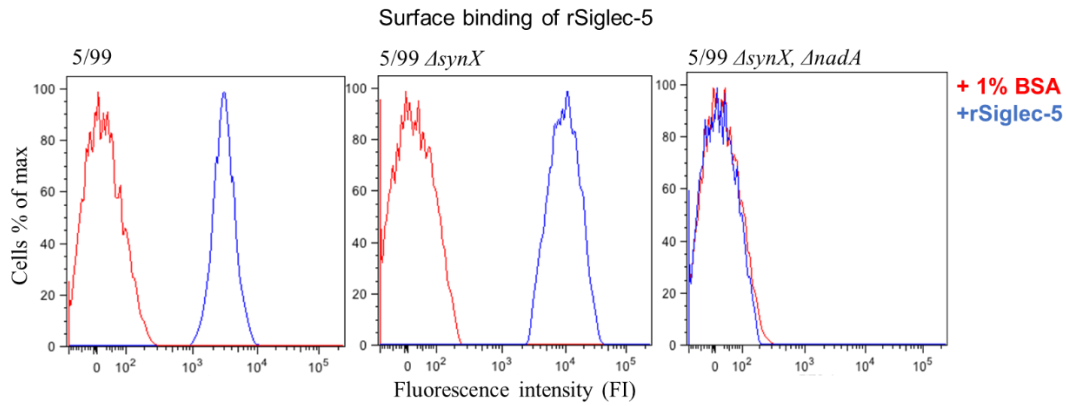
To further validate NadA-dependent binding to Siglecs, we tested the binding of recombinant Siglecs ectodomains in the context of whole bacterium. *Neisseria meningitidis* strain 5/99 was selected for the higher levels of NadA expression (Fagnocchi *et al.* 2013). Moreover, NadA expression was compared among wt 5/99 strain and the isogenic bacterium unencapsulated (*ΔsynX*) and deleted of the *NadA* gene. NadA expression was detected by using an mouse anti-NadA polyclonal antibody. Although NadA is well recognized by the antibodies in capsulated strain, protein surface exposition increases with the lack of the capsule, as shown in figure 9 A. In the KO strain, anti-NadA antibodies did not bind bacteria.

The abovementioned strains were incubated with 15 µg/ml of soluble recombinant extracellular domain of Siglec-5 and surface attachment was detected by flow cytometry (Figure 9 B). 5/99 and 5/99 *ΔsynX* strains showed a shift in fluorescence intensity, proportional to NadA surface exposition. We further confirmed Siglec-5 attachment on *N. meningitidis* by confocal microscopy (figure 9 C). In conclusion, *N. meningitidis* binds to recombinant Siglec-5 in NadA dependent manner since this interaction was not detected in the NadA KO strain.

A



B



C

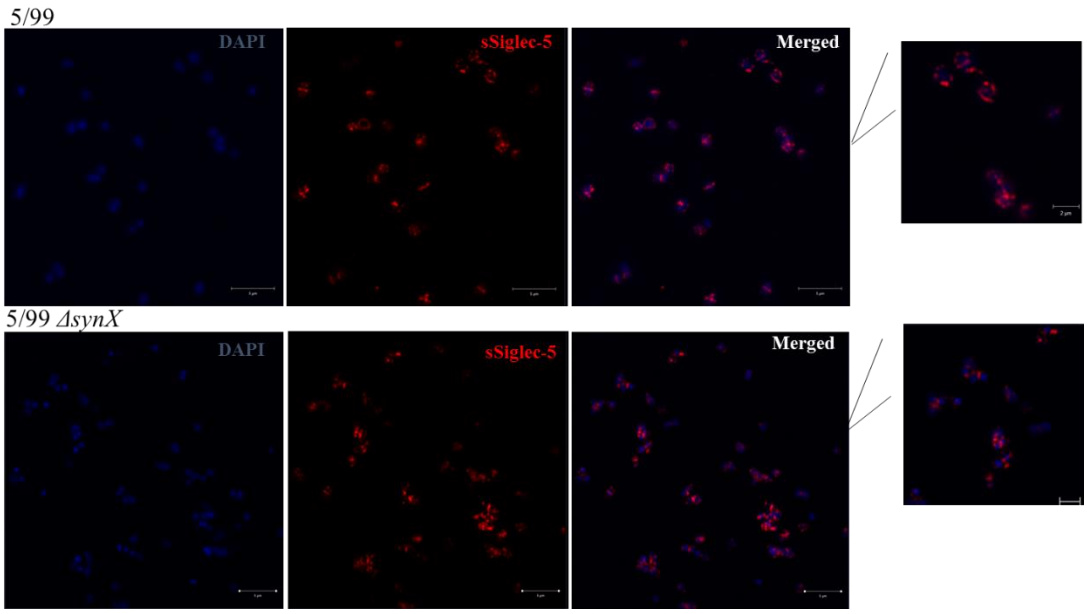


Figure 9A. Surface expression of NadA on *N. meningitidis* strains. Flow cytometric analysis (FACS) of 5/99 , *ΔsynX* and *ΔsynX*, *ΔnadA* strains stained with mouse polyclonal anti-NadA antibody followed by anti-mouse Ig FITC labeled secondary antibodies. A representative FACS profile obtained by plotting histograms of fluorescence intensity (FI) is shown. **9B. Surface binding of soluble Siglec-5 ectodomain on 5/99 *N. meningitidis* strains.** Flow cytometric analysis (FACS) of 5/99, *ΔsynX* and *ΔsynX, ΔnadA* strains incubated with 1% Bovine Serum Albumin (red peak) or 6XHis tagged sSiglec-5 (15 ug/ml) (blue peak) for 15 minutes at 37°C in agitation. After incubation and washes, bacteria were stained with mouse polyclonal anti-Histidine tag antibody followed by anti-mouse Ig FITC labeled secondary antibodies. In panels A and B, the red lines represent the control with only the buffer (1% BSA).
7C. Surface binding of Siglec-5 was also observed by confocal microscopy, following similar procedures to those used for figure 7B. Bacterial chromosome was stained with DAPI (blue) and Siglec-5 with anti-His tag antibody (red). Images were acquired by confocal microscope (Zeiss, LSM 700 in AiryScan System), magnification 100X.

3.2 rNadA is internalized by monocytes

NadA interaction with monocytes and macrophages has already been reported in literature (Tavano *et al.* 2008). To investigate the binding of recombinant NadA to Siglecs expressed by cells myeloid origin, we used THP-1 immortalized cells.

First, we checked the expression of Siglec-5 and -14 on this cell line by immunoblotting (figure 10 A). We found both Siglec-5 (molecular weight 72kDa) and Siglec-14 (45kDa), expressed at comparable levels.

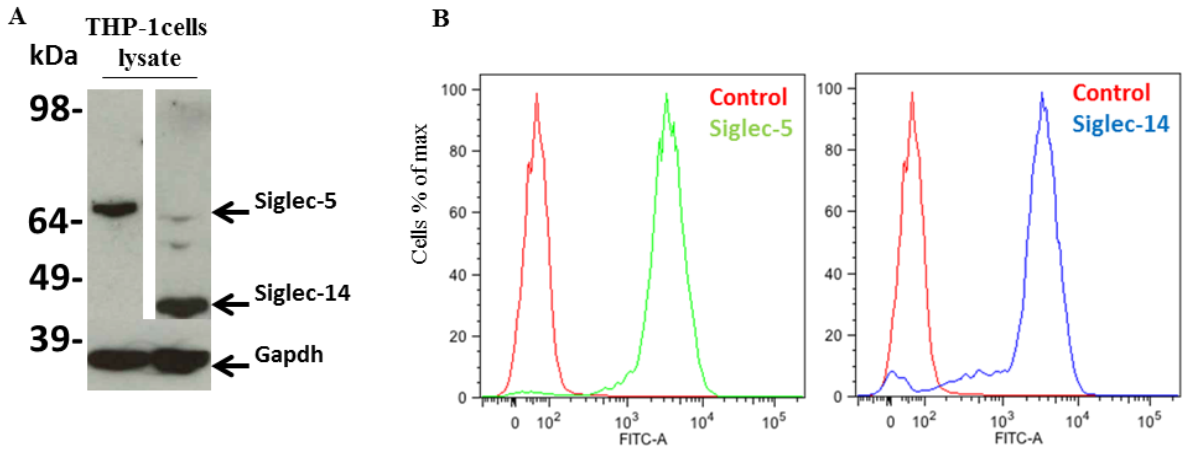


Figure 10. Expression of Siglec-5 and -14 on THP-1 cells.
A) Cell lysates of THP-1 cells were analyzed by immunoblotting. The expression was revealed by using monoclonal anti-Siglec-5 and -14 antibodies respectively.
B) Flow cytometry analysis of surface expression of Siglec-5 and -14 by THP-1 cells. In red, cells incubated with secondary antibodies alone.

THP-1 cells were incubated with 200 µg/ml of rNadA or PBS for 30 min at 37°C. In parallel, the surface staining and intracellular staining were performed. In the latter case, cells were fixed and permeabilized before the incubation with anti-NadA antibody. In non-permeabilized conditions, cells were directly stained with anti-NadA monoclonal and the correspondent secondary antibody. Fluorescence readings were collected by flow cytometry analysis. As shown in figure 9, in both conditions a shift in fluorescence intensity (blue) with respect to the control (red) was observed, although it was more pronounced in permeabilized cells (figure 11 B). This observation suggests that the recombinant NadA is quickly internalized by monocytic cells. Surprisingly, pre-incubation of THP-1 cells with increasing concentration of antibodies against Siglec-5 or Siglec-14 (25µg/ml, 50µg/ml, 75µg/ml) did not neutralize the observed NadA signal (figure 11 C, D).

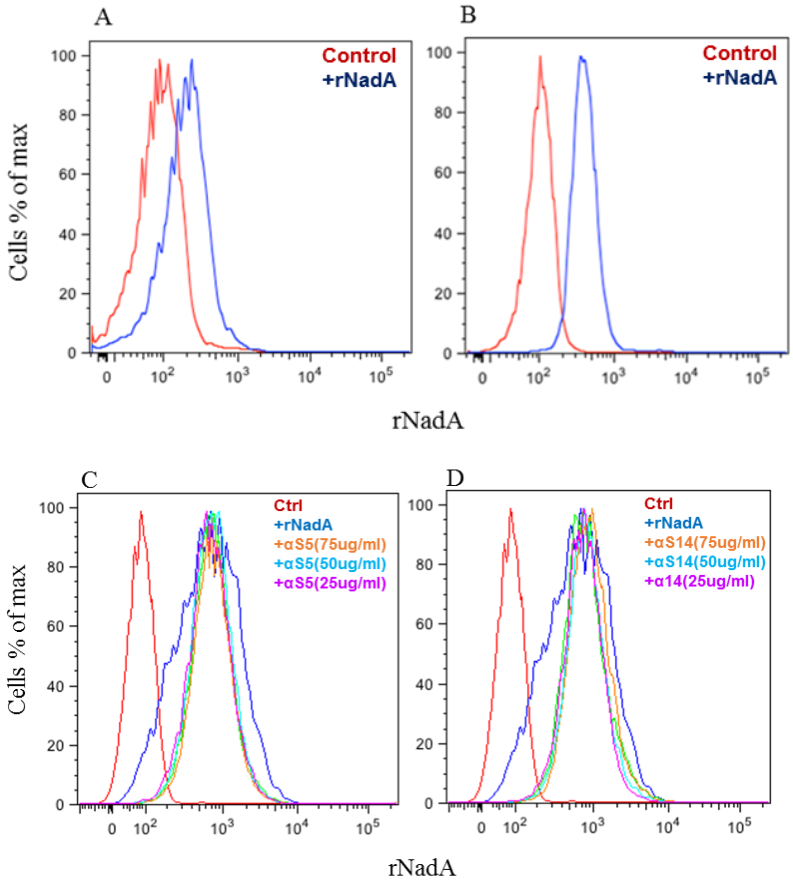


Figure 11. Characterization of the binding of rNadA to human monocytes: human immortalized THP-1 monocytic cells were incubated with rNadA (200 µg/mL) for 30 minutes at 37°C. **A)** Cells were incubated with primary antibodies against NadA and fixed. **B)** cells were fixed and permeabilized with 0.1% Triton, 1% BSA in PBS and incubated with primary antibodies against NadA. **C-D)** THP-1 cells were pre-incubated with increasing concentrations of anti-Siglec-5 and -14 antibodies before the addition of recombinant NadA. Fluorescence readings were collected by flow cytometry.

3.3 N. meningitidis wt and ΔnadA on monocytes and macrophages

In literature, the role of NadA in *N. meningitidis* association with phagocytes was studied by employing *E. coli* expressing NadA (Tavano *et al.* 2008). We sought to verify the NadA-dependent binding of the *N. meningitidis* to monocytes and macrophages using unencapsulated *N. meningitidis* strain 5/99 wt or *ΔnadA*. Bacteria were incubated with THP-1 monocytic cells for 1, 3 and 24 hours at 37°C before washing out and proceeding to cell lysis and Colony Forming Unit (CFU) count. Results are shown in figure 12.

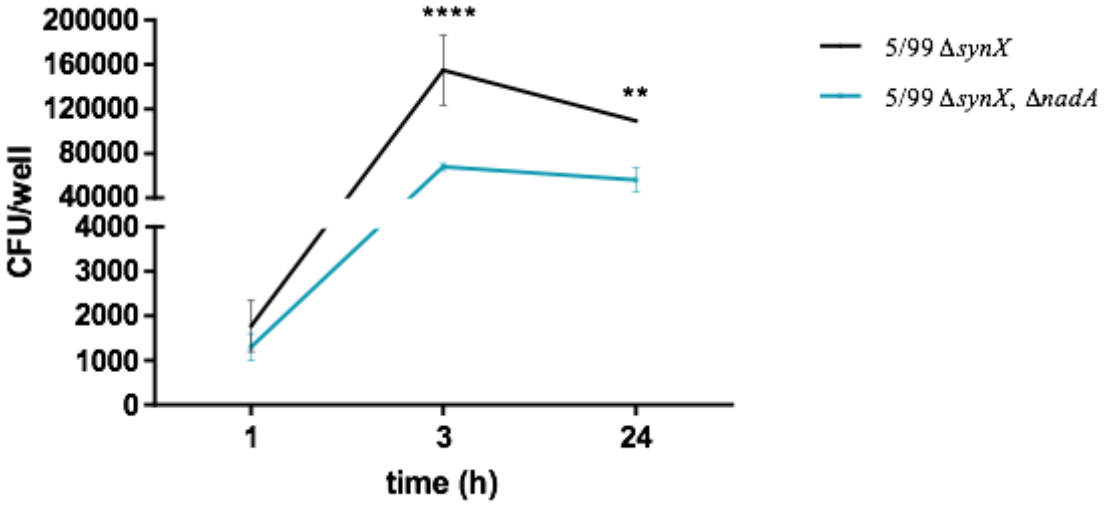


Figure 12. Time course infection of THP-1 cells with unencapsulated *N. meningitidis* wt and Δ*nadA* strains. THP-1 cells were challenged for different time points (1 ,3 ,24h) and at each time point monocytes were lysed with 1% saponin. The number of live bacteria associated to cells was determined after serial dilutions, agar plating and CFU counting. Data represent the mean (SD) of 3 experiments performed in duplicate (Reported p value **** < 0.0001, **< 0.01(two -way ANOVA test).

