Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

BIOLOGIA CELLULARE E MOLECOLARE

Ciclo XXVII

Settore Scientifico Disciplinare: 05/E2
Settore Concorsuale di afferenza: BIO/11

EXPLORING HOST-PATHOGEN INTERACTIONS THROUGH PROTEIN MICROARRAY

Large-scale protein microarray analysis revealed novel human receptors for the staphylococcal immune evasion protein FLIPr and for the neisserial adhesin NadA

Presentato da: **Luigi A. D. Scietti**

Coordinatore Dottorato
Chiar.mo Prof. **Davide Zannoni**

Tutor
Chiar.mo Prof. **Vincenzo Scarlato**

Relatore
Dott.ssa **Sabrina Liberatori**

Co-relatore
Dr. **Mikkel Nissum**

Esame finale anno 2015

To my family and Ali,

because with their love everything is possible

"If we become increasingly humble
about how little we know,
we may be more eager to search"

"Quanto più consideriamo con umiltà
il poco che sappiamo,
tanto più saremo desiderosi
di ricercare ulteriormente"

Sir John Templeton

Preface

I was writing my Master thesis in Pavia when by chance someone told me about PhD fellowships in Novartis Vaccines Siena. I almost didn't even know what a PhD fellowship was, but foremost I didn't know what a PhD studentship meant. When Dr. Sabrina Liberatori, my "one-hundred-idea per second supervisor" to whom I'm grateful for all the support and the suggestions provided during these three years, on 2nd January 2012, the first day of my new experience, started to describe to me the detailed matrix structure I had started to belong to, all the people I had to work with, and all the data generated by former members of the laboratory I started realizing that those three years would be a very tough life experience. And the expectations were not deluded.

This thesis is the result of a work in the laboratories of the Novartis Vaccines Research Center in Siena, where I had the great chance to work and understand the organization that underlie the company's way of doing research. My work in Siena was supported by a fellowship from Univeristà degli studi di Bologna, represented in the person of Prof. Vincenzo Scarlato, always kind and available for scientific exchange, life advice, and, not minor, for solving bureaucracy issues.

The work you will read would not be possible without a great number of people in the different Novartis departments. The first part of my period in Siena was under the "maternal" supervision of Dr. Erika Bartolini, the person who introduced me to the world of protein microarray. In the same way, the chapter three of this thesis exist thanks to the hospitality and patience of Dr. Meike Scharenberg, Dr. Seguinde Arora and Dr. Xavier Leber from NIBR (Novartis Institute for Biomedical Research) in Basel (CH), who hosted and carefully supervised me during the protein array screening for two long and intense weeks in July '13.

All this work would not be possible also without the tireless and precious work of the people from Cloning and Expression Unit and Purification Unit in Novartis Siena (managed respectively by Dr. Domenico Maione and Dr. Elena Cartocci) who cloned and purified the large bacterial libraries used in chapter 2. In a similar way, the work of the Protein Science laboratory at GNF (Genomic Institute of the Novartis Research Foundation) in San Diego (CA) was essential to provide such a large number of human recombinant proteins used in both chapter 2 and 3. Within the thesis you will also see several experiments performed using label-free technologies with a constant exchange of ideas, suggestions and opinions with Dr. Werner Pansegrau and Dr. Paola Lo Surdo from Biophysical characterization Unit in Novartis Siena and Dr. Hendrick Wuensche from Pall bioscience. A particular acknowledgment also to Dr Vincenzo Nardi Dei for technical assistance in DLS measurements, Alessia Liguori (PhD student) for providing protein constructs used in chapter 3 and Barbara Benucci (PhD student) for plasmid production used for cell transfection in FACS experiments.

All the project was additionally supervised by Dr. Mikkel Nissum, who I want to thank for always providing me what is used to call the "global picture", which is not easy to keep in mind during daily work.
I would also acknowledge the team of people working on *S. aureus* in Siena and the project leader Dr. Fabio Bagnoli, for keeping me in track with the "biological meaning" of the data. Closely linked to this a particular acknowledgment also to Prof. Pietro Speziale and Dr. Simonetta Rindi from Molecular Medicine at Università di Pavia for whole blood assay in chapter 2.
I am also grateful to Novartis Academy in the person of Dr. Ilaria Ferlenghi for giving me the opportunity to perform a PhD in a company like Novartis.
Of course these three years would be much different and harder without all the present and past members (Dr. Benedetta Lombardi, Ambra Marongiu, Dr. Christina Schluepen, Chiara Carnemolla, Silvia Dalba, Dr. Katia Sampieri and Irene Pinzuti) of the Protein-Protein Interaction group to which I had the honor to belong, people who I want to personally thank in this preface for their essential and continuative knowledge sharing and experimental support all over my PhD experience.
I hope my work and the work of all the people mentioned could constitute a small brick in the pyramid of knowledge both for scientific technical advance and better theoretic understanding of the mechanism regulating bacterial pathogenesis and immune evasion, but also as a concrete improvement for faster and easier vaccine antigens discovery and development.
A final note to the reader; if you are reading this line after the others, I'm absolutely grateful to you since you at least read two page of my thesis. I hope the next ones will be enough interesting to keep you reading as well.

Luigi Scietti,
Siena and Graffignana,
February 2015

Contents

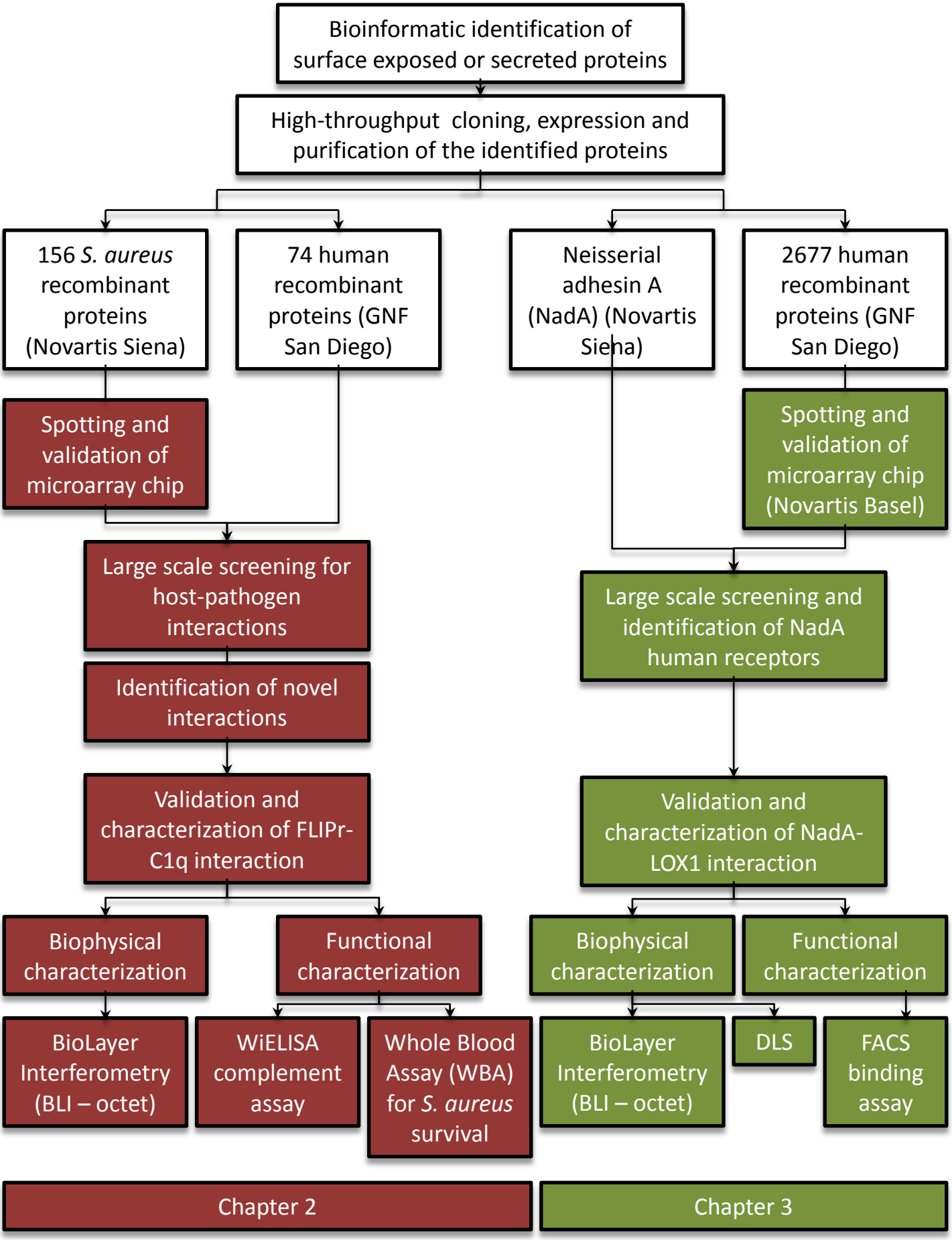
Abstract

Adhesion, immune evasion and invasion are key determinants during bacterial pathogenesis. Pathogenic bacteria, indeed, possess a wide variety of surface exposed and secreted proteins which allow them to adhere to tissues, escape the immune system and spread throughout the human body. Therefore, extensive contacts and a broad crosstalk between the human and the bacterial extracellular proteomes take place at the host-pathogen interface at the protein level. Recent researches emphasized the importance, from a therapeutic point of view, of a global and deeper understanding of the molecular mechanisms which underlie bacterial immune evasion and pathogenesis. Through the use of a large-scale, unbiased, protein microarray-based approach and of wide libraries of human and bacterial purified proteins, novel host-pathogen interactions were identified. A scheme of this approach can be found on page xviii.