After 1h of challenge, no significant differences in bacterial binding was detected between NadA wt and Δ*nadA* strain. Instead, after 3 hours of infection, the strain which expresses NadA adhere more than the KO NadA strain (p value **** < 0.0001). After long term contact between *N. meningitidis* and monocytic cells, this difference is maintained.

THP-1 monocytic cells can be differentiated into macrophages by adding phorbol 12-myristate 13-acetate (PMA) for 72 hrs. The differentiation consists in the acquisition of adherent phenotype and changes in morphology. The adherent THP-1 cells were also challenged with *N. meningitidis* strains for 15, 30, 60 and 180 minutes (figure 13). In this condition, the difference between wt and NadA KO was observed only within 3 hours of infection while at longer incubation times the number of bacteria recovered were equivalent for each strain (16 and 24 hrs not shown).

To verify whether the different level of adhesion observed on macrophages at 1 hr of infection correlated with the presence of Siglecs receptors, we pre-incubated cells with polyclonal antibody anti-Siglec-5/14 before adding wt bacteria or NadA KO. The pre-incubation with antibodies determined a decrease of adhesion of the 5/99 strain expressing NadA (*< 0.05) while for the isogenic strain deprived of NadA there was not difference (figure 14). These results suggest that Siglecs receptors could contribute *to N. meningitidis* association to monocytic cells when NadA is expressed.

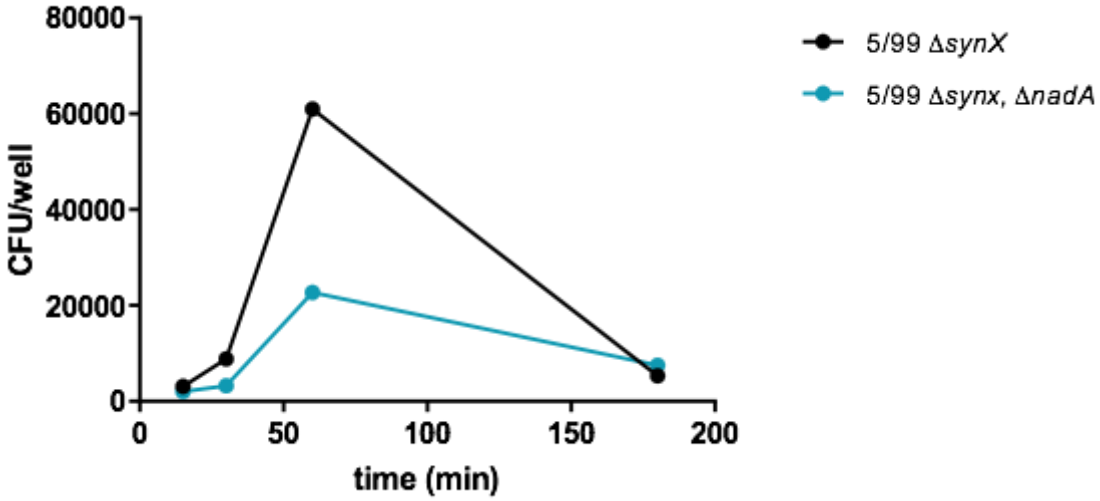


Figure 13. Time course infection of macrophages derived from THP-1 cells with unencapsulated *N. meningitidis* wt and *ΔnadA* strains. THP-1 cells were challenged for different time points (15, 30, 60 ,180 min) and at each time point monocytes were lysed with 1% saponin. Live bacteria associated to cells were determined after serial dilutions, agar plating and CFU counting.

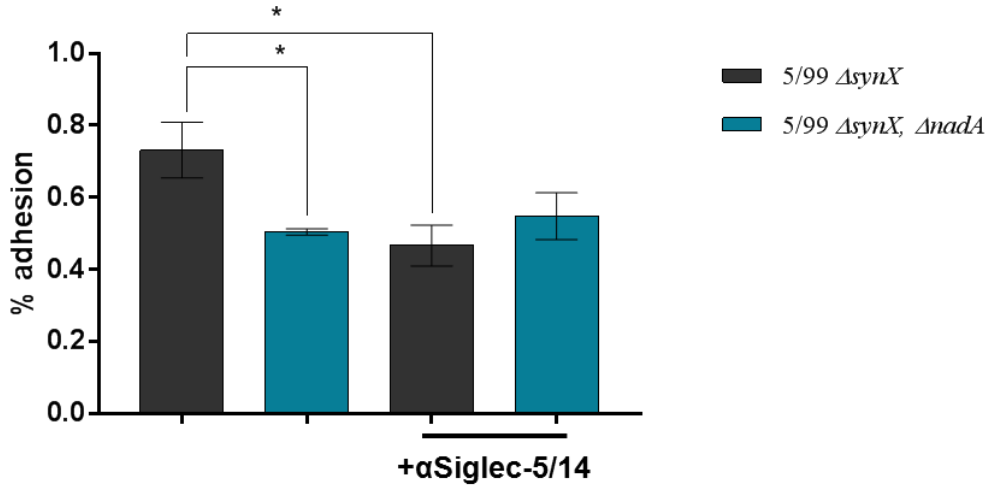
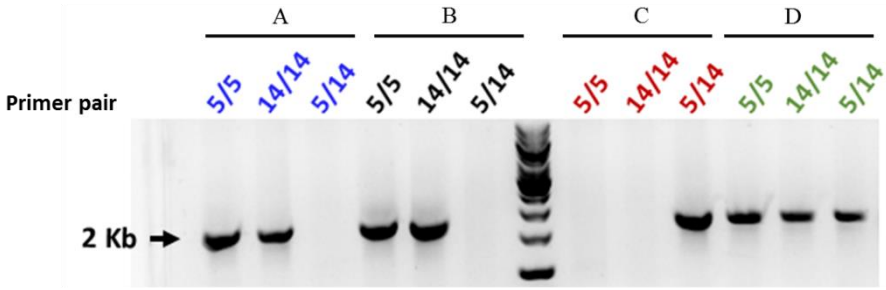


Figure 14. Inhibition of adhesion on macrophages derived from THP-1 cells infected with unencapsulated *N. meningitidis* wt and *ΔnadA* strains. Cells were pre-incubated with polyclonal antibody anti-Siglec-5/14 for 30 min at 37°C. Then, cells were incubated with *N. meningitidis* strains for 1h. After extensive washings, cells were lysed with 1% saponin. Live bacteria associated to cells were determined after serial dilutions, agar plating and CFU counting. Data represent mean (SD) of 3 experiments performed in duplicate (Reported p value *< 0.05).

3.4 Stimulation of monocytes and macrophages from donors with N. meningitidis OMVs

Outer membrane vesicles (OMVs) are blebs from bacterial membranes released to overwhelm the immune cells (Post *et al.* 2005) This membranous material contains several membrane proteins including NadA. To the aim of checking if NadA interaction with eukaryotic Siglecs receptors evoked a host response, OMVs derived from *N. meningitidis* strain 5/99 wt and Δ*nadA* were used to stimulate monocytes and macrophages and to measure the cellular response in terms of secreted cytokines. The studies with primary monocytes and macrophages from donors were conducted in collaboration with Doctor P. Italiani (CNR, Naples) by using OMVs purified *in house.*

As described before, Siglec-5 and Siglec-14 locus is polymorphic in human population meaning that a single cell can express both activatory (Siglec-5) and inhibitory (Siglec-14) receptors or only inhibitory (Siglec-14/5 fusion). These expression patterns might influence the cytokine release from immune cells when facing a foreign particle. For these reasons, we screened the Siglecs genotypes of monocytes from donors and performed the experiments on all three of them. Genotyping of the SIGLEC14 locus was performed as described previously by Yamanaka *et al.* 2009. In brief, a set of three PCR reactions, specifically amplifying SIGLEC14, SIGLEC5, and SIGLEC14/5 fusion genes, were run in parallel using genomic DNA from each donor as template. As representative, a screening on PBMC from 4 healthy donors is shown in figure 13. Genomic DNA from homozygous wt individuals yields products only in 2 reactions +/+/−; that from heterozygous individuals yields products in all three reactions (+/+/+); and that from homozygous null individuals yields product only in the third (−/−/+). (Summary in the table of figure 15). In conclusion, we confirmed the presence of three patterns of Siglecs gene arrangement in the human population and performed our studies on all the three genotypes.



Donor	SIGLEC-5	SIGLEC-14	SIGLEC-14/5	Genotype
A	+	+	-	WT
B	+	+	-	WT
C	-	-	+	NULL
D	+	+	+	WT/NULL

Figure 15. Genotyping of monocytes from donors. Genomic PCR results of a set of individuals (A, B, C, D). The primer pair 5R/5F specifically amplifies a part of the *SIGLEC5* gene, and the primer pair 14R /14F a part of the SIGLEC14 gene. Genomic PCR with the primer pair 14F / 5R yields a product only when the SIGLEC14/5 fusion gene is present. The table below summarize the genotypes found.

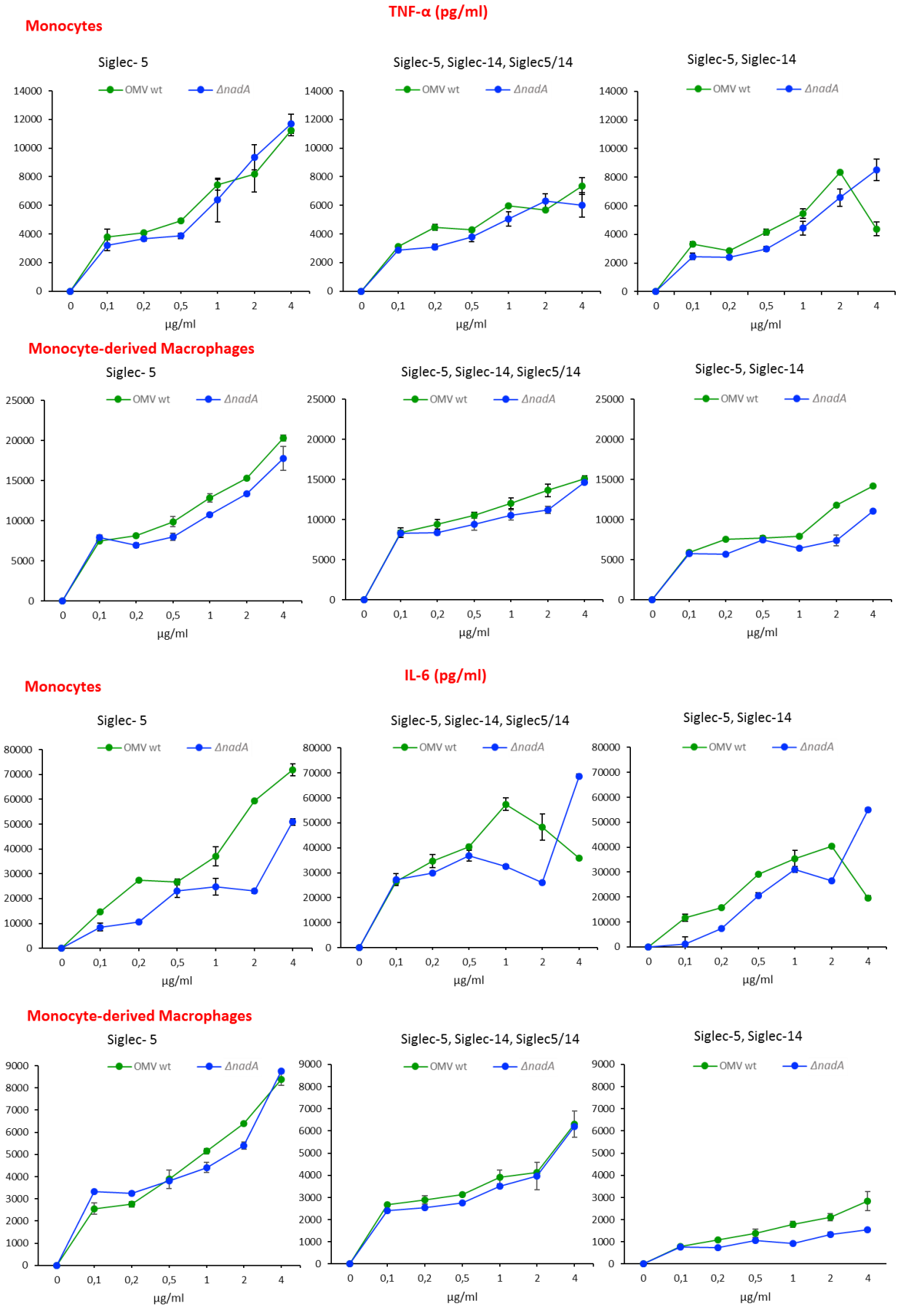
Monocytes and macrophages were stimulated with OMVs derived from *N. meningitidis* wt and *ΔnadA*. Increasing concentrations of vesicles preparation (0.1-4 μg/ml) were added on phagocytes O/N at 37°C. After incubation, supernatants were collected. A pattern of secreted cytokines was investigated by ELISAs. Data shown in figure 16 (A, B, C) illustrates the complete analysis of pro- and one anti-inflammatory cytokines.

Tumor necrosis factor α, interleukin-6 and interleukin-1β were tested as pro-inflammatory cytokines. In donors lacking the activatory Siglec-14 (indicated as Siglec-5 null donors), we expected the levels of pro-inflammatory cytokines to decrease following the binding of NadA to the cellular receptors. Unexpectedly, no substantial differences were obtained between the two types of OMVs.

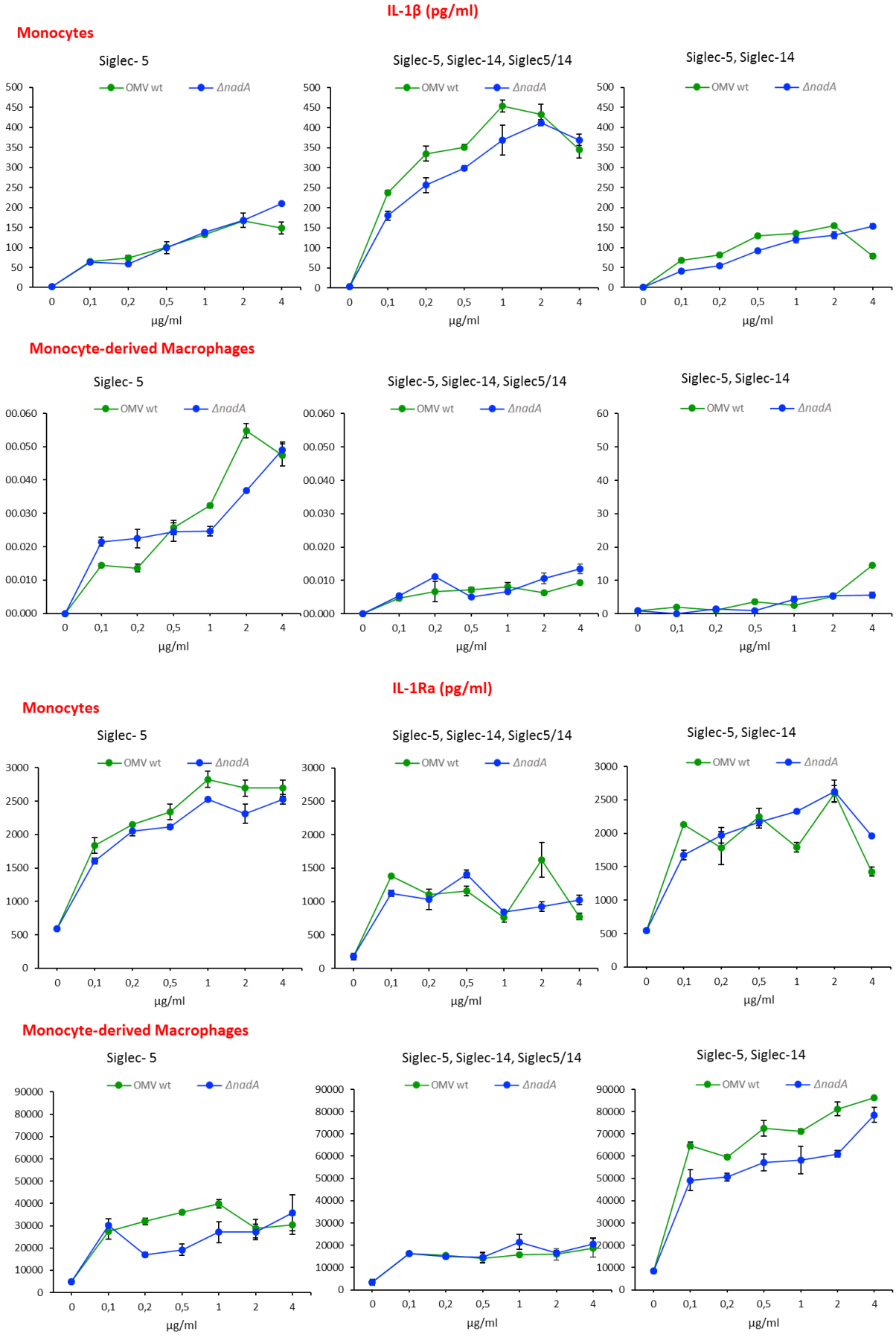
The IL-1 family encompasses 11 cytokines (agonists and antagonists) and 10 receptors (membrane/soluble receptors, co-receptors and decoy receptors). The main cytokine of the family is IL-1β, strictly regulated by its natural inhibitor, IL-1Ra, and soluble receptors IL-1R2 (sIL-1R2) (Boraschi *et al.* 2018). Here, we measured the level of IL-1β, IL-1Ra and sIL-1R2 in the supernatants of human primary monocytes and monocyte-derived macrophages stimulated with increasing doses of OMVs wt, and OMVs *ΔnadA* (range from 0.1µg/ml to 4µg/ml). Fresh monocytes were isolated from donors with different genotypic arrangement for Siglec receptors (*Siglec5, Siglec 14, Siglec14-null* or *Siglec14/5*) and related macrophages were differentiated *in vitro* as described in materials & methods section. We observed a reduction of IL-1β production by monocytes upon stimulation with OMVs lacking NadA independently on Siglec receptor genotyping. Moreover, this reduction has been observed only in macrophages isolated from donor Siglec5.

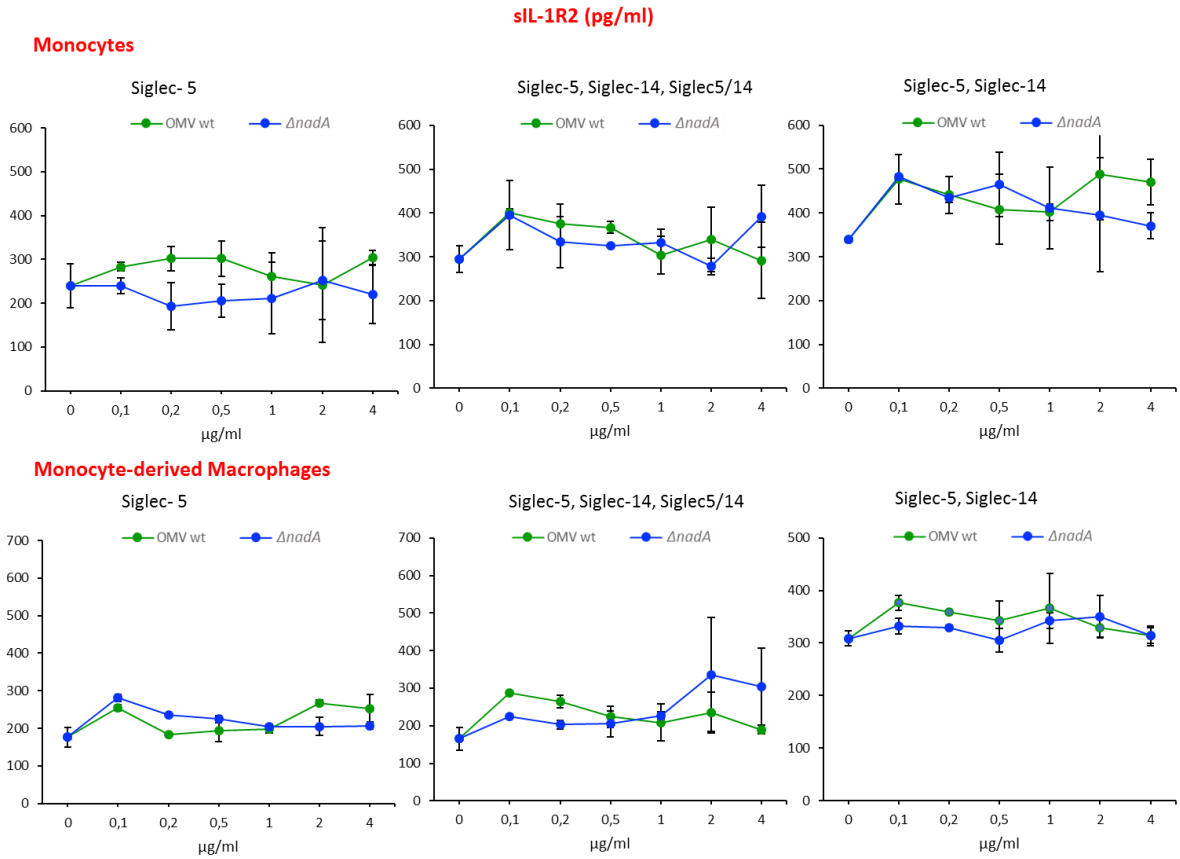
No differences were observed in IL-1Ra and sIL-1R2 production, neither after stimulation with the different types of OMVs. IL-10 was analyzed as representative inhibitory cytokine. Although the levels this inhibitory cytokine secreted by the different genotypes varies, again no difference among the two types of OMVs were observed following stimulation.

A


TNF-α (pg/ml)
Monocytes
Siglec- 5
Siglec-5, Siglec-14, Siglec5/14
Siglec-5, Siglec-14
OMV wt
ΔnadA
µg/ml
Monocyte-derived Macrophages
IL-6 (pg/ml)

B


IL-1β (pg/ml)
Monocytes
Siglec- 5
Siglec-5, Siglec-14, Siglec5/14
Siglec-5, Siglec-14
OMV wt
ΔnadA
µg/ml
Monocyte-derived Macrophages
IL-1Ra (pg/ml)
Monocytes
Monocyte-derived Macrophages


sIL-1R2 (pg/ml)
Monocytes
Siglec- 5
Siglec-5, Siglec-14, Siglec5/14
Siglec-5, Siglec-14
OMV wt
ΔnadA
µg/ml
Monocyte-derived Macrophages
Siglec- 5
Siglec-5, Siglec-14, Siglec5/14
Siglec-5, Siglec-14
OMV wt
ΔnadA
µg/ml

C

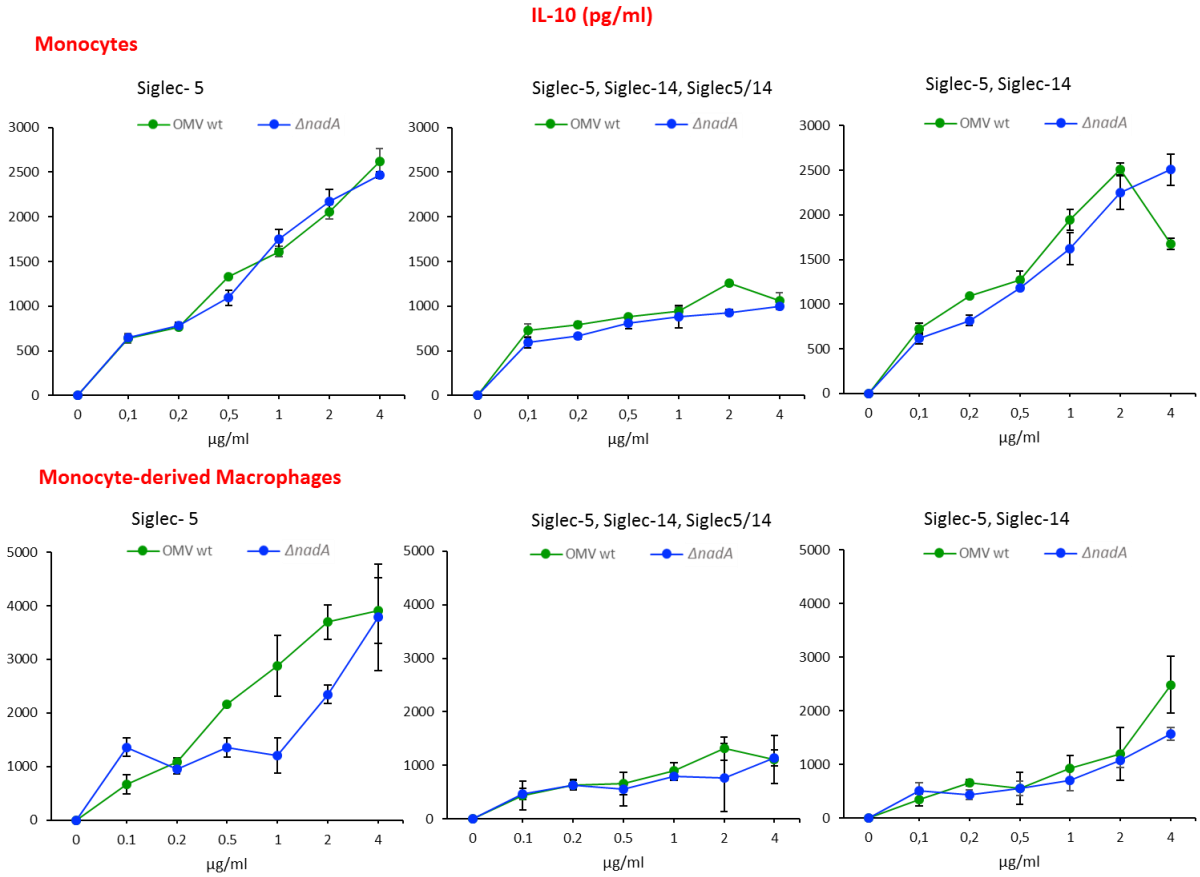

IL-10 (pg/ml)
Monocytes
Siglec- 5
Siglec-5, Siglec-14, Siglec5/14
Siglec-5, Siglec-14
OMV wt
ΔnadA
µg/ml
Monocyte-derived Macrophages
Siglec- 5
Siglec-5, Siglec-14, Siglec5/14
Siglec-5, Siglec-14
OMV wt
ΔnadA
µg/ml

Figure 16 A. Induction of pro-inflammatory cytokines TNF-α and IL-6 by OMVs wt and *ΔnadA*. Monocytes and macrophages with the three Siglecs genotypes were incubated with increasing concentration (from 0.1 to 4 ug/ml) of OMVs wt (green) and Δ*nadA* (blue) for 24 hours. TNF-α and IL-6 production in the cell-free supernatants were determined by ELISA. Cytokines concentration in pg/ml (y-axis) was obtained by interpolation of absorbance values against a standard curve. **B. Induction of IL-1β, IL-1Ra and sIL-1R2 cytokines by OMVs wt and Δ*nadA*.** Monocytes and macrophages with the three Siglecs genotypes were incubated with increasing concentration (from 0.1 to 4 ug/ml) of OMVs wt (green) and Δ*nadA* (blue) for 24 hours. IL-1β , IL-1Ra and sIL-1R2 production in the cell-free supernatants were determined by ELISA. Cytokines concentration in pg/ml (y-axis) was obtained by interpolation of absorbance against a standard curve. **C. Anti-inflammatory cytokine IL-10 release after OMVs wt and Δ*nadA* stimulation.** Monocytes and macrophages with the three Siglecs genotypes were incubated with increasing concentration (from 0.1 to 4 ug/ml) of OMVs wt (green) and Δ*nadA* (blue) for 24 hours. IL-10 production in the cell-free supernatants was determined by ELISA. Cytokines concentration in pg/ml (y-axis) was obtained by interpolation of absorbance values against a standard curve.

In conclusion, the fluctuations in the release of cytokines on the different genotypes is not dependent on NadA presence on the OMVs. In homologous system Siglec-5 and -14 do not seem to bind NadA on monocytes/macrophages membrane.

4. Effects of NadA binding to soluble Siglec-5

Although Siglecs are type I transmembrane receptors, data shown suggested a weak NadA-Siglecs interaction on cell membrane in more physiological contexts. Numerous works have reported that plasma contains higher amounts of CD33 related Siglecs, such as Siglec-5, -14 and -9, physiologically released as soluble proteins. Further, the amount of the soluble species can increase 3-4fold under pathological conditions (Biedermann *et al.* 2007). Soluble forms of Siglecs in supernatants of cultured cells, such as neutrophils, monocytes and macrophages, were found as well (Matsubara *et al.* 2015, Zeng et al. 2017). For these reasons, we checked first whether soluble Siglecs, were produced by cells used in this study and then if the presence of soluble siglecs influences the cycle of infection of *N. meningitidis*.

4.1 Siglecs are shed by monocytes and macrophages

Culture media collected from confluent monocytes/macrophages from donors, THP-1 cells and macrophages derived were checked for Siglecs shedding. Moreover, pooled human sera were included in the analysis, to assess the *ex vivo* amount of Siglecs and *in vitro* release by cell cultures. Samples were analyzed by sandwich ELISA as follow. ELISA plates were coated with capture antibody against Siglec-5 and -14 and incubated with sera and culture supernatants. Binding was revealed by a mouse anti Siglec-5/14 biotin labeled detection antibody and the appropriate avidin-HRP secondary antibody. Siglec-5/14 concentrations in each condition was calculated by plotting the absorbance (450 nm) against a standard curve obtained with recombinant human Siglecs (figure 17).