This approach was first applied to *Staphylococcus aureus* (chapter two of this thesis), a human commensal gram-positive bacterium also cause of a wide variety of diseases ranging from skin infections to endocarditis and sepsis. The screening led to the identification of several novel interactions between the human and the *S. aureus* extracellular proteomes. Among all, the interaction between the *S. aureus* immune evasion protein FLIPr (formyl-peptide receptor like-1 inhibitory protein) and the human complement component C1q, key players of the offense-defense fighting, was characterized using label-free techniques and functional assays.

The same approach was also applied to *Neisseria meningitidis* (chapter three of this thesis), a gram-negative encapsulated bacterium major cause of bacterial meningitis and fulminant sepsis worldwide. The screening led to the identification of several potential human receptors for the neisserial adhesin A (NadA), an important adhesion protein and key determinant of meningococcal interactions with the human host at various stages. The interaction between NadA and human LOX-1 (low-density oxidized lipoprotein receptor) was confirmed using label-free technologies and cell binding experiments *in vitro*.

Taken together, these two examples provided not only concrete insights into *S. aureus* and *N. meningitidis* pathogenesis, but also identified protein microarray coupled with appropriate validation methodologies as a powerful large scale tool for host-pathogen interactions studies.


Bioinformatic identification of surface exposed or secreted proteins
High-throughput cloning, expression and purification of the identified proteins
156 S. aureus recombinant proteins (Novartis Siena)
74 human recombinant proteins (GNF San Diego)
Neisserial adhesin A (NadA) (Novartis Siena)
2677 human recombinant proteins (GNF San Diego)
Spotting and validation of microarray chip
Spotting and validation of microarray chip (Novartis Basel)
Large scale screening for host-pathogen interactions
Large scale screening and identification of NadA human receptors
Identification of novel interactions
Validation and characterization of FLIPr-C1q interaction
Validation and characterization of NadA-LOX1 interaction
Biophysical characterization
Functional characterization
Biophysical characterization
Functional characterization
BioLayer Interferometry (BLI – octet)
WiELISA complement assay
Whole Blood Assay (WBA) for S. aureus survival
BioLayer Interferometry (BLI – octet)
DLS
FACS binding assay
Chapter 2
Chapter 3

Aim of the thesis

Pathogenic bacteria express on the surface and secrete a wide variety of proteins that specifically mediate their adhesion, evasion and invasion into the host body. These contacts occur extensively at the host-pathogen interface between the bacterial and host extracellular proteomes. Usually, bacterial immune evasion molecules and all the surface-exposed molecules constitute excellent vaccine antigens. Therefore, the identification and characterization of novel host-pathogen interactions are essential for both the understanding of molecular mechanism of pathogenesis, and for therapeutic purposes. The large number of interactions discovered so far, sometimes promiscuous and involving multiple components of the human defense apparatus, seems to suggest a very complex picture where additional interactions may take place at the host-pathogen interface. Aim of this study is to apply an un-biased systematic large scale protein microarray-based approach to identify novel interactions between bacterial and the human extracellular proteomes.

Chapter ONE

Bacterial immune evasion and host-pathogen interactions: a protein perspective

Introduction

Every day, a real molecular battle between pathogenic bacteria and their hosts take place. Each of them exploits *in toto* their fully equipped molecular arsenal to finally be the survivor. Evading the assorted molecular attack of the host immune system constitutes a key feature for bacterial survival. Thus it is not surprising that bacteria evolved several mechanisms for host colonization, immune system evasion and host invasion. These crucial events are usually mediated by protein-protein interactions at the host-pathogen interface. Immune recognition and host colonization processes are characterized, in fact, by wide and extended interactions between the host and bacterial extracellular proteomes, constituted by all the surface exposed and secreted proteins that are the key determinants in the host-pathogen interplay. The co-evolution between host and bacterial pathogens led to the development of bacterial proteins able to recognize and exploit specific host receptors, enabling bacteria to adhere and penetrate into the host. In the same way, once entered, several proteins are specifically produced to counteract both the innate immune response, constituted by complement system and antimicrobial peptides, and cell-mediated response, mainly constituted by professional phagocytes such as neutrophils, monocytes and macrophages. Here is presented a detailed description of the human complement system, a key effector in bacterial recognition. In addition, the examples of *Staphylococcus aureus* and *Neisseria meningitidis* will be provided to describe the main strategies for complement evasion.

The human complement system

The human complement system is the main component of the innate immune response and accomplishes numerous functions like the recognition of foreign cells, activation of adaptive immunity and the removal of cellular debris [1]. It is thereby considered the first barrier against pathogenic bacteria and viruses that enter in the bloodstream. It is composed by more than 30 proteins present in human serum and tissue fluid as well as on cell surfaces [2, 3]. The complement system is usually described as a double-edged sword, since it is very easily activated, but at the same time tightly regulated in each step. The immune system has in fact to keep a tight balance between attack on foreign surfaces and protection of host ones through the use of several regulators that prevent complement activation [4, 5]. From a molecular point of view, the complement system is characterized by serin-proteases that specifically cleave the next factor, constituting a proteolytic cascade resulting in bacterial opsonisation and lysis [6]. The complement action can be divided in three main steps: the initiation, the cascade amplification and the effector production. The initiation step is characterized by proteins able to recognize a wide variety of molecular substrates which lead to the activation of the first serine proteases. The complement cascade is then amplified:

each serin-protease is activated by the previous and activates the next through a proteolytic cleavage [7, 8]. During the proteolytic cleavage several peptides called anaphylatoxin are generated. These constitute the effectors. They are able to trigger a wide variety of responses including inflammation, phagocytosis and B-cells and T-cells stimulation. Based on the type of molecules involved in the activation step, three different activation pathways can be distinguished: the classical, the lectin and the alternative pathways. All the three pathways converge at a central step, the cleavage of complement component C3.