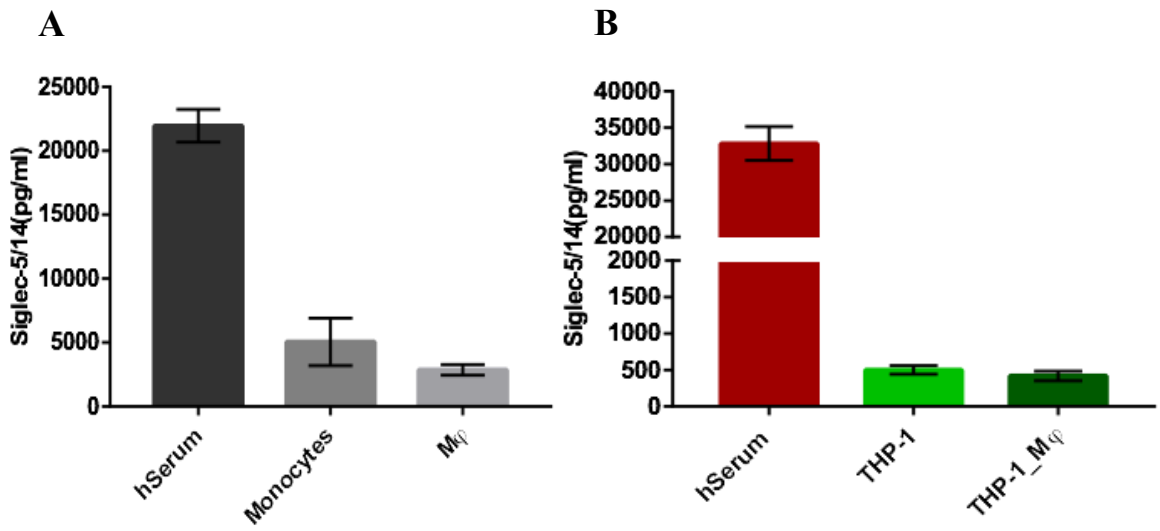


Figure 17. Siglec-5 and 14 released by Monocytes /Macrophages (Mφ) from donors (A) and THP-1 cells (B). ELISAs performed on media collected from cultured confluent cells. Concentration in pg/ml was obtained by interpolation of absorbance values against a standard curve. Graphs are representative of three detections performed in duplicate.

Although human serum contains the expected high levels of sSiglecs, phagocytes from human donors also release noticeable levels higher than THP-1 cells and THP-1-derived macrophages.

4.2 N. meningitidis OMVs effect on Siglecs release

Since monocytes from donors released high levels of Siglec-5/14, we checked the release *status* during stimulation with OMVs. Monocytes from the three Siglecs genotypes were incubated with increasing concentration of OMVs. Obtained supernatants were analyzed for soluble Siglec-5/14 by sandwich ELISA as described in 4.1 paragraph. Dose response-curve of Siglecs release are shown in figure 18. In resting state, the level of soluble Siglecs seems to correlate with the different genotypes. Notably, when only Siglec-5 is expressed, the levels of shed receptor are lower. It is important to mention that it is not possible to discriminate sSiglec-5 and -14 in solution due to the high homology of the ectodomain (99% of homology) and the lack of commercial specific antibodies. For these reasons, we cannot confirm which is the most represented Siglec in solution both in resting and stimulated monocytes.

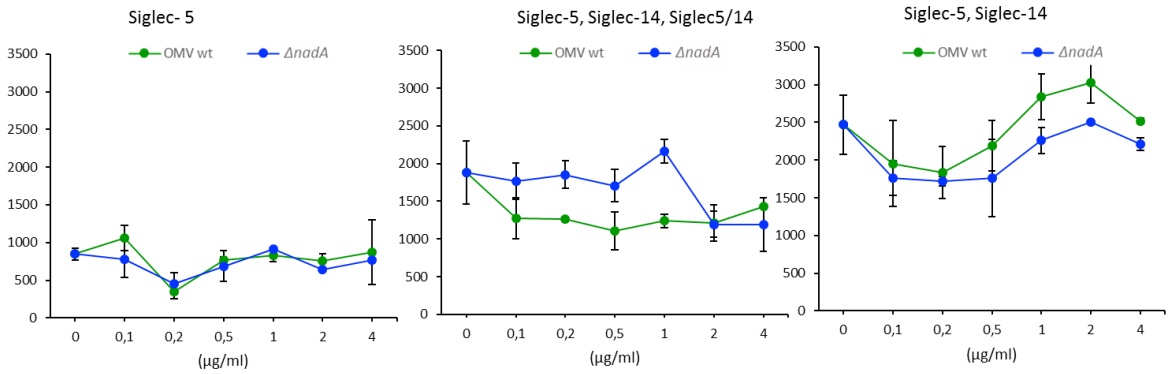


Figure 18. Dose response of Siglec-5/14 release in monocytes medium after stimulation with OMVs preparations obtained from *N. meningitidis* 5/99 wt strain (green) and *ΔnadA (blue)*. Supernatant of monocytes from different genotypes (null, heterozygous and wt, respectively) were tested for sSiglec-5/14 content. Concentration in pg/ml was obtained by interpolation of absorbance values against a standard curve. Graphs are representative of three measurements performed in duplicate.

Even though the stimulation with OMVs shows a genotype-dependent trend, a correlation with NadA expression did not emerge. Indeed, behavior of OMVs from *N. meningitidis ΔnadA* appeared to be similar to the wt.

4.3 N. meningitidis binds sSiglecs shed by monocytes in NadA dependent manner

As described in paragraph 3.1, *N. meningitidis* binds recombinant purified Siglec ectodomain in solution, in NadA dependent manner. We asked whether *N. meningitidis* 5/99 strain expressing NadA capture soluble Siglecs from THP-1 media. To test this hypothesis, we collected supernatants after 1h of challenge and looked for the binding of Siglecs on *N. meningitidis* wt or *ΔnadA*. Free-bacteria in the supernatants of infection were pelleted, fixed and stained with a goat- anti Siglec-5/14 antibody. Then appropriate conjugate secondary antibodies were added. As shown by confocal microscopy analysis, bacteria expressing NadA (red) are covered by Siglec-5/14 (green) and *ΔnadA* strain did not (figure 19). This evidence suggests a possible influence of shed Siglecs during contact of *N. meningitidis* with phagocytic cells. Moreover, we found that this novel interaction is strictly NadA dependent.

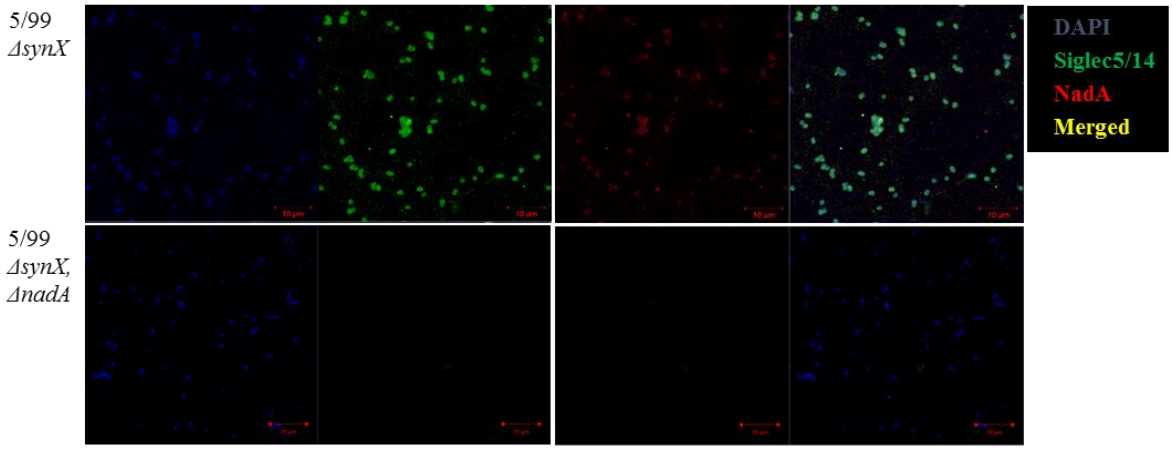


Figure 19. Soluble Siglec 5/14 in THP-1 supernatant specifically bind to *N. meningitidis* expressing NadA. Confocal microscopy acquisition of supernatant content from THP-1 cells challenged for 1h with *N. meningitidis* strains 5/99 Δ*synX* and Δ*synX* Δ*nadA*. After extensive washings, bacteria in the supernatant were stained with goat polyclonal anti-Siglecs antibody and mouse polyclonal anti-NadA antibody followed by Alexa-Fluor 488, 647-conjugated secondary antibodies and DAPI to stain bacterial chromosome. Immunofluorescence allowed to visualize the Siglec-5/14 bound to bacterial surface (green) and NadA (red). The co-localization of Siglec-5/14 on 5/99 strain *ΔsynX* is indicated in yellow. In the Δ*nadA* strain, the presence of Siglecs on bacterial surface was not detectable. Images are representative of at least 3 experiments showing similar results. Images were acquired by confocal microscope (Zeiss, LSM 700), magnification 100X.

4.4 rSiglec-5 influence on N. meningitidis association with and internalization by THP-1 cells

In the previous paragraph, extracellular Siglecs were shown to associate with *N. meningitidis* in NadA dependent manner. On the basis of this evidence, we sought to elucidate whether this association may have significant biological consequences. As first, we examined whether the binding of exogenous soluble Siglec-5 to *N. meningitidis* had an impact on the bacterial-phagocyte interaction. To this end, we pre-incubated *N. meningitidis* with soluble recombinant Siglec-5 and analyzed the effect on adhesion to and uptake by THP-1 monocytic cells.

Allophycocyanin (APC) labelled bacteria were pre-incubated with or without 15 µg/ml of soluble Siglec-5 for 30 minutes at 37°C. THP-1 cells were added and the incubation was conducted for 30 minutes at 37° in agitation. In parallel, identical samples were incubated with antibiotic (gentamicin at 200 µg/ml) for 45 min at 37° to exclude non-internalized bacteria. Finally, samples were fixed and analyzed by flow cytometry. The mean fluorescence intensity (MFI) of THP-1 cells APC^+ is shown in figure 20. As shown in figure 20A, the MFI indicates the total associated bacteria derived from non-antibiotic treated samples. When THP-1 cells are incubated with 5/99 strain expressing NadA (figure 20, black) covered by exogenous Siglec-5, the MFI increased. This result could indicate an enhancement in bacterial association when Siglec-5 is on bacterial surface. This difference in bacterial attachment was not observed with the isogenic strain KO NadA (figure 20, blue). In figure 20B, cells were treated with gentamicin following incubation with labelled bacteria. In this condition, the addition of soluble protein Siglec-5 exerted a reduction of APC^+ phagocytes compared to the condition without Siglec. These results indicated that Siglec-associated bacteria adhere better to THP-1 cells but are less internalized by these cell line. The phenomenon appears to be NadA dependent, since incubation of THP-1 cells with the strain lacking NadA did not show any difference in both conditions.

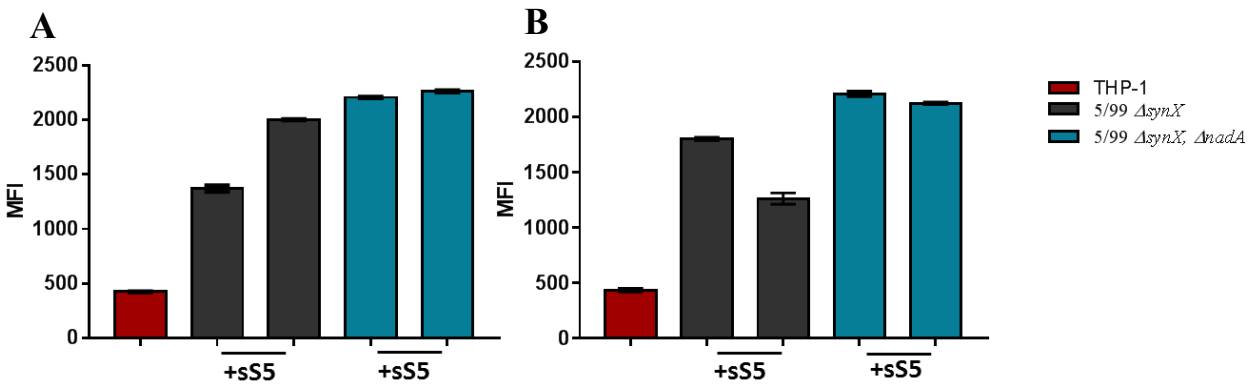


Figure 20. Association and internalization of *N. meningitidis* by THP-1 monocytic cells. A: MFI of APC+ cells after incubation with 5/99 strain *ΔsynX* (black columns) and *ΔsynX ΔnadA* strains (blue columns). In red is indicated the baseline fluorescence of THP-1 cells alone, without adding bacteria. **B**: MFI of APC+ cells challenged as in A and then treated with the antibiotic gentamicin. Graphs are the mean (SEM) of 3 acquisitions.

4.5 Recombinant NadA inhibits rSiglec-5 binding to C1q

During the dissemination into the bloodstream or at the interface with resident macrophages in the nasopharynx, we can suppose that meningococcus can be covered by Siglecs. In the complex milieu of bloodstream, *N. m*eningitidis encounters soluble factors such as complement cascade components. The activation of complement factors was shown mainly to lead to both the recognition of foreign particles by phagocytes and to the killing by the membrane attack complex (MAC) pore formation (Schneider *et al*. 2007). Some human receptors of the immunoglobulin superfamily such as RAGEs [receptor for AGEs (advanced glycation end-products)] and Siglec-3 receptors were found to bind complement component 1 (C1q), the first protein involved in the activation of classical pathway (Ma *et al.* 2012; Son *et al*. 2017). Thus, we asked whether soluble Siglec-5 and -14 tightly bound to *N. meningitidis* could have a correlation with complement cascade. The first step was to look for possible interactions between Siglec-5 and -14 and complement factors. In particular, we checked if Siglec-5 could recognize C1q by mean of the extracellular immunoglobulin-like domain such as Siglec-3 (Son *et al.* 2017). Direct evidence of C1q-sSiglec-5 interaction was investigated by ELISA. C1q (10µg/ml) from serum was coated on a plate and incubated for 1h at 37°C with increasing concentrations of soluble recombinant Siglec-5. Following extensive washing, Siglec-5/C1q interaction was

revealed as captured C1q on plates by using mouse anti-Siglec-5 antibody. A dose-range binding activity towards C1q was observed (figure 21).

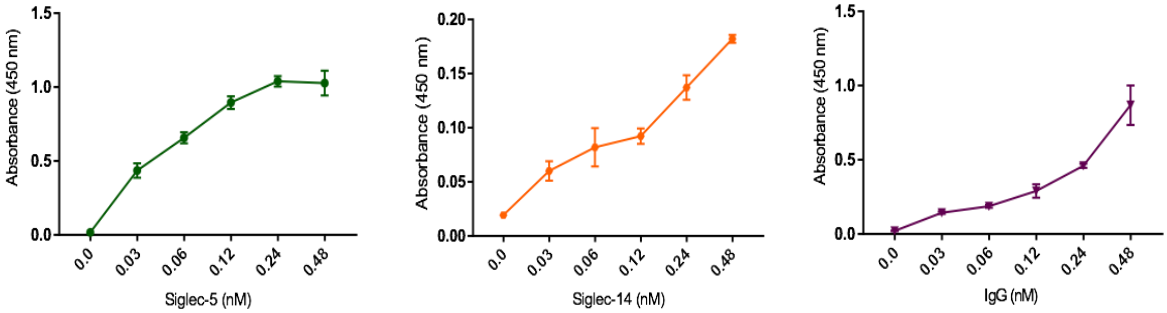


Figure 21. Recombinant Siglec-5 and 14 binding to human complement C1q. ELISA plates coated with 10 µg/ml of purified human complement component C1q were incubated with a range of dilutions (1:2) of recombinant Siglec-5 (0.03 nM-0.48 nM) for 1-hour. Immunoglobulins G were used as control. Siglec-5 binding to immobilized C1q is represented as absolute absorbance. Graphs are representative of three detections performed in duplicate. Error bars indicate SD.