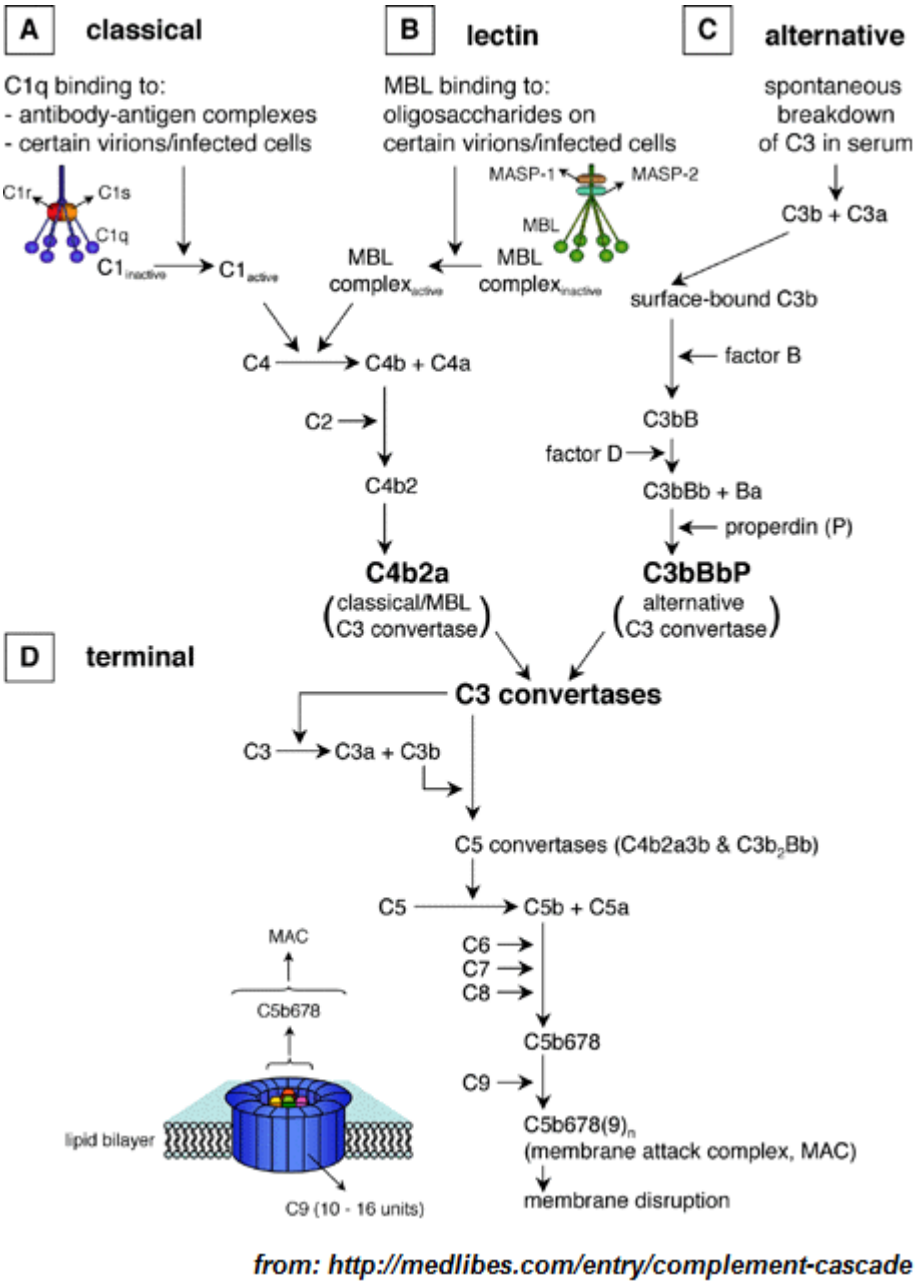


Figure 1.1 The complement cascade. Complement classical **(A)**, lectin **(B)** and alternative **(C)** pathway are shown. Classical and lectin pathways converge in the cleavage by C1s and MASP of C4 and C2 which constitutes the C3 convertase. The terminal pathway **(D)** with the formation of MAC is also shown.

The classical pathway (CP)

The initial step that triggers complement classical pathway activation is the recognition of non-self molecules by the complement component C1 (**figure 1.1A**). C1 is a 790 kDa complex formed by the association of a recognition protein, C1q, and a Ca^{2+}-dependent tetramer composed by two copies of two proteases, C1r and C1s [9, 10]. C1q is a 450KDa multiprotein complex characterized by three subcomponents (C1qA, C1qB and C1qC) organized in heterotrimers, which are then super-organized to form an examer with a peculiar bouquet-like structure (**figure 1.2A-B**). The overall structure is characterized by an N-terminal collagen-like "stalk" and C-terminal globular heads (**figure 1.3A**) which are involved in the recognition function of C1q. C1q has the striking ability to recognize abnormal structures from self and the crystal structure of the globular heads provides a basis for its versatile recognition properties [11].

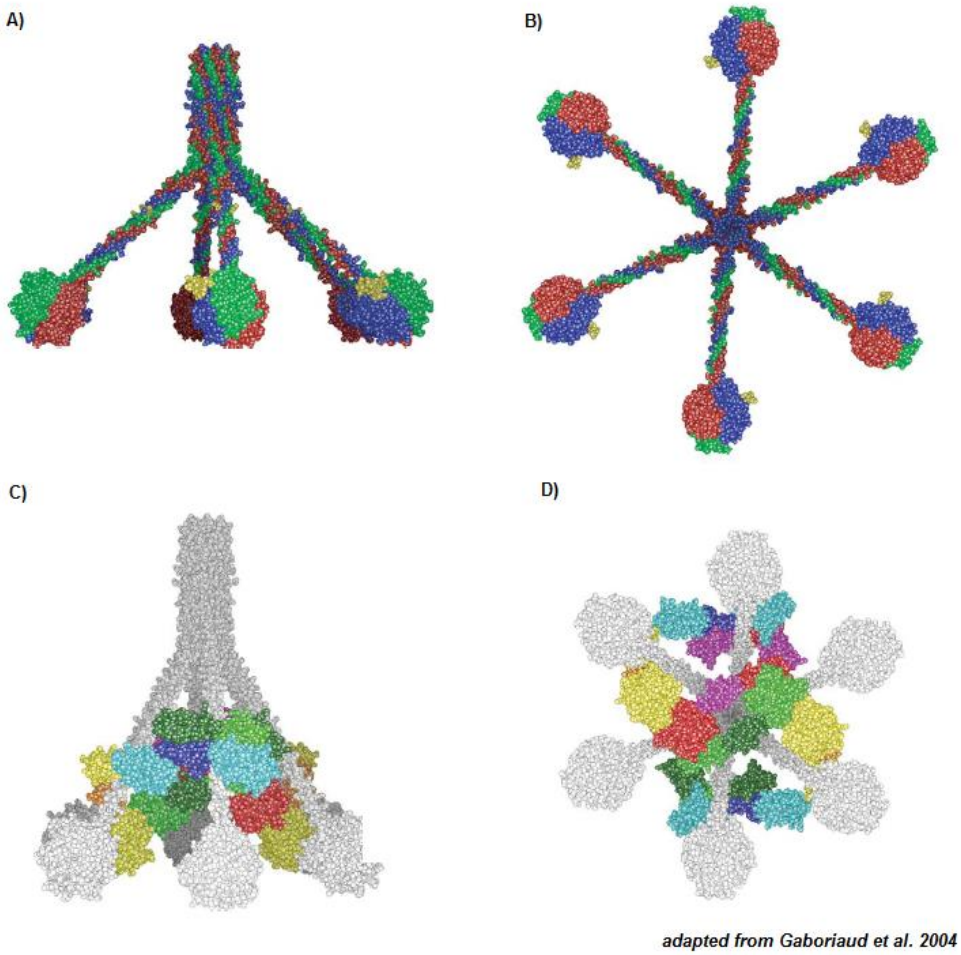


Figure 1.2 Three-dimensional structural model of the human C1 complex. (A-B) Side and bottom view of the C1q molecule. The C1qA (blue), C1qB (green) and C1qC (red) subcomponents are shown**. (C-D)** Side and bottom view of the C1 complex. C1 is depicted in the resting state, in which C1q is proposed to have a 'closed' conformation. C1r and C1s are shown and interact with C1q collagen-like stalk

Usually complement activation is associated with the recognition by C1q of aggregated antibodies or C-reactive protein (CRP). There is increasing evidence, however, that C1q possesses a broader range of recognition including b-amyloid fibrils [12, 13] the pathological form of the prion protein [14, 15] and apoptotic cells [16], consequently acting as a key factor in immune tolerance. Binding of C1q to a target cell or molecule is thought to elicit a signal that triggers self-activation of C1r, which converts proenzyme C1s into a highly specific serin-protease that cleaves complement components 4 and 2 (C4 and C2), thereby activating the classical complement pathway. C1r and C1s have similar domain architectures, since they are characterized by five non-catalytic modules: the N-terminal CUB domain is followed by an epidermal growth factor (EGF)-like module, a second CUB domain and a tandem repeat of complement control protein (CCP) modules. The very C-terminal domain is the catalytic chymotrypsin-like SP (Serin-Protease) domain (**figure 1.3B**) [17]. Two copies of C1r and two of C1s associate with the C1q collagen-like stalk, folding into a compact '8-shaped' conformation, enabling contact between the catalytic regions of C1r and C1s, a prerequisite for C1s activation [18] (**figure 1.2C-D**).