To assess the possible involvement of NadA in this interaction, a competitive ELISA-like assay was performed in the same conditions, i.e. with fixed C1q on plates and soluble Siglec-5, by adding increasing concentration of recombinant NadA with recombinant Siglec-5 at fixed concentration (0.12 nM). As control, we used IgG fixation to C1q and verify if the adhesin interfered with the capture,

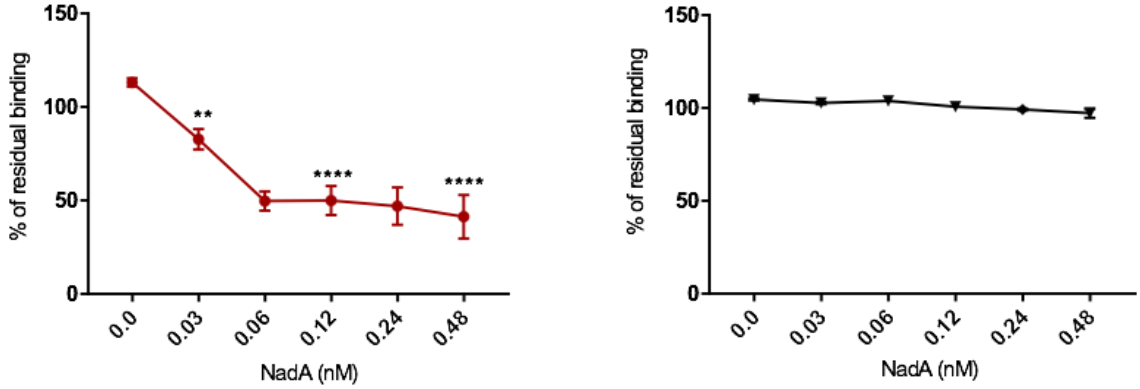


Figure 22. NadA interference of Siglec-5 binding to human complement C1q. (**A**) ELISA plates coated with 10 µg/ml of purified human complement component C1q were incubated with several dilutions of recombinant NadA (0.03 nM-0.48 nM) toghether with a fixed concentration of Siglec-5 (0.12 nM) for 1 hour at 37 C°. Incubation of IgG with NadA was used as control. NadA competition with Siglec-5 binding is shown as % of residual absorbance, indicating Siglec-5 binding. Siglec-5 alone was set as 100% of binding to calculate the % of residual binding. Graphs are representative of three detections performed in duplicate. Bars indicate SD (p values ** =0.008, ****< 0.0001, One-way ANOVA test)

As shown in figure 22, in presence of NadA, the signal of bound Siglec-5 decreased below 50% at the maximal NadA concentration stoichiometrically equivalent at 1:1 to fixed Siglec-5. IgG fixation did not

show any reduction following addition of NadA (figure 22) consistent with a specific interference of NadA to the Siglec-5 region of binding to C1q.

4.6 C1q deposition on N. meningitidis "decorated" with rSiglec-5

C1q is the apical factor in the activation of classical pathway (CP) of complement cascade. C1q can directly bind to bacterial surface structures (such as Lipid A) or to clustered IgG or IgM on bacteria. Upon C1q binding, its associated proteases (C1r and C1s) become activated and form the C1 complex. This event initiates the complement cascade via the classical pathway that proceeds with the C3b opsonization of the pathogen and the associated enhanced phagocytosis. To deeply investigate the NadA interference on Siglec-5/C1q complex observed in solution, the analysis proceeded on the whole bacterium. *N. meningitidis* 5/99 strains wt or *ΔnadA* were incubated with C1q (20 μg/ml) with or without recombinant Siglec-5. Bound C1q was detected by flow cytometry on capsulated (figure 23) and unencapsulated strains (figure 24). Representative histograms depicts the detection in the different conditions. Graphs with columns represent geometric mean fluorescence derived from flow cytometry measurements. In both cases, an increased C1q deposition is observed in presence of anti-NadA antibodies (figure 23-24). When sSiglec-5 was added, a decrease of C1q dependent antibodies deposition was observed. This evidence was not found on the strains deprived of NadA. These data suggest an active role of NadA in counteracting complement deposition on *N. meningitidis* during infection.

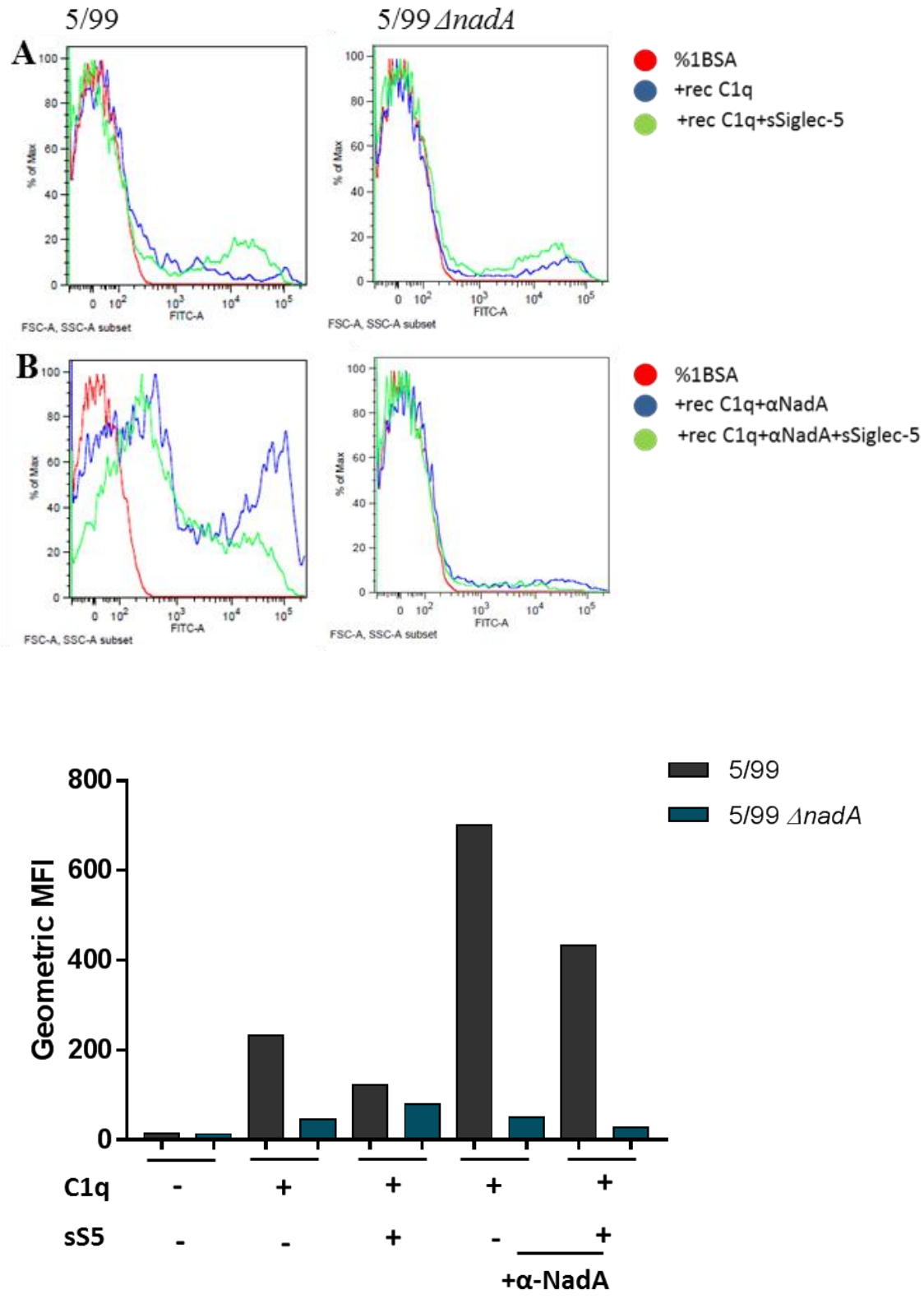


Figure 23. C1q deposition on capsulated *N. meningitidis* by antibodies against NadA and sSiglec-5. (A) 5/99 and 5/99 *ΔnadA* strains were incubated with buffer alone (PBS/1% BSA, in red), purified C1q (blue) and recombinant Siglec-5 (green). **(B)** Bacteria were incubated with purified C1q and mouse Ab Anti-NadA (blue) or with recombinant Siglec-5 (green). Incubation with respective mixes were conducted for 30 min at 37C°. After washings, bacteria were incubated with FITC conjugated anti-C1q polyclonal antibody. Bound C1q was detected by flow cytometry. Column graph indicates the geometric MFI derived from the histograms.

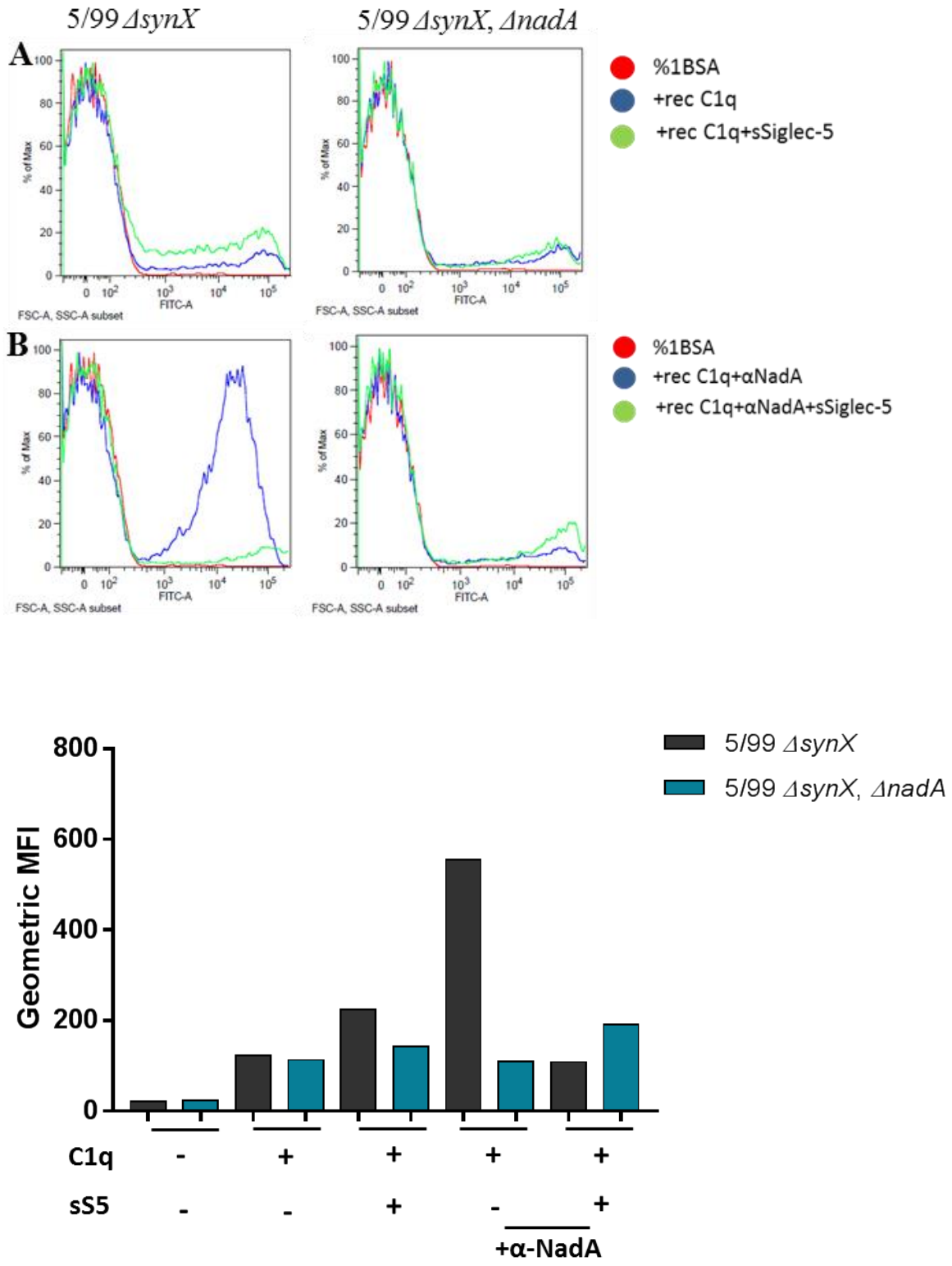


Figure 24. C1q deposition on unencapsulated *N. meningitidis* by antibodies against NadA and sSiglec-5. (A) The isogenic strains 5/99 *ΔsynX* and 5/99 *ΔsynX*, *ΔnadA* strains were incubated with buffer alone (PBS/1% BSA, in red), purified C1q (blue) and recombinant Siglec-5 (green). **(B)** Bacteria were incubated with purified C1q and mouse Ab Anti-NadA (blue) or with recombinant Siglec-5 (green). Incubation with respective mixes were conducted for 30 min at 37C°. After washings, bacteria were incubated with FITC conjugated anti-C1q polyclonal antibody. Bound C1q was detected by flow cytometry. Column graph indicates the geometric MFI derived from the histograms.

4.7 Serum depletion of Siglec-5/14: consequences on N. meningitidis survival

The activation of CP, primed by C1 complex formation, leads to the C3b deposition on bacteria. C3b is an essential component of convertases which initiates the assembly of the late complement components (C5b through to C9), leading to the formation of the membrane attack complex (MAC). The MAC is a multiprotein structure that inserts into lipid bilayers, causing membrane disruption and cell lysis (Schneider *et al*. 2007). Since the presence of Siglec-5 interferes with the C1q deposition mediated by anti-NadA antibodies, we proceeded to test whether this blockage can interfere with the activation of classical pathway and therefore with the survival of bacterium in the human serum. To verify this hypothesis, human serum bactericidal activity (hSBA) assays were conducted. The SBA assay measures the ability of circulating antibodies to lyse meningococci in the presence of complement, otherwise known as complement-mediated killing via the CP of the immune response. The bactericidal titer is defined as the dilution of the test anti-serum/antibody that results in a 50% decrease in colony forming units (CFUs) per mL of bacteria. As such, it is a functional assay in that it measures the killing of live bacteria (reviewed in McIntosha *et al.* 2014).

To verify whether soluble Siglec-5/14 in human serum plays a role in bactericidal activity, we prepared a sSiglec-5/14 depleted serum. sSiglecs depletion were obtained by incubating human serum on a plate coated with anti-Siglecs antibodies. After three consecutive incubations, concentration of sSiglecs were determined by ELISA and compared with the non-treated serum (figure 25, hSerum *vs* ΔSiglecs).

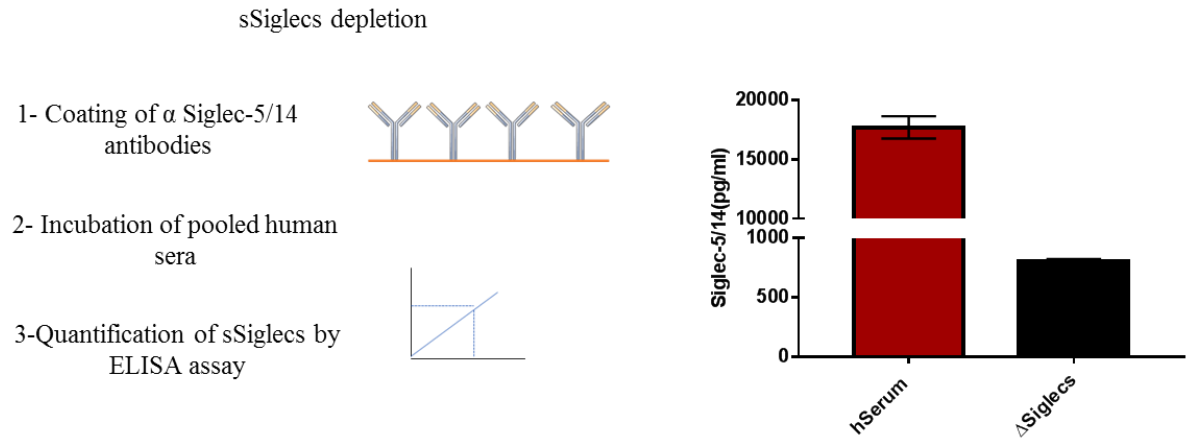


Figure 25. Quantification of residual Siglec-5/14 in pooled human sera after depletion protocol. ELISA quantification of Siglecs before and after depletion (flowchart on the left). Concentration in pg/ml was obtained by interpolation of absorbance values against a standard curve. Graphs show the result obtained for the first depleted sera pool, similar results were obtained with other pools .

The calculated residual Siglecs in the treated serum shown in figure 25 (black column) was the 9% respect to the untreated. Different pools gave similar results in all cases resulting in a percentage of depletion higher than 90%. With this tool, we proceed with the hSBA assay. Bacteria were incubated with both sera, as source of complement and antibodies against NadA. Moreover, we used the anti-capsule polyclonal antibody, since sialic acids decorations on capsule are described to be involved in a moderate binding with Siglec-5 (Jonas *et al.* 2003). Experiments performed with 5/99 strain did not show strong differences in hSBA titers in the depleted serum respect to the untreated (data not shown). We speculated that this result may be due to the amount of free-Siglecs in sera, found in the range of

nanograms (fig. 17), could not be enough to saturate the bacterial exposed adhesin due to the high expression of NadA on this strain. Consequently, the antibodies against NadA overcomes the partial "masking" effect of Siglecs on the antigen. For these reasons, we selected the strain *N. meningitidis* 96217 which express lower levels of NadA with respect to the strain 5/99.

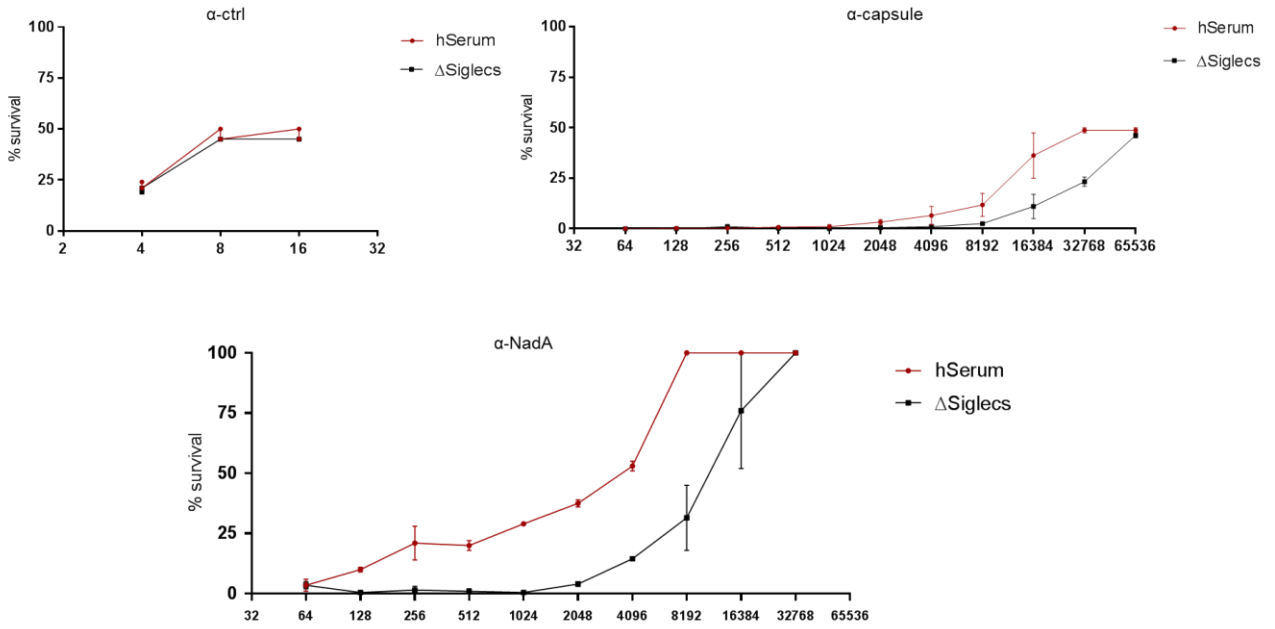


Figure 26. Human SBA performed on *N. meningitidis* 96217 strain.
Percentage of survival of *N. meningitdis* 96217 strain with anti-serum against NadA, antibody against capsule or anti-serum against a surface exposed antigen used as control (ctrl). Graphs show percentage of bacteria survival at different concentrations of antibodies in untreated serum (red) and ΔSiglecs serum (black).

Preliminary data from the hSBA performed are shown in figure 26. When bacteria were incubated with depleted Siglecs serum, 50% of survival was reached two titers lower than the untreated serum, suggesting an interference of NadA with complement deposition. Anti-control antibody driven killing did not show variation between the two sera. Interestingly, the anti-capsule antibody was more efficient in hSBA when ΔSiglecs serum was used as complement source, indicating that Siglec-5/14 play an active role in complement deposition on bacterial capsule. These data indicate that the presence of NadA on the bacterium may be protective against human complement and depends on the relative concentration of sSiglec 5/14 in the serum.

Together, these results may indicate that the capsulated *Neisseria meningitidis* decorated by soluble Siglecs receptors could confer a survival advantage to the bacterium when facing antibodies against NadA and the effectors of the complement cascade.

Supplementary results

I: Biochemical characterization of NadA-FcγRIIA receptor binding

Surface plasmon resonance (SPR) analysis

To assess the binding kinetics between NadA and FcγRIIA, SPR (Biocore) technology was used. Recombinant soluble ectodomain of FcγRIIA (figure S1) was first immobilized onto a Ni-NTA sensor chip, via its His-tag. In order to check the activity of FcγRIIA, human IgGs were injected. Binding level of > 1300 RU is reached demonstrating that Fcγ RIIA was active (figure 1A). Upon injection of 100 nM NadA no activity is seen suggesting no interactions beween NadA and Fcγ RIIA occurs (figure 1.1 B). The binding analysis was also conducted in inverted configuration using Fcγ RIIA as analyte on immobilized NadA. To test the functional integrity of immobilized NadA, we use the known NadA ligand NadA-R1. As expected, a strong SPR signal was revealed, instead injecting Fcγ RIIA no signal was visible (data not shown). Capturing of Fcγ RIIA via its His tag resulted in an active immobilized protein, as demonstrated by the interaction with human IgG. However, no interaction was observed with NadA in any of the two configurations. In summary, these analyses showed no evidence of a NadA-Fcγ RIIA interaction, in contrast with the finding of the protein array.

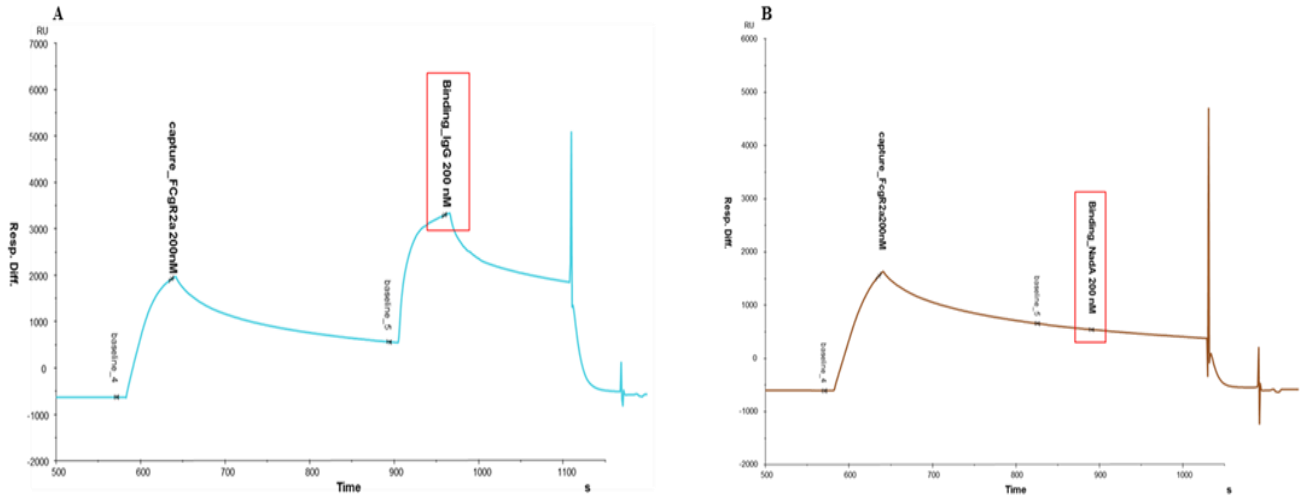


Figure I. Interaction betwen ectodomain of FcγRIIA and human IgG. (A) A representive sensorgram is plotted, showing the response (RU) versus time. Human IgG were incubated with immobilized sFcγRIIA. Fcγ RIIA (100 nM) in HBS-P+ was injected for 60 sec at 10 ul/min to reach a capture level around 1500 RU, followed by a stabilization phase of 60 sec. To check activity of captured Fcγ RIIA, human IgG (100 nM in HBS-P+) was injected for 120 sec at 30 ul/min. Followed by the dissociation phase (120 sec). Binding level of > 1300 RU was reached (red square). **(B)** sNadA was injected for 120 sec at 30 ul/min, as human IgG. The response was <1000 RU as the baseline level.

Discussion and conclusions

The main aim of this work was to find human interactors of Neisseria adhesin A (NadA), a proteic recombinant antigen included in Bexsero, a vaccine against *Neisseria meningitidis* serogroup B. NadA was extensively described as promoter of both adhesion to and invasion into human epithelial cells (Comanducci *et. al* 2002, Capecchi *et al.* 2005, Bozza *et al.* 2014). From one of this early study (Capecchi *et al*. 2005) emerged that cells pre-treatment with pronase abolished NadA binding to epithelial cells, suggesting that NadA may engage a proteic species on the cell membrane. Nagele *et al.* (2011) described Integrin β protein as NadA receptor by mean of heterologous expression systems (Nagele *et al.* 2011), an evidence that could not be confirmed in our laboratories (M. Merola, personal communication). The secreted chaperone Heat shock protein 90 (Hsp90) was found to be an extracellular interactor of NadA, both on epithelial cells and monocytes (Montanari *et al.* 2012, Cecchini *et al.* 2011). Nevertheless, this interaction seems to be not relevant for cell binding. More recently, the endothelial scavenger receptor Lox-1 was found to bind recombinant NadA, although the biological significance has never been explored (Scietti *et al.* 2016). Protein microarrays represent a powerful tool for large-scale screenings, as this technology has been successfully applied to the identification of novel protein-protein interactions in different organisms (Margarit *et al*. 2009). In this work, we searched for NadA receptors by a large-scale approach using NadA as probe on a library of almost 6500 surface-bound and secreted human proteins. Among the positive hits obtained, putative receptors expressed on epithelial cells did not emerge.The screening disclosed three interactors worth to be investigated. We found proteins expressed by cells of the myeloid lineage, such as Siglec-5, Siglec-14 and FcγrIIa. We firstly screened the consistency of these candidates and validated the interaction by means of biochemical tools. Label free methods were used, such as biolayer interferometry for Siglecs and Surface Plasmon Resonance for FcγRIIA. In regard of FcγRIIA, (supplementary results) the binding to NadA was not confirmed, whereas biolayer interferometry allowed the determination of a strong affinity binding between rNadA and Siglec -5 and -14. The NadA residues involved in the complex with Siglec-5 were mapped by hydrogen–deuterium exchange (HDX)-Mass Spectometry. Two non-consecutive peptides (Val^{36}-Gly^{50} and Ile^{60}-Asp^{75}), located in NadA N-terminal head were identified as crucial. The *in silico* 3D reconstruction highlighted a conformational nature of Siglec-5 and NadA interaction. Interestingly, the region identified is overlapping with the epitopes recognized by murine monoclonal bactericidal antibody 33E8 (Malito *et al.* 2014).

By using recombinant proteins and heterologous expression systems, we obtained further validations. NadA properly expressed on *E. coli* bound recombinant Siglec-5 and -14 in the context of bacterial membrane. The recombinant adhesin strongly bound to CHO-K1 cells transfected with full length Siglec-5 and Siglec-14 but not Siglec-9, tested as negative control. The latter system was also used to investigate adhesion of *Neisseria Meningitidis* strains. Whereas wild-type NadA strain shown a significant increase of adhesion, the isogenic knock out strain did not. Accordingly, pre-treatment of cells with anti-Siglecs antibodies abolished binding.

Therefore, we looked for the biological relevance of Siglecs-NadA binding on cells naturally expressing Siglecs, such as monocytes and macrophages. In the frame of *N. meningitidis* disease, interaction between the bacterium and monocytes could happen during sepsis, when the bacterium crosses the nasopharynx barrier and enters the blood stream. Tissue resident macrophages are encountered by *N. meningitidis* in the nasopharingeal mucosa (Corbett *et al*.2004). To determine the presence of a direct interaction between NadA and Siglecs in the context of monocytes and macrophages membranes, we used THP-1 monocytic cells. Firstly, the recombinant NadA binding was explored. We found a rapid internalization of the protein after 30 minutes of incubation, as suggested in a previous work with primary monocytes (Franzoso *et al.* 2008). The pre-incubation of monocytic cells with antibodies against Siglec-5 and -14 did not abolish the association of NadA protein,

even at increasing concentrations. This evidence suggests that Siglecs did not directly participate to NadA cellular association or internalization. The contribute of NadA in association with monocytes and macrophages was explored with *N. meningitidis* strains wt and *ΔnadA* for the first time. The challenge was performed for different time points of infection. On THP-1 monocytic cells, a difference in association after 3 hours of challenge was found, which decreased after 24 hours. In adherent macrophages a difference in associated bacteria was observed after 1h. With these systems we cannot suggest a conclusive contribution of NadA and Siglecs interaction in *N. meningitidis* association to and internalization by phagocytes. Several other receptors are expressed on the surface of these cells which could have a role in terms of recognition and internalization (Johswich *et al.* 2017). On the pathogen side, several other major and minor adhesins dictate the first contact with, and survival within, host cells. Probably, these redundancies in host-pathogen factors complement the role of NadA.