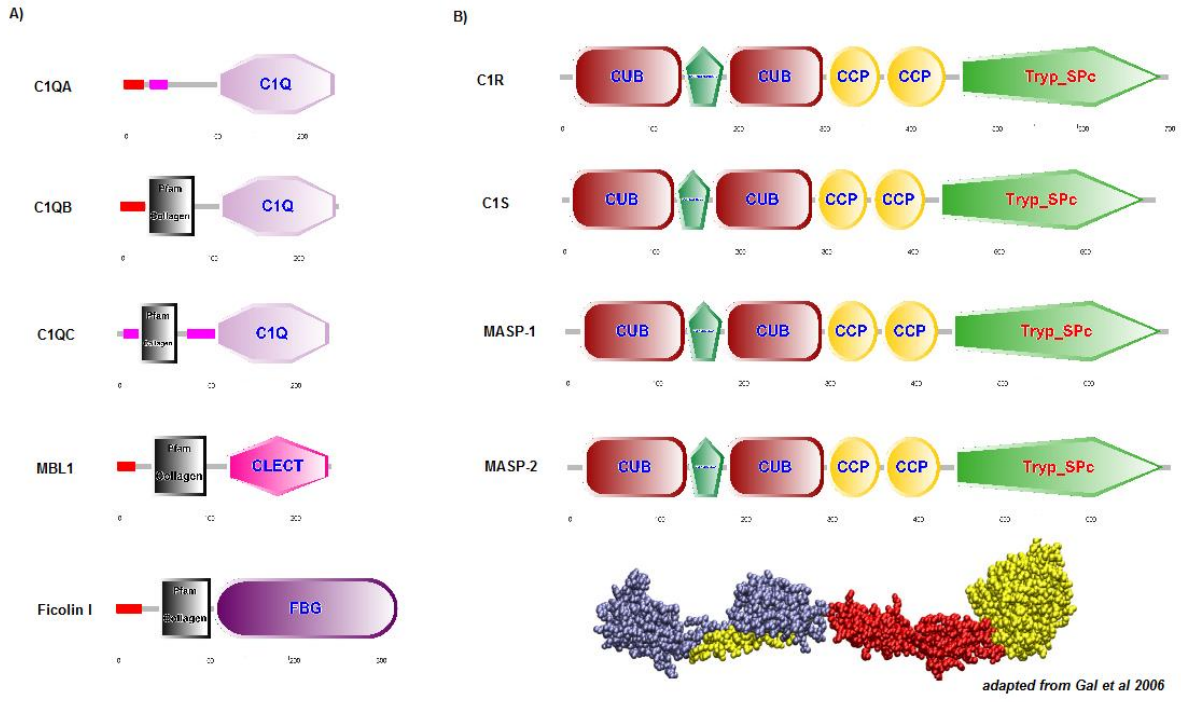


Figure 1.3 Domain organization similarities between the classical and lectin pathways. (A) Domain prediction of the C1qA, C1qB and C1qC proteins compared with MBL and Ficolin I. Collagen domain (black), C1q globular head (grey), C-type lectin domain (CLECT - pink) and fibrinogen binding domain (FBG - violet) are shown. **(B)** Domain prediction of the C1r, C1s, MASP-1 and MASP-2 serine proteases. The six domains are shown: the N-terminal CUB domain (red), the epidermal growth factor (EGF)-like domain (dark green), a second CUB domain, a tandem repeat of complement control protein (CCP) modules (yellow) and the catalytic chymotrypsin-like serin protease domain (Tryp_SPc - light green) at the very C-term. Overall structure is also shown (adapted from Gal et al. 2006). Domain prediction was performed using SMART-EMBL online software (http://smart.embl-heidelberg.de)

The lectin pathway (LP)

The triggering point of the lectin pathway is, from a structural and functional point of view, very similar with the classical pathway. It is activated by many types of recognition molecules including mannose-binding lectin (MBL) [19] and ficolins (H, L and M-types also called type I, II and III) [20] which associates with MASP (MBL-Associated Serine Protease), resembling the C1q/C1r/C1s complex (**figure 1.1B**). MBL is, from a structural point of view, very similar to C1q, except that it is composed by only one type of polypeptide chain and it binds to pathogen-associated molecular patterns (PAMPs) on the surface of microbes (**figure 1.4**) [21, 22]. MBL is in fact characterized by a collagen-like region and a C-type carbohydrate recognition domain (CRD) at the C-terminal of the protein, able to recognize monosaccharides such as glucose, mannose and N-acetyl-glucosamine. In the same way, ficolins contain a short N-terminal, a collagen-like domain and a C-terminal fibrinogen-like domain (FBG) instead of the CRD domain as recognition portion (**figure 1.3A**) [23]. MASP-1, MASP-2 and MASP-3 form with C1r and C1s the family of serine-proteases and share identical domain organization and similar overall structure (**figure 1.3B**).

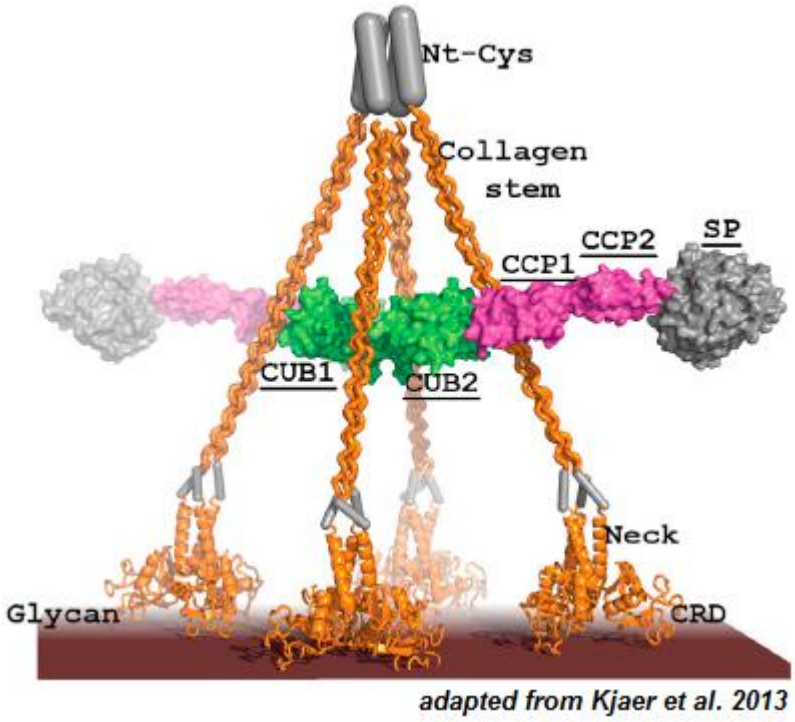


Figure 1.4 The assembled model of a MASP protease in complex with a tetramer of MBL trimers. MBL collagen stem and CRD domain are shown (orange). MASP dimer is also shown.

The C3 cleavage and the terminal pathway

Both the classical and the lectin pathway, after the activation of the C1s and MASP serine proteases respectively, converge in a unique pathway which starts with the highly specific cleavage of complement components 2 and 4 (C2 and C4) (**figure 1.1D**). The resulting cleavage lead to the production of two C2 fragments, C2a and C2b, and two C4 fragments, C4a and C4b. In a spatiotemporal order, C4 is first cleaved by C1s or MASP, than C4b binds to C2, which is then cleaved in C2a and C2b. The fragments C2a and C4b remain complexed together to form the C4b2a complex, also known as C3 convertase. This molecule is in fact able to specifically cleave the complement component C3 in C3a and C3b, driving to pathway amplification. C3a, C2b and C4a are not involved in complement amplification but constitute the so-called anaphylatoxins, potent pro-infammatory molecules which target specific activator receptors on phagocytic cells.

The C3b fragment, instead, constitute the key molecule for complement fixation and amplification. It is in fact able to bind extraneous surfaces (like bacteria) driving to cell opsonization. In addition, when present at high concentrations, C3b can also complex with C4b2a (C3 convertase) to form the C4b2a3b complex, also known as C5 convertase, since it's able to cleave complement component C5 into C5a and C5b, directly driving to terminal pathway activation [24-27]. C5b is a very unstable molecule which can be however stabilized by complement component C6. The C5b6 complex attracts complement component C7 and C8 yielding to the C5b678 complex. The proteins in this complex acquire a highly hydrophobic character and can insert into cell membranes generating pores of 10Å diameter. The binding of multiple copies of C9 to the C5b678 complex yields the final Membrane Attack Complex (MAC) with a diameter of around 70Å (**figure 1.5**) [28-32].

The alternative complement pathway (AP)

In addition to the classical and lectin, a third pathway called alternative pathway was also described (**figure 1.1C**). It is based on spontaneous cleavage of C3 in C3a and C3b naturally occurring in serum. C3b, once cleaved, can complex both with the C3 convertase (C4b2a) forming the C5 convetase (C4b2a3b) as described above, or with complement factor B, driving to alternative pathway activation. The factor B, once complexed with C3b to form the C3bB complex, is activated by factor D to form the C3bBb complex, a C3 convertase [8, 33, 34]. Upon increasing production of C3b, it can bind C3bBb becoming C3bBb3b with C5 convertase properties and the addition of complement factor P (or properdin) stabilizes the complex leading to terminal pathway activation [35].