In a collaboration with a laboratory in CNR of Naples, we investigated the NadA/Siglecs interaction role on primary monocytes and macrophages, in terms of cytokines secretion. Outer Membrane Vesicles from *N. meningitidis* wt and *ΔnadA* purified *in house* were used as stimulants on primary cells, analyzing the three possible Siglec-5 and -14 genotypes. The release of cytokines did not reveal a correlation with NadA expression on the Outer membrane Vesicles. Further studies are currently ongoing to investigate other aspects of interaction between NadA/Siglecs on phagocytes from human donors.

Siglecs are transmembrane receptors that could be released as soluble proteins. Recently, several works have reported that human plasma contain high amounts of CD33 related Siglecs such as Siglec-5, -14 and -9. Further, the amount of the soluble species can increase 3-4 folds under pathological conditions (Biedermann *et al.* 2007, Forster *et al*. 2018, Zeng *et al.* 2017). Soluble forms of Siglecs in supernatants of cultured cells, such as neutrophils, monocytes and macrophages were found, as well (Matsubara *et al.* 2015, Zeng *et al*. 2017). We checked in our cell lines whether Siglec-5 and -14 were released in culture media. Pooled human sera were included in the analysis to assess the *ex vivo* amount of Siglecs and *in vitro* release by cell cultures. Accordingly, to reported values (Angata *et al*. 2014, Huang *et al*.2018), sSiglecs in human serum were found in a range of concentration between 30-100 ng/ml. Primary monocytes and macrophages release ~2.5-5 ng/ml while immortalized monocytic (THP-1) cells release 0.5 ng/ml. THP-1 derived macrophages shed a less amount respect to monocytic cell line, as observed for macrophages differentiated from primary monocytes. In resting state, the level of soluble Siglec-5 and -14 seemed to correlate with the different genotypes. Notably, when only Siglec-5 is expressed, the levels of released receptor were lower, indicating that fixed amounts of each Siglec are released following a genotype-dependent rule. Nevertheless, Siglec-5 and -14 released from these cells are not influenced by NadA exposed on OMVs.

The presence of soluble Siglecs in human serum and in the media of cultured phagocytes could open speculations on a new role of NadA in meningococcus pathogenesis. Interestingly, from our analysis emerged that meningococci are captured by soluble Siglecs from THP-1 media, in NadA dependent manner. The addition of exogenous Siglecs, during interaction with monocytes, suggested an enhancement of *N. meningitidis* attachment on surface and a reduction of internalization. This phenotype was observed for the NadA expressing strain. The mechanism of this phenotype is not known and neither are its downstream effects. However, a similar phenotype was observed with another soluble lectin, the Galectin-3 (Quattroni *et al.* 2012) which interacts with *Neisseria meningitidis* as well. In a similar manner, we speculate that Siglec-5/14 presence on bacteria could confer different characteristic of the membrane surface, which may expand the type/number of molecular interactions with the host surface proteome. Further investigations are needed to clarify the significance of these evidences.

During the phase of blood spreading, *N. meningitidis* could face free soluble Siglecs and factors of the complement cascade. The capability of Siglec-3, the most known member of CD33-related Siglecs, to recognize the complement factor C1q (Son *et al.* 2017) prompted us to explore the binding with Siglec-5 and

14. We found that recombinant Siglec-5 and Siglec-14 are able to bind to the C1q factor in solution. Interestingly, when NadA is introduced during the contact between Siglec-5 and C1q, the binding is abolished. To date, we do not have evidences on the precise regions of these multiples interactions and how NadA interferes with them. As observed for Siglec-3, we assume that the binding between Siglecs and C1q could fall in the immunoglobulin-like extracellular domain of Siglecs which potentially recognize C1q head. Indeed, the globular head is involved in the recognition of Immunoglobulins G and M, the prototypical interactors of C1q (Thielens *et al*.2017). To reveal the precise region of interaction, in depth investigations are ongoing.

We attempted to observe these *in vitro* findings on the whole bacterium. Host immune response against bacteria is initiated with C1q binding to the Fc region of antibodies surrounding the bacterial surface. This event triggers the complement cascade through the classical pathway that proceeds with the C3b opsonization of the pathogen and the associated enhanced phagocytosis (Schneider *et al*.2007). We explored the influence of Siglecs on C1q deposition in presence of polyclonal antibodies against NadA. We observed that the presence of recombinant Siglec-5 blocked the antibody dependent deposition of C1q. The effect is more evident in unencapsulated strains. This difference could be due to the exposition of NadA, available for Siglec-5 binding. When we checked the killing of meningococci in human sera used as source of complement, we observed that in Siglec-5/14 depleted serum, bacteria were more sensitive to killing. Thus, NadA capture of Siglec-5/14 could influence the survival of *N. meningitidis* in the bloodstream, co-operating with other well-characterized systems of serum resistance, such as recruitment of negative complement regulators to the bacterial surface (Schneider *et al.* 2007*)*. It should be considered that C1q/antibody opsonization of bacteria could influence and enhance phagocytosis. Indeed, we are looking for possibile influence of sSiglec/NadA in opsonophagocytosis, another defense mechanism of innate immunity that could be encountered by *N. meningitidis.*

In summary, this work revealed two new human interactors for NadA that could be found not only on phagocytes surfaces (monocytes, macrophages and neutrophils) but also free in the bloodstream. On the host side, we characterized NadA as a new proteic target for Siglecs on *N. meningitidis* bacterial surface. Importantly, the C1q binding to Siglec-5 and 14 was revealed for the first time, opening new questions on Siglecs biology.

On the basis of the evidences revealed in this work, we propose a model (figure 28) which could explain the function of NadA soluble Siglecs interaction during infection cycle of *N. meningitidis.* We propose that the interaction could occur during meningococcal infection at sites where Siglecs, shed by immune cells, accumulate and allow binding to the surface of bacteria. A consequence of this interaction would be the increased bacterial adhesion to the surface of phagocytes, which could lead to enhanced meningococcal survival, for istance by preventing internalization (figure 28 A). NadA/Siglecs interaction could influence the classical pathway activation by two possible mechanisms. NadA could compete with Siglecs/C1q interaction (Figure 28 B, I), whereas the presence of soluble Siglecs on *N. meningitidis* surface could mask epitopes recognized by bactericidial antibodies against NadA (Figure 28 B, II). These two strategies could influence complement cascade activation, confering a survival advantage to the bacterium in the extracellular milieu.

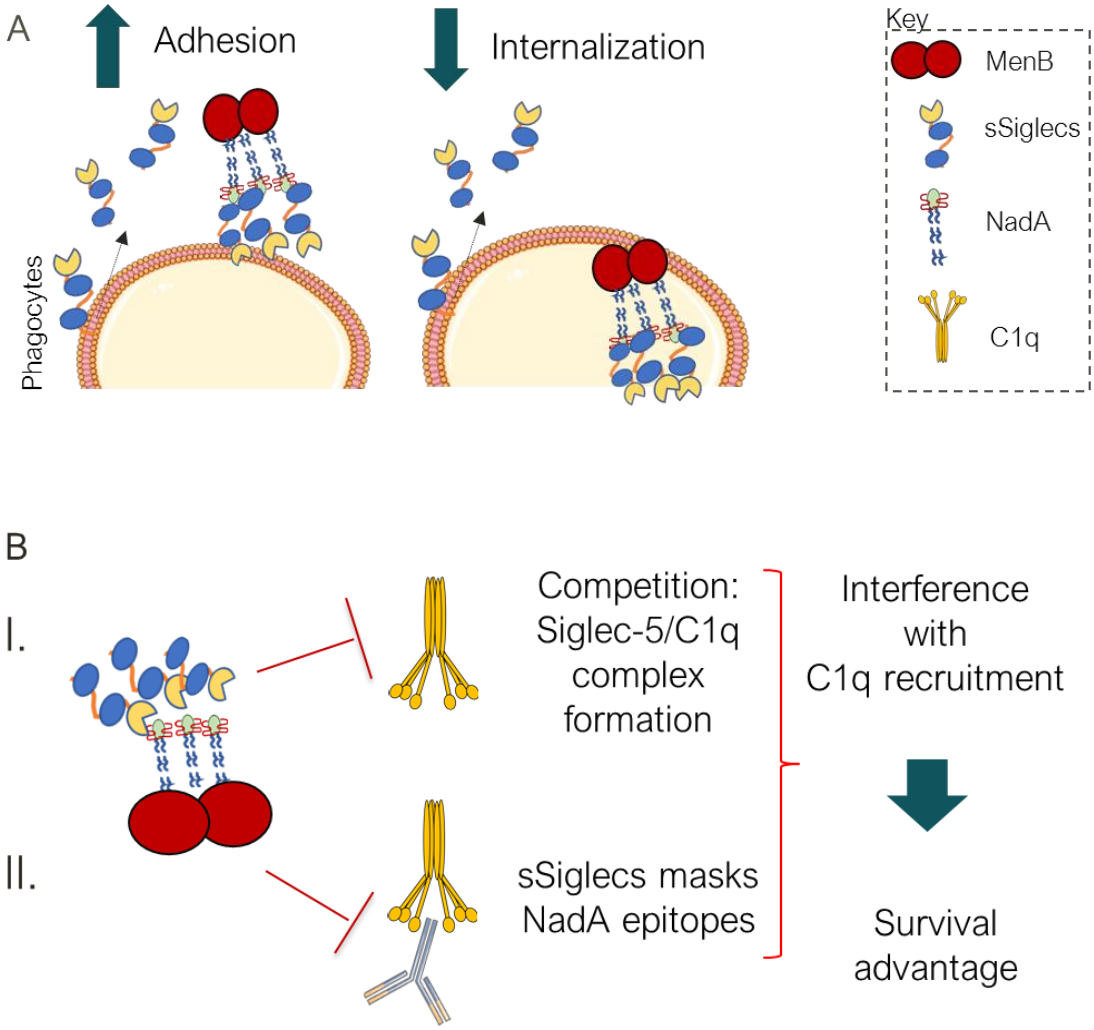


Figure 28. *N. meningitidis* covered by soluble Siglecs: biological consequences during cycle of infection:

A) At the interface with phagocytes

B) In the bloodstream phase

Material and methods

Experimental model

Mammalian Cell lines and culture conditions

Chinese hamster ovary CHO-K1 cells (ATCC CCL-61) were grown in F-12K Medium (Gibco) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 10% iFBS and maintained at 37°C in a controlled humidified atmosphere containing 5% CO2.
Human THP-1 monocytic leukemia cells (ATCC TIB-202) were cultured at 37°C under 5% CO_2 at a density of 2–8 × 10^5/mL. The cell culture medium was RPMI-1640 (Gibco) supplemented with 1 mM Sodium pyruvate, 10% iFBS, 100 U/mL penicillin, 100 mg/mL streptomycin. Differentiation was achieved by suspending cells in complete growth medium with addition of 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma). Differentiated, adherent THP-1 cells, derived from 72-h treatment, were submitted to recovery conditions for further 24-h in PMA-free complete medium before proceeding with experiments.
Human peripheral monocytes were obtained from discarded buffy coats of healthy blood donors, provided by the Transfusional Center of the Hospital "Policlinico Federico II" of Naples (Italy) and treated in accordance with GSK Internal guidelines and applicable Italian legislation. Monocytes were obtained by isolating PBMCs on Ficoll-Paque PLUS gradients (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) and subsequent separation with Monocyte Isolation kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to manufacturer's instructions. Only preparations with > 98% purity and viability were used. The purity of isolated cells was determined microscopically after cytocentrifugation and differential staining with a modified Wright-Giemsa dye (Diff Quik, Medion Diagnostics AG, Düdingen, Switzerland). Viability was determined by trypan blue dye exclusion. Monocytes were also analyzed for cell-surface CD14 and CD16 antigen expression by flow cytometry. The subsets of purified monocytes were: 80% $CD14^{++}CD16^{-}$ cells, 2-6% $CD14^{++}CD16^{+}$ cells, and 7-10% $CD14^{dim}CD16^{+}$ cells, fully reflecting the distribution of whole blood monocyte subsets (Wong *et al.* 2012).

Bacterial strains and growth conditions

Escherichia coli BL21(DE3)T1R (New England BioLabs) was the strain used to express full-length NadA (variant 3) following transformation with pET21b-NadA, as previously described (Capecchi *et al.* 2005), or the empty vector as control. *E. coli* was cultured at 37°C in Luria–Bertani (LB) broth supplemented with 100 μg/mL ampicillin. Surface protein expression for full-length NadA was achieved without addition of IPTG exploiting the expression due to the leakage in the induction system.
N. meningitidis strains used in this study: 5/99 and 96217, serogroup B (NadA allele 3). 5/99 Knock-down NadA strains were generated *in house* (not published). The deletion in *synX* gene to prevent capsule formation was performed as described elswhere (Fisseha *et. al* 2005).

N. meningitidis was routinely grown on chocolate agar polyvitex (Biomerieux) at 37 C° , 5% CO_2 for 16 hours. In each experiment bacteria were grown in GC base medium (OD_{600} start: 0.05) until OD_{600} 0.5 (2.5hrs), except for Serum Bactericidial Assay, SBA (until OD_{600} 0.25) and when specified.

Method detail

Expression and purification of soluble proteins

The cDNA encoding the human predicted extracellular domain of Siglec 5 (residues 1-437) and FcγRIIA (residues 38-216) were subcloned into a pSecTag2 B vector (Invitrogen) containing sequences encoding the murine Ig k-chain V-J2-C signal peptide and a myc/6xHis tag. Constructs were expressed into Expi293F cells according to the manufacturer's instructions (Life Technologies). Briefly, 30 μg of DNA were transfected into 30 mL culture containing 75 x 10^6 Expi293F cells using ExpiFectamine 293 Reagent. Cells were incubated at 37°C, 125 rpm, 8% CO2 and after 24 h, ExpiFectamine 293 Transfection Enhancer 1 and 2 were added. Seventy-two and 144 h after transfection, cell cultures were centrifuged at 800 rpm for 8 min and the protein-containing supernatants were harvested, pooled, clarified by centrifugation, filtered through a 0.22 μm filter, and stored at 4°C until purification. His-tagged recombinant ectodomains were purified by affinity chromatography by using HiTrap™ TALON® crude column (GE Healthcare Life Sciences). Fractions of interest were pooled and were concentrated by using 10 kDa cutoff spin concentrator (Millipore Amicon Ultra); sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) was performed to check purity and samples aliquotated and stored at -20°C for further analysis.

Protein array

Protein microarrays (GNF PATH) were generated by spotting about 7000 recombinant soluble proteins (6500 human and 500 mouse proteins) (0.5mg/ml) from the GNF library, which were produced as described by Gonzalez *et al.* 2010. Each protein was spotted in duplicates per array onto ultra-thin nitrocellulose coated glass slides (PATH slides; Grace Bio Labs) using the ink-jet spotter Marathon (Arrayjet) (spots of ~150 μm in diameter). Printing was performed in a cabinet with controlled humidity and temperature (55-60% and 12°C respectively). Nonspecific binding was minimized by preincubating arrays with a blocking solution (BlockIt, ArrayIt) for 1 hour. rNadA was diluted in Nap-PBS at final concentration of 1μM and overlaid on the arrays at RT for 1 h. After washing with 0.1% Tween 20 in PBS buffer (TPBS), arrays were incubated with rabbit polyclonal serum against NadA (diluted 1:2000) at RT for 1 h. Slides were washed again as before and interactions were detected by incubating with an AlexaFluor647-conjugated anti-rabbit IgG secondary antibody (Jackson Immunoresearch). Fluorescence images were obtained using Power scanner (Tecan Trading AG, Switzerland) and the 16-bit images were generated with PowerScanner software v1.2 at 10 μm/pixel resolution and spot fluorescence intensities were determined using ImaGene 7.0 software (Biodiscovery Inc.). Microarray data analysis was performed using *in-house* developed software. For each protein, the mean fluorescence intensity (MFI) of replicated spots was determined, after subtraction of the background value surrounding each spot. Proteins with a high coefficient variation (CV) between the two replicates were discarded. All obtained MFI score (excluding the ones found in the control microarray where the AlexaFluor 647-conjugated anti-rabbit IgG alone were probed to determine its non-specific binding to the printed proteins) were classified in four categories: (a) High reactivity: MFI>30000; (b) Medium reactivity 15000>MFI>30000; (c) Low reactivity 5000>MFI>15000; (d) No reactivity MFI<5000.