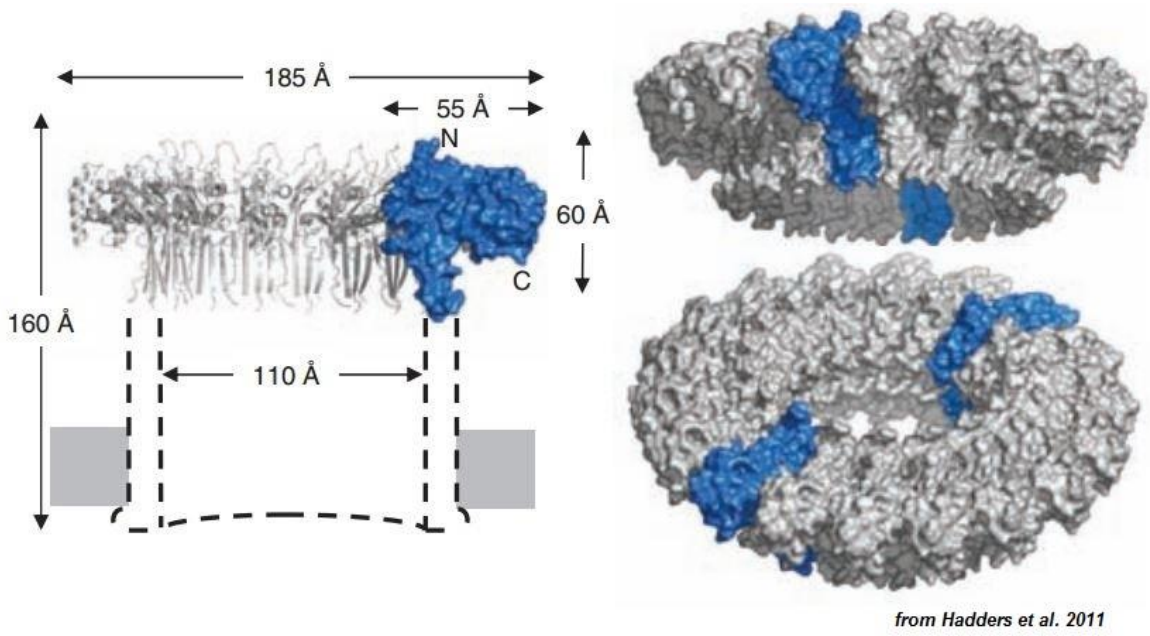


Figure 1.5 Hypothetical model of the C9 pore. A model of MAC is shown. The model is derived from a ring of 18 monomers of C8. Single C8 is highlighted in blue.

The complement regulators

Since the complement system can be easily and rapidly activated through the triggering of molecules of the three pathways, it needs to be finely tuned and regulated. Therefore several membrane bound and soluble Regulators of Complement Activity (RCA) are exposed or secreted by host cell to prevent self-attack. Among the soluble regulators, complement factor H (CFH), complement factor I (CFI), C4 binding protein (C4BP) and vitronectin constitute the key effectors in complement regulation. CFH is constituted by 20 Short Complement Regulator (SCR) domains, each of length about 61 residues. It binds to the C3 convertase (C3bBb) driving to the displacement of factor Bb and to convertase inactivation [36-38]. CFI, instead cleaves C3b bound to CFH into C3c and C3d, preventing re-complexing of C3b and therefore convertase formation [39, 40]. C4BP instead acts on C4, accelerating the decay of the C3 convertase (C4b2a). In addition acts as a cofactor for complement factor I in C3 cleavage [41-44]. Vitronectin (also called S-protein or serum spreading factor) is an important extracellular matrix molecule and inhibitor of the complement terminal complex. It can inhibit the terminal complement complex at various stages through the occupation of the membrane binding site of the C5b-7 complex to form SC5b-7 complex [45] and through the prevention of C9 polymerization and MAC complex formation [46].

The decay-accelerating factor (DAF or CD55), the membrane cofactor protein (MCP or CD46), the complement receptor type I (CR1 or CD35) and the complement receptor of the immunoglobulin superfamily (CRIg) constitute the most popular examples of membrane bound complement regulators. DAF is able to recognize C4b and C3b fragments preventing

cleavage of respectively C2 and factor B, thus avoiding complement activation. It is expressed on the plasma membranes of all cell types that are in close contact with plasma complement proteins [47-51]. MCP binds to C3b and C4b and functions as a cofactor of complement factor I for their cleavage [52, 53].

Usually both soluble and membrane bound RCA consist of strings of Complement Control Protein (CCP) domains, which from a functional point of view, are the direct responsible for complement regulation [54]. Viruses and bacteria often express proteins with an overall structure similar to the CCP domains, mimicking the mechanisms of host protection. Bacterial pathogens have also evolved proteins that bind the soluble regulators, which prevents complement mediated cell lysis and phagocytosis.

Bacterial immune adhesion and evasion: the *Staphylococcus aureus* and *Neisseria meningitidis* examples

The bacterial adhesion, evasion and dissemination processes take place though extensive protein-protein interactions between the human and bacterial extracellular proteomes. Among these three, the evasion process requires specific proteins to counteract the host immune system. Several bacteria, in fact, produce proteins which target key effectors of the immune system. *Staphylococcus aureus* and *Neisseria meningitidis* among others represent peculiar example for complement evasion and common evasion strategies are already known and well described in literature.

Mechanism of immune evasion

Despite the wide variety of bacterial proteins targeting the large panel of human proteins involved in immunity, three main evasion approaches can be identified: the recruitment or mimicking of complement regulators, the inactivation by enzymatic degradation (proteolysis) and the modulation or inhibition of complement proteins by direct interactions (**figure 1.6**) [55]. The key determinants for self and non-self determination are the RCA present on cell surfaces. Several pathogens have found the way to stably bind RCA that circulates in human plasma therefore preventing the complement action [56]. This process is mediated by common structural features present on RCA, the short consensus repeat (SCR) domains (like the CCP domain previously described) that allow the same bacterial protein to recruit different host RCA. In addition some viral proteins were described to be structurally similar to RCA, closely mimicking their function [57, 58]. The degradation of complement components

into non-functional proteins is the main feature of several bacterial proteases which targets a wide variety of human substrates [59-62]. Furthermore, the bacterial acquisition and activation of host proteases is an additional alternative to the enzymatic attenuation of complement. Complement is a very finely tuned system where a small change in regulation can break the delicate balance and complement efficiency. Therefore, surprisingly, only few bacterial proteins have been identified to directly bind complement components, inhibiting its action. Most of the identified proteins have been discovered in *S. aureus* even if also *B. burgdorferi*, streptococci and human parasites as *Schistosoma* and *Trypanosoma* have been shown to produce complement inhibitors [63, 64].

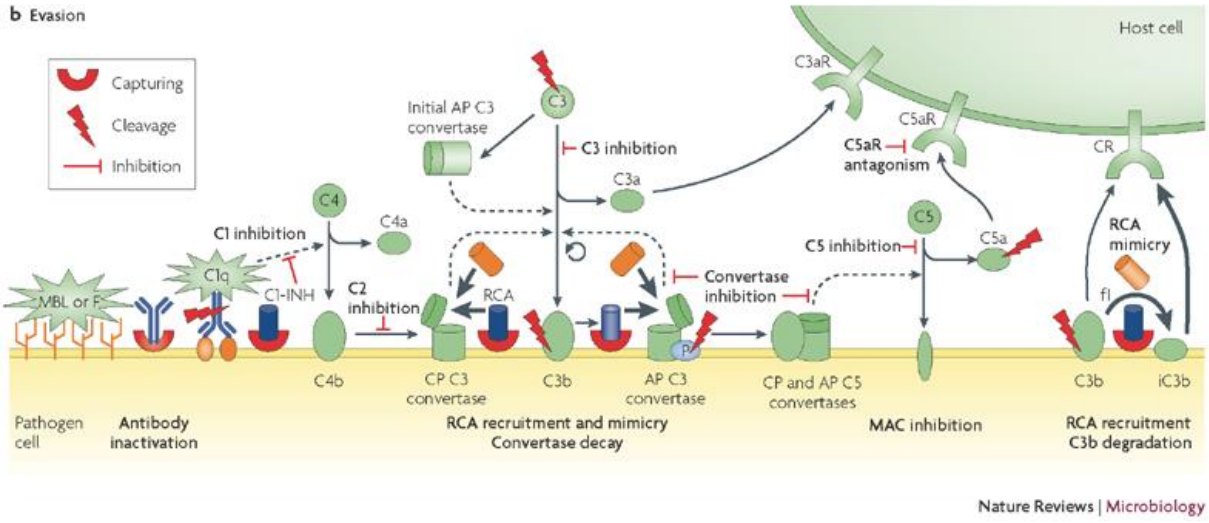


Figure 1.6 Mechanisms of bacterial immune evasion. Three main strategies can be distinguished: the captuding or mimicking of complement regulators, the inactivation by cleavage (proteolysis) and the modulation or inhibition of complement proteins by direct interactions. (*Picture from Lambris 2008*)

S. aureus and N. meningitidis, masters of complement evasion

Staphylococcus aureus is a gram-positive bacterium and opportunistic pathogen living as commensal in human skin and nasal cavities in 20% of humans [65, 66]. *S. aureus* constitutes, from a molecular point of view, a prototype for the study of immune evasion strategies. Despite the invasion and colonization molecular bases were well established [67, 68], novel mechanisms of immune evasion and the relative immune-evading molecules have been discovered and described only recently, drawing *S. aureus* as an intriguing model of host-pathogen interplay [59, 69-71]. Most of the identified evasion processes target the complement initiation, amplification and pro-inflammatory molecules, since *S. aureus* peptidoglycan-rich cell wall protect it from MAC formation and complement mediated cell-lysis [72]. Several proteins, in fact, impairs initiation of the classical pathway. Staphylococcal protein A (SpA) is one of the extensively studied and characterized protein in *S. aureus*. This protein binds Fc portion of IgG preventing recognition by C1q and FcγR thereby preventing classical complement and macrophage activation respectively [73, 74]. Less characterized

than SpA, but not less relevant in pathogenesis are the staphylococcal binder of immunoglobulin (Sbi) and superantigen-like 10 (SSL-10) proteins, able to bind IgG deposited on the surface of the bacterium [71, 75, 76]. In addition, *S. aureus* has developed a way to use host proteases to inhibit complement. This is the case of staphylokinase, which, through the recruitment and activation of plasmilogen in plasmin, a wide spectrum serine-protease, degrades circulating and surface bound C3 and human IgG, thereby reverting their opsonization effect [77, 78]. Several *S. aureus* proteins are capable of direct complement inhibition at C3 and C5 level. Efb (extracellular fibrinogen-binding protein) is able to bind C3b and inhibit opsonophagocytosis [79]. Through structural analysis, an Efb-homologous protein (Ehp) has been identified. It shares with Efb the ability to bind C3b inducing conformational changes which prevent complement amplification. Ehp however binds two C3b molecules simultaneously and possesses a greater complement inhibition capacity [80, 81]. SCIN (staphylococcal complement inhibitor) and its homologues proteins SCINB and SCINC stabilize and inhibit the C4b2a and C3bBb convertases. The structure of the SCIN-C3bBb complex has shown that SCIN sterically blocks the formation of the C3-C3bBb complex, preventing C3 cleavage [82-85]. SSL-7 (staphylococcal superantigen-like protein-7), instead, binds C5 with high affinity, resulting in complement terminal pathway inhibition [86]. In addition to these complement regulator proteins, *S. aureus* secretes a plethora of proteins acting on monocytes and neutrophils preventing their activation. CHIPS (chemotaxis inhibitory protein of *S. aureus*) is an antagonist of C5a receptor [87, 88]; SSL5 blocks both the interaction between the P-selectin glycoprotein ligand 1 and P-selectin and the activation of GPCRs preventing neutrophils extravasation [89]. Very recently, in addition, FLIPr (formyl peptide receptor like-1 inhibitory protein) and FLIPr-like proteins have been described to have immune evasion properties. These proteins were first identified as inhibitors of formyl-peptide receptor (FPR) and formyl-peptide receptor like-1 (FPRL1), two chemoattractant receptors on neutrophyls [90, 91]. In further studies these proteins have been described as potent FcγR antagonist on macrophages showing their ability in preventing opsonization and phagocytosis [92].

Neisseria meningitidis is a gram-negative encapsulated bacterium carried asymptomatically in the nasopharynx of approximately 5–10% of the human population [93]. For still not completely understood reasons it may become pathogenic and can cause meningococcal meningitis and septicemia. The ability to evade the host immune system is mainly due to cellular structures like capsule and LOS and LPS (lipo-oligosaccharides and lipo-polysaccharides) [94]. Capsule is made of repeating saccharide units which are used to divide *N. meningitidis* into 12 immunologically distinct groups among which A, B, C, W, X, and Y are the most virulent. From a functional point of view, the capsular structure prevents

complement deposition and complement-mediated bacterial killing [95]. Sialic acid on LOS and LPS, instead, is thought to mimic sialic acid on host cells and subvert the complement alternative pathway by enhancing interactions of CFH with surface bound C3b [96]. Despite these two non-proteinaceous structures *N. meningitidis* is able to recruit, through surface proteins, several complement regulators to block activation of complement at several key steps. Key virulence factors for *N. meningitidis* are fHbp (factor H binding protein) and NspA (neisserial surface protein A), two proteins exposed on the bacterial surface able to bind complement factor H and inhibit complement [97, 98]. Based on its amino acid sequence, fHbp is divided, into either three variant groups or two sub-families [99, 100]. fHbp is also part of the recently released 4 component meningococcal vaccine (4cMenB) against type B meningococcus [101, 102]. In addition to fHbp and NspA, several other proteins have been described to target complement regulators. PorA (porin A) is a membrane protein which binds to C4bp; however, strong binding occurred only under hypotonic conditions [103]. The phase variable protein Opc (opacity protein) and the meningococcal surface fibril (Msf also known as NhhA and Hsf) bind to vitronectin allowing the decrease in MAC deposition, enhancing resistance to complement mediated killing and the binding to human brain endothelial cells through the cell-binding RGD domain of vitronectin [104, 105].

Concluding remarks

Within the last decade, significant progress has been made in the understanding of the different aspects of bacterial pathogenesis, mainly thanks to important technologic advances like whole genome sequencing and bioinformatic approaches. Furthermore, in the last years, important findings have been made to further detail the description of both the human immune system mechanism of function and of the modulation of bacterial response to environmental stimuli. Despite that, there are still several gaps in our knowledge on how bacteria pass from being commensal harmless to highly virulent and life threatening organisms and we are only beginning to appreciate the layers of complexity of bacterial evasion mechanisms. Large-scale approaches in the molecular biology, immunology, epidemiology and bioinformatic fields, with the years to come will provide a more accurate and detailed picture of host-pathogen interactions. Among all, the discovery of novel host-pathogen interactions at the protein level can provide not only specific functional information on bacterial determinants, but also novel insights for the discovery and development of potentially effective therapeutic compounds and vaccine candidates.

References

1. Carroll, M.V. and R.B. Sim, *Complement in health and disease.* Adv Drug Deliv Rev, 2011. **63**(12): p. 965-75.
2. Walport, M.J., *Complement. First of two parts.* N Engl J Med, 2001. **344**(14): p. 1058-66.
3. Walport, M.J., *Complement. Second of two parts.* N Engl J Med, 2001. **344**(15): p. 1140-4.
4. Kirkitadze, M.D. and P.N. Barlow, *Structure and flexibility of the multiple domain proteins that regulate complement activation.* Immunol Rev, 2001. **180**: p. 146-61.
5. Zipfel, P.F., *Complement and immune defense: from innate immunity to human diseases.* Immunol Lett, 2009. **126**(1-2): p. 1-7.
6. Nonaka, M., *Evolution of the complement system.* Subcell Biochem, 2014. **80**: p. 31-43.
7. Forneris, F., J. Wu, and P. Gros, *The modular serine proteases of the complement cascade.* Curr Opin Struct Biol, 2012. **22**(3): p. 333-41.
8. Gros, P., F.J. Milder, and B.J. Janssen, *Complement driven by conformational changes.* Nat Rev Immunol, 2008. **8**(1): p. 48-58.
9. Arlaud, G.J., et al., *Structural biology of C1: dissection of a complex molecular machinery.* Immunol Rev, 2001. **180**: p. 136-45.
10. Cooper, N.R., *The classical complement pathway: activation and regulation of the first complement component.* Adv Immunol, 1985. **37**: p. 151-216.
11. Gaboriaud, C., et al., *The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties.* J Biol Chem, 2003. **278**(47): p. 46974-82.
12. Rogers, J., et al., *Complement activation by beta-amyloid in Alzheimer disease.* Proc Natl Acad Sci U S A, 1992. **89**(21): p. 10016-20.
13. Tacnet-Delorme, P., S. Chevallier, and G.J. Arlaud, *Beta-amyloid fibrils activate the C1 complex of complement under physiological conditions: evidence for a binding site for A beta on the C1q globular regions.* J Immunol, 2001. **167**(11): p. 6374-81.
14. Klein, M.A., et al., *Complement facilitates early prion pathogenesis.* Nat Med, 2001. **7**(4): p. 488-92.
15. Mabbott, N.A., et al., *Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie.* Nat Med, 2001. **7**(4): p. 485-7.
16. Navratil, J.S., et al., *The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells.* J Immunol, 2001. **166**(5): p. 3231-9.
17. Gaboriaud, C., et al., *Structure and activation of the C1 complex of complement: unraveling the puzzle.* Trends Immunol, 2004. **25**(7): p. 368-73.
18. Arlaud, G.J., et al., *Structural biology of the C1 complex of complement unveils the mechanisms of its activation and proteolytic activity.* Mol Immunol, 2002. **39**(7-8): p. 383-94.
19. Holmskov, U., S. Thiel, and J.C. Jensenius, *Collections and ficolins: humoral lectins of the innate immune defense.* Annu Rev Immunol, 2003. **21**: p. 547-78.
20. Matsushita, M. and T. Fujita, *Ficolins and the lectin complement pathway.* Immunol Rev, 2001. **180**: p. 78-85.
21. Gal, P., et al., *Serine proteases of the classical and lectin pathways: similarities and differences.* Immunobiology, 2007. **212**(4-5): p. 267-77.

22. Vorup-Jensen, T., J.C. Jensenius, and S. Thiel, *MASP-2, the C3 convertase generating protease of the MBLectin complement activating pathway.* Immunobiology, 1998. **199**(2): p. 348-57.
23. Kjaer, T.R., S. Thiel, and G.R. Andersen, *Toward a structure-based comprehension of the lectin pathway of complement.* Mol Immunol, 2013. **56**(3): p. 222-31.
24. Arlaud, G.J., M.G. Colomb, and J. Gagnon, *A functional model of the human C1 complex Emergence of a functional model.* Immunol Today, 1987. **8**(4): p. 106-11.
25. Porter, R.R. and K.B. Reid, *The biochemistry of complement.* Nature, 1978. **275**(5682): p. 699-704.
26. Ziccardi, R.J. and N.R. Cooper, *Direct demonstration and quantitation of the first complement component in human serum.* Science, 1978. **199**(4333): p. 1080-2.
27. Gaboriaud, C., et al., *Deciphering the fine details of c1 assembly and activation mechanisms: "mission impossible"?* Front Immunol, 2014. **5**: p. 565.
28. Muller-Eberhard, H.J., *The membrane attack complex of complement.* Annu Rev Immunol, 1986. **4**: p. 503-28.
29. Tschopp, J., H.J. Muller-Eberhard, and E.R. Podack, *Formation of transmembrane tubules by spontaneous polymerization of the hydrophilic complement protein C9.* Nature, 1982. **298**(5874): p. 534-8.
30. Tschopp, J., *Ultrastructure of the membrane attack complex of complement. Heterogeneity of the complex caused by different degree of C9 polymerization.* J Biol Chem, 1984. **259**(12): p. 7857-63.
31. DiScipio, R.G. and T.E. Hugli, *The architecture of complement component C9 and poly(C9).* J Biol Chem, 1985. **260**(27): p. 14802-9.
32. Hadders, M.A., D.X. Beringer, and P. Gros, *Structure of C8alpha-MACPF reveals mechanism of membrane attack in complement immune defense.* Science, 2007. **317**(5844): p. 1552-4.
33. Pangburn, M.K. and H.J. Muller-Eberhard, *The C3 convertase of the alternative pathway of human complement. Enzymic properties of the bimolecular proteinase.* Biochem J, 1986. **235**(3): p. 723-30.
34. Xu, Y., S.V. Narayana, and J.E. Volanakis, *Structural biology of the alternative pathway convertase.* Immunol Rev, 2001. **180**: p. 123-35.
35. Forneris, F., et al., *Structures of C3b in complex with factors B and D give insight into complement convertase formation.* Science, 2010. **330**(6012): p. 1816-20.
36. Pangburn, M.K., R.D. Schreiber, and H.J. Muller-Eberhard, *Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution.* J Exp Med, 1977. **146**(1): p. 257-70.
37. Vik, D.P., et al., *Factor H.* Curr Top Microbiol Immunol, 1990. **153**: p. 147-62.
38. Barlow, P.N., G.S. Hageman, and S.M. Lea, *Complement factor H: using atomic resolution structure to illuminate disease mechanisms.* Adv Exp Med Biol, 2008. **632**: p. 117-42.
39. Roversi, P., et al., *Structural basis for complement factor I control and its disease-associated sequence polymorphisms.* Proc Natl Acad Sci U S A, 2011. **108**(31): p. 12839-44.
40. Nilsson, S.C., et al., *Complement factor I in health and disease.* Mol Immunol, 2011. **48**(14): p. 1611-20.
41. Hillarp, A. and B. Dahlback, *The protein S-binding site localized to the central core of C4b-binding protein.* J Biol Chem, 1987. **262**(23): p. 11300-7.

42. Hofmeyer, T., et al., *Arranged sevenfold: structural insights into the C-terminal oligomerization domain of human C4b-binding protein.* J Mol Biol, 2013. **425**(8): p. 1302-17.
43. Nagasawa, S., et al., *Human C4b-binding protein, C4bp. Chymotryptic cleavage and location of the 48 kDa active fragment within C4bp.* FEBS Lett, 1983. **164**(1): p. 135-8.
44. Blom, A.M. and S. Ram, *Contribution of interactions between complement inhibitor C4b-binding protein and pathogens to their ability to establish infection with particular emphasis on Neisseria gonorrhoeae.* Vaccine, 2008. **26 Suppl 8**: p. I49-55.
45. Preissner, K.T., R. Wassmuth, and G. Muller-Berghaus, *Physicochemical characterization of human S-protein and its function in the blood coagulation system.* Biochem J, 1985. **231**(2): p. 349-55.
46. Podack, E.R., K.T. Preissner, and H.J. Muller-Eberhard, *Inhibition of C9 polymerization within the SC5b-9 complex of complement by S-protein.* Acta Pathol Microbiol Immunol Scand Suppl, 1984. **284**: p. 89-96.
47. Post, T.W., et al., *Structure of the gene for human complement protein decay accelerating factor.* J Immunol, 1990. **144**(2): p. 740-4.
48. Lublin, D.M. and J.P. Atkinson, *Decay-accelerating factor: biochemistry, molecular biology, and function.* Annu Rev Immunol, 1989. **7**: p. 35-58.
49. Holers, V.M., et al., *Human C3b- and C4b-regulatory proteins: a new multi-gene family.* Immunol Today, 1985. **6**(6): p. 188-92.
50. Law, S.K., *C3 receptors on macrophages.* J Cell Sci Suppl, 1988. **9**: p. 67-97.
51. Sim, R.B., et al., *Structure and specificity of complement receptors.* Immunol Lett, 1987. **14**(3): p. 183-90.
52. Adams, E.M., et al., *Contribution of the repeating domains of membrane cofactor protein (CD46) of the complement system to ligand binding and cofactor activity.* J Immunol, 1991. **147**(9): p. 3005-11.
53. Liszewski, M.K. and J.P. Atkinson, *Membrane cofactor protein.* Curr Top Microbiol Immunol, 1992. **178**: p. 45-60.
54. Serruto, D., et al., *Molecular mechanisms of complement evasion: learning from staphylococci and meningococci.* Nat Rev Microbiol, 2010. **8**(6): p. 393-9.
55. Lambris, J.D., D. Ricklin, and B.V. Geisbrecht, *Complement evasion by human pathogens.* Nat Rev Microbiol, 2008. **6**(2): p. 132-42.
56. Zipfel, P.F., R. Wurzner, and C. Skerka, *Complement evasion of pathogens: common strategies are shared by diverse organisms.* Mol Immunol, 2007. **44**(16): p. 3850-7.
57. McKenzie, R., et al., *Regulation of complement activity by vaccinia virus complement-control protein.* J Infect Dis, 1992. **166**(6): p. 1245-50.
58. Rosengard, A.M., et al., *Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement.* Proc Natl Acad Sci U S A, 2002. **99**(13): p. 8808-13.
59. Rooijakkers, S.H. and J.A. van Strijp, *Bacterial complement evasion.* Mol Immunol, 2007. **44**(1-3): p. 23-32.
60. Oda, T., et al., *Inactivation of chemotactic activity of C5a by the serratial 56-kilodalton protease.* Infect Immun, 1990. **58**(5): p. 1269-72.
61. Chmouryguina, I., et al., *Conservation of the C5a peptidase genes in group A and B streptococci.* Infect Immun, 1996. **64**(7): p. 2387-90.

62. Tsao, N., et al., *Streptococcal pyrogenic exotoxin B cleaves properdin and inhibits complement-mediated opsonophagocytosis.* Biochem Biophys Res Commun, 2006. **339**(3): p. 779-84.
63. Inal, J.M. and R.B. Sim, *A Schistosoma protein, Sh-TOR, is a novel inhibitor of complement which binds human C2.* FEBS Lett, 2000. **470**(2): p. 131-4.
64. Deng, J., et al., *Inhibition of the complement membrane attack complex by Schistosoma mansoni paramyosin.* Infect Immun, 2003. **71**(11): p. 6402-10.
65. Peacock, S.J., I. de Silva, and F.D. Lowy, *What determines nasal carriage of Staphylococcus aureus?* Trends Microbiol, 2001. **9**(12): p. 605-10.
66. Lowy, F.D., *Staphylococcus aureus infections.* N Engl J Med, 1998. **339**(8): p. 520-32.
67. Patti, J.M., et al., *MSCRAMM-mediated adherence of microorganisms to host tissues.* Annu Rev Microbiol, 1994. **48**: p. 585-617.
68. Foster, T.J. and M. Hook, *Surface protein adhesins of Staphylococcus aureus.* Trends Microbiol, 1998. **6**(12): p. 484-8.
69. Chavakis, T., K.T. Preissner, and M. Herrmann, *The anti-inflammatory activities of Staphylococcus aureus.* Trends Immunol, 2007. **28**(9): p. 408-18.
70. Foster, T.J., *Immune evasion by staphylococci.* Nat Rev Microbiol, 2005. **3**(12): p. 948-58.
71. Rooijakkers, S.H., K.P. van Kessel, and J.A. van Strijp, *Staphylococcal innate immune evasion.* Trends Microbiol, 2005. **13**(12): p. 596-601.
72. Frank, M.M., *Annihilating host defense.* Nat Med, 2001. **7**(12): p. 1285-6.
73. Cedergren, L., et al., *Mutational analysis of the interaction between staphylococcal protein A and human IgG1.* Protein Eng, 1993. **6**(4): p. 441-8.
74. Gouda, H., et al., *Three-dimensional solution structure of the B domain of staphylococcal protein A: comparisons of the solution and crystal structures.* Biochemistry, 1992. **31**(40): p. 9665-72.
75. Zhang, L., et al., *A second IgG-binding protein in Staphylococcus aureus.* Microbiology, 1998. **144 (Pt 4)**: p. 985-91.
76. Itoh, S., et al., *Staphylococcal superantigen-like protein 10 (SSL10) binds to human immunoglobulin G (IgG) and inhibits complement activation via the classical pathway.* Mol Immunol, 2010. **47**(4): p. 932-8.
77. Lahteenmaki, K., P. Kuusela, and T.K. Korhonen, *Bacterial plasminogen activators and receptors.* FEMS Microbiol Rev, 2001. **25**(5): p. 531-52.
78. Rooijakkers, S.H., et al., *Anti-opsonic properties of staphylokinase.* Microbes Infect, 2005. **7**(3): p. 476-84.
79. Lee, L.Y., et al., *Inhibition of complement activation by a secreted Staphylococcus aureus protein.* J Infect Dis, 2004. **190**(3): p. 571-9.
80. Hammel, M., et al., *A structural basis for complement inhibition by Staphylococcus aureus.* Nat Immunol, 2007. **8**(4): p. 430-7.
81. Hammel, M., et al., *Characterization of Ehp, a secreted complement inhibitory protein from Staphylococcus aureus.* J Biol Chem, 2007. **282**(41): p. 30051-61.
82. Katschke, K.J., Jr., et al., *Structural and functional analysis of a C3b-specific antibody that selectively inhibits the alternative pathway of complement.* J Biol Chem, 2009. **284**(16): p. 10473-9.
83. Janssen, B.J., et al., *Structure of compstatin in complex with complement component C3c reveals a new mechanism of complement inhibition.* J Biol Chem, 2007. **282**(40): p. 29241-7.

84. Rooijakkers, S.H., et al., *Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor.* Nat Immunol, 2009. **10**(7): p. 721-7.
85. Rooijakkers, S.H., et al., *Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases.* Nat Immunol, 2005. **6**(9): p. 920-7.
86. Langley, R., et al., *The staphylococcal superantigen-like protein 7 binds IgA and complement C5 and inhibits IgA-Fc alpha RI binding and serum killing of bacteria.* J Immunol, 2005. **174**(5): p. 2926-33.
87. de Haas, C.J., et al., *Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent.* J Exp Med, 2004. **199**(5): p. 687-95.
88. Postma, B., et al., *Chemotaxis inhibitory protein of Staphylococcus aureus binds specifically to the C5a and formylated peptide receptor.* J Immunol, 2004. **172**(11): p. 6994-7001.
89. Bestebroer, J., et al., *Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling.* Blood, 2007. **109**(7): p. 2936-43.
90. Prat, C., et al., *A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1.* J Immunol, 2006. **177**(11): p. 8017-26.
91. Prat, C., et al., *A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from Staphylococcus aureus (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR.* J Immunol, 2009. **183**(10): p. 6569-78.
92. Stemerding, A.M., et al., *Staphylococcus aureus formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcgammaR antagonists that inhibit IgG-mediated effector functions.* J Immunol, 2013. **191**(1): p. 353-62.
93. Stephens, D.S., B. Greenwood, and P. Brandtzaeg, *Epidemic meningitis, meningococcaemia, and Neisseria meningitidis.* Lancet, 2007. **369**(9580): p. 2196-210.
94. Lewis, L.A. and S. Ram, *Meningococcal disease and the complement system.* Virulence, 2014. **5**(1): p. 98-126.
95. Uria, M.J., et al., *A generic mechanism in Neisseria meningitidis for enhanced resistance against bactericidal antibodies.* J Exp Med, 2008. **205**(6): p. 1423-34.
96. Kajander, T., et al., *Dual interaction of factor H with C3d and glycosaminoglycans in host-nonhost discrimination by complement.* Proc Natl Acad Sci U S A, 2011. **108**(7): p. 2897-902.
97. Lewis, L.A., et al., *The meningococcal vaccine candidate neisserial surface protein A (NspA) binds to factor H and enhances meningococcal resistance to complement.* PLoS Pathog, 2010. **6**(7): p. e1001027.
98. Madico, G., et al., *The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance.* J Immunol, 2006. **177**(1): p. 501-10.
99. Masignani, V., et al., *Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870.* J Exp Med, 2003. **197**(6): p. 789-99.
100. Fletcher, L.D., et al., *Vaccine potential of the Neisseria meningitidis 2086 lipoprotein.* Infect Immun, 2004. **72**(4): p. 2088-100.
101. Serruto, D., et al., *The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens.* Vaccine, 2012. **30 Suppl 2**: p. B87-97.

102. Pizza, M., et al., *Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing.* Science, 2000. **287**(5459): p. 1816-20.
103. Jarva, H., et al., *Binding of the complement inhibitor C4bp to serogroup B Neisseria meningitidis.* J Immunol, 2005. **174**(10): p. 6299-307.
104. Hubert, K., et al., *Opc expression, LPS immunotype switch and pilin conversion contribute to serum resistance of unencapsulated meningococci.* PLoS One, 2012. **7**(9): p. e45132.
105. Griffiths, N.J., et al., *Meningococcal surface fibril (Msf) binds to activated vitronectin and inhibits the terminal complement pathway to increase serum resistance.* Mol Microbiol, 2011. **82**(5): p. 1129-49.

Chapter TWO

High-throughput protein microarray screening revealed a novel *Staphylococcus aureus* C1q-binding and complement inhibitor protein

Introduction

Staphylococcus aureus is a gram-positive bacterium and opportunistic pathogen living as commensal in human skin and nasal cavities in 20% of humans [1, 2]. In some cases, *S. aureus* can infect humans causing a broad range of diseases, from superficial skin to invasive potentially life-threatening infections like endocarditis, pneumonia and sepsis. In addition, antibiotic treatment is often ineffective due to the development of many antibiotic resistant strains, the hypervirulent MDRS (Multi Drug Resistant Strains) which are spreading in many hospitals [3].

S. aureus, like many other pathogenic bacteria such as *N. meningitides* and group A and B *Streptococcus* (GAS and GBS