Bio-layer interferometry

Octet QKe (ForteBio, Pall Science) was used for binding studies. Streptavidin (SA) biosensors (ForteBio) were used to immobilize biotinylated proteins in a 600 seconds loading. The equilibration time was 300 seconds to achieve a sufficient baseline signal. Immediately before analysis, tips were prewet in kinetic buffer HBS-P, composed by 10 mM HEPES, 150 mMNaCl, 0,02% P-20 Surfactant, pH 7.4 (GE Healthcare Life technology). To calculate affinity of rSiglec5 and rSiglec14 on immobilized biotinylated rNadA (25 μg/mL in kinetic buffer), serial dilutions (from 500 nM to 7,81nM) were tested. Kinetic was then monitored. A ligand-loaded

biosensor was used in association with kinetic buffer as baseline. During the entire kinetic assay the sample plate was kept at 30°C shaking 1000 rpm. Reference subtracted BLI response curves were used for the affinity constant determination. Inter-step correction and Y-alignment were used to minimize tip-dependent variability. KD was calculated at response at 1190-1195 seconds, using the Data Analysis Software v7.1 (Forte Bio).

Surface plasmon resonance analysis (SPR)

SPR was used to validate the NadA binding with FcγRIIA. The SPR experiments were performed with Biacore T200 instrument at 25 °C (GE Healthcare). A Ni-NTA sensor chip was used to immobilize soluble FcγRIIA (GE Healthcare) via its C-terminal 10-His tag. To check activity of captured Fcγ RIIA, human Immunoglobulin G (used as positive control) or NadA (100 nM in HBS-P+) were separately injected for 120 sec at 30 ul/min. Following dissociation for 120 sec, the chip is regenerated by injection of 350 mM EDTA for 60 sec at 10 ug/ml. SPR data were analyzed using the Biacore T200 Evaluation software (GE Healthcare).

Hydrogen-deuterium exchange-MS

The hydrogen-deuterium exchange (HDX) experiments, with and without recombinant protein (Siglec 5), were performed in the same experimental session. The antigen/interactor complex was formed by adding 200 pmolrNadA to the recombinant protein, using a molar ratio of 1:1. The method followed is described in Bertoldi *et al.* 2017.

Plasmid construction and transfection method

The full-length coding region of Siglec 5, Siglec 14 and Siglec 9 (GenBank accession N° NP_003821.1; NP_001092082.1; NP_055256.1 respectively) were obtained from GeneCopoeia as pEZ-M14 FLAG-tag vectors while the full-length coding region of human and mouse FcγRIIA (GenBank accession N° NP_001129691.1 and N° XP_006496722.1 respectively) were obtained from GeneArt as pCDNA3.1 Strep-tag vector. Transfection of CHO-K1 cells with Siglec plasmids was performed using Lipofectamine LTX Reagent with PLUS Reagent (Life technologies) following the manufacturer's instructions. 1.5x10^5 cells plated in a 24 well were transfected with a mix of lipofectamine and appropriate purified plasmid. After 24 hours post-transfection cells were used for binding experiments and adhesion assays.

Outer membrane vesicles purification from Neisseria meningitidis supernatants

Neisseria meningitidis wilt-type 5/99 and the isogenic Δ*nadA* strains were cultured in GC-based (Difco) agar medium with Kellogg's supplement I at 37°C in presence of 5% CO_2 for 16h. Then, bacteria were resuspended to 0.15 OD_{600nm} in 50ml of in MCDMI-Meningitidis Chemical Define Medium I- medium and grown at 37°C, 180 rpm until stationary phase. The bacterial cells were pelleted by centrifugation for 30 min at 4000 rpm at 4°C and the culture supernatants filtered (pore size 0.22 μm). Finally, OMV were collected from the filtered supernatants by ultracentrifugation for 3 h at 96000xg at 4°C (Optima XPN-100 ultracentrifuge, Beckman Coulter,USA). Obtained pellets were washed in PBS (1 h at 96000xg at 4°C), resuspended in PBS and quantified by Lowry assay (DC protein assay, BioRad).

Binding assays with recombinant proteins, Flow cytometry and Confocal Microscopy Immunostaining

Binding of rSiglec 5 and rSiglec 14 onto *E. coli*–NadA and *E. coli*–pET was conducted as follows. Bacteria grown overnight were diluted and resuspended in PBS 1% BSA (Sigma) buffer and rSiglec 5 or rSiglec 14 added to a final concentration of 200μg/mL. After incubation for 1 hour at RT, bacteria were washed extensively with buffer and spread on polylysine-coated slides (Thermo Scientific). Samples were then fixed

in 0.5 % paraformaldehyde (PFA,ThermoFisher), washed and blocked with PBS + 3% BSA for 1 h at room temperature. After multiple washings, samples were incubated with rabbit polyclonal anti-Siglec 5 or 14 antibodies for 1 h at room temperature (Anti-Siglec 5 (N-term) antibody produced in rabbit (SAB1303597 Sigma); Anti-Siglec 14 (N-term) antibody produced in rabbit (SAB1303894 Sigma)). Washings were followed by incubation with Alexa Fluor 647 donkey anti-rabbit IgG. Labelled preparations were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), analysed with a Zeiss LSM-710 confocal microscope and images were captured using ZEN software (Carl Zeiss, Oberkochen, Germany).
CHO-K1 and THP-1 binding assay with recombinant NadA was performed as previously described (Comanducci *et al*. 2002). For inhibition of the binding experiments cells were incubated with different concentrations of antibody prior to rNadA incubation. Cells were analysed with a FACS-Scan flow cytometer (Beckton-Dickinson). The mean fluorescence intensity (MFI) for each population was calculated using FlowJo software (version 9.8; Tree Star, Inc, Ashland, OR).

Binding assay with *Neisseria meningitidis* strains with recombinant proteins (rSiglec-5 produced *in house*), or C1q purified from complement (Sigma) was conducted as follows. Bacteria were washed, diluted from 0.5 to 0.25 0D/ml in buffer BSA 1%. Bacterial suspension was added to a 96 well plate and reagents at the appropriate concentration were added. Incubation lasted for 30' at 37°C. After extensive washings, bacteria were blocked with buffer for at least 30' at RT. Samples were incubated for 1h at RT with appropriate primary antibodies. For Siglec-5 binding detection, mouse antibody anti-Histidine tag was used (Thermo Fisher), followed by incubation with (APC)-conjugated goat $F(ab)_2$ antibody to mouse Ig (dilution 1:400, Thermo Scientific). For studies with C1q (10ug/ml), a mix of anti-NadA antibody (5ug/ml) and Siglec-5 (20ug/ml) were added to bacteria for 30 min at 37°C in gentle agitation. C1q binding to *N. meningitidis* was detected by FITC conjugated rabbit anti-C1q antibody (Abcam) diluted 1:200. After washings, pellets were resuspended in 1% PFA and incubated for 1h at RT. Cells were analysed with a FACS-Scan flow cytometre (Beckton-Dickinson).The mean fluorescence intensity (MFI) was calculated using FlowJo software (version 9.8; Tree Star, Inc, Ashland, OR).

Adhesion assays

For adhesion assay $1{,}5x10^5$ CHO-K1 cells were seeded onto 24-well tissue culture plates and transfected as describe before. At least 2 hours before infection protocol, medium was changed with fresh infection medium (IM, F-12K medium with 1% FBS, antibiotic free). Meningococci strains were grown in GC medium (OD start: 0.05) until optical density 0.5 (2.5hrs). Bacteria were pelleted and resuspended in infection medium (MOI 200:1). Bacterial suspension was added to the apical surface of the cultures for 3 hours at 37°C and 5% CO_2. After removal of non-adherent bacteria by extensive washings, cells were lysed with 1% saponin and serial dilutions of the suspension were plated onto GC agar to count the CFU.
For adhesion/internalization assays with THP-1 cells, $3x10^5$ THP-1 cells were seeded onto 24-well tissue culture plates and treated with PMA for 72 hours. Meningococci, grown as already mentioned , were pelleted and resuspended in infection medium with a MOI 40:1. Bacteria were added to the apical surface of the cultures for different time points at 37°C and 5% CO_2. After removal of non-adherent bacteria by several washing, cells were lysed with 1% saponin and serial dilutions of the suspension were plated onto GC agar to count the CFU. The internalization of bacteria was determined using gentamicin antibiotic for 30 minutes at 37 C°, 5% CO_2 (200 ug/ml) to eliminate extracellular adherent bacteria. After washings with warm medium cells were re-incubated for 45 minutes. Internalized bacteria were released using 1% saponin and count CFU as above. In some experiments *N. meningitidis* was labelled before infection to detect attachment and internalization by flow cytometry. Bacteria were labelled with Alexa Fluor™ 647 NHS Ester (Succinimidyl Ester, Thermo Fisher) for 15 minutes at 37 C°, 5% CO_2. Then, bacteria were washed and added to each well and, when required, pre-incubated with recombinant Siglec-5 (15 ug/ml). The infection was conducted for 30 minutes

and stopped with extensive washings. In the end, cells/bacteria pellets were fixed with 4% PFA for 30 minutes and the day after analyzed by flow cytometer.

Dose-response stimulation with outer membrane vesicles

For dose-response experiments, monocytes and macrophages were seed at the density of 5 x 10^5 cells/well in 1 ml of complete medium and stimulated for 24 h with outer membrane vesicles produced from *Neisseria meningitidis* wilt-type 5/99 and the isogenic Δ*nadA* strains (concentration range 0,1- 4 ug/ml) . Culture supernatants were collected and stored at -80°C.

Enzyme linked immunosorbent assay (ELISA)

Cytokines production in the cell-free supernatants were determined by ELISA, according to the manufacturer's instructions (R&D Systems, MN, USA). The levels were measured spectrophotometrically at 450 nm using an ELISA microplate reader (Jupiter, Asys, Austria).
Siglec-5 and 14 detection in human sera and cell-free supernatants was determined by Duo Set Kit, following Manufacture's instructions (R&D Systems, MN, USA).
Cell- free supernatants were collected from confluent cells, centrifuged and stored at -80 C° until use. For investigations on C1q, a general protocol of ELISA was followed. Briefly, Nunc MaxiSorp PS Immuno F96-plates (ThermoFisher, USA) were coated overnight at 4 °C with 100 µl human C1q (Sigma, USA) at the final concentration of 10 µg/ml. Plates were washed three times with 0.05% PBS-T (Tween-20) and blocked with 1% (w/v) BSA + 0.05% PBS for at least 1 hour at RT. Recombinant Siglec-5 and 14 ectodomain proteins were two-fold serially diluted in PBS, then added to the collagen/C1q-coated wells and incubated for 1h at 37°C. Following incubation, wells were washed with 0.05% PBS-T. Biotinylated mouse anti-human Siglec-5/14 antibody (1:200 in PBS/BSA) was added to each well and incubated for 2 hours at RT. Wells were washed before adding HRP-conjugated streptavidin antibody (1:200), in PBS/BSA for 20 minutes at RT. After three washings, substrate solution (TMB Single solution, Life technologies) was added to each well for 20 minutes at RT in the dark. 50 ul of stop solution (2N H_2SO_4) was added. Optical density at 450 nm was determined using a microplate reader. Competitive ELISAs were conducted by adding increasing concentration of NadA (from 0.03-0.48nM) to a fixed concentration of soluble Siglec-5 (0.12 nM) and incubated for 1h at 37°C.

Serum depletion of Siglecs and Serum bactericidal activity assay (SBA)

Pooled human serum deprived of soluble Siglecs were obtained by three consecutive incubations on a plate coated with anti-Siglecs antibodies (capture antibody of DuoSet Kit. R&D Systems, MN, USA). Bacteria grown until early log phase (OD_{600} of ~0.25) were diluted in Dulbecco's Phosphate Buffered Saline (DPBS) containing 1% bovine serum albumin (BSA) at the working dilution of 10^4–10^5 and incubated with serial two fold dilutions of tested antibodies starting from a concentration of 1 µg/ul. Pooled human serum used as a complement source for hSBA was obtained from three volunteer donors under informed consent.

Statistical analysis

At least three independent experiments, run under the same conditions, were performed, except for some preliminary data. Results were analyzed with Excel or GraphPad Prism Software by applying the one-way ANOVA or two-way ANOVA when requested. ($P \leq 0.05$ was considered statistically significant (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$,****$P \leq 0.0001$).

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Workshops and Courses Attended

"RNA sequencing & Single-Cell-Sequencing workshop". Promoted and organized by Associazione culturale bioinformatica per la medicina molecolare (Bx2M), March 25th -29th 2019, Turin (Italy)
"School of Immunology"(course advances) promoted and organized by SIICA (Società italiana di Immunologia, Immunologia clinica e allergologia) on July 20th and 22th 2018 at Villa Pace, Messina (Italy)

"Vth workshop on *in vitro* alternatives"10th-11th January 2018, TLS, Siena promoted and organized by IV tech s.r.l.

Posters

Spinello Z., Guidotti S.,Censini S, Rossi-Paccani S, Pezzicoli A. Next generation *in vitro* modeling of the human airway mucosa as an experimental approach to study respiratory infections. GSK Vaccines, Siena **.** Research & Development days- 26th -27th June 2019.

Sampieri K, **Spinello Z.**, Maccari S, Rossi-Paccani S, Pezzicoli A. 3D reconstruction of the human airway mucosa *in vitro* as an experimental model to study COPD exacerbations. 3D Tissue Infection Symposium 5th-7th April , 2019, Wurzburg,Germany.

Spinello Z., Benucci B, Rossi-Paccani S, Merola M. Characterization of *Neisseria meningitidis adhesin A* (NadA) binding to specific receptors on monocytes/macrophages."10th PhD students workshop, November 2017. GSK Vaccines, Siena (Italy).

Spinello Z., Benucci B, Rossi-Paccani S, Merola M. Characterization of *Neisseria meningitidis adhesin A* (NadA) binding to specific receptors on monocytes/macrophages. European Initiative for basic research in microbiology and infectious Diseases (EIMID) 14th Annual Meeting held at Oxford, United Kingdom on 27th -29th Sept. 2017

Presentations

"Post-doc and PhD Students Workshop. Global edition", 28th and 29th November 2019. GSK Vaccines, Siena (Italy). Presentation title:"Interactions of Neisseria adhesin A with Siglec-5 and 14".
"11th PhD students workshop", 10th and 11th December 2018. GSK Vaccines, Siena (Italy). Presentation title: "Interaction of Neisseria adhesin A with Siglec-5 and Siglec-14".

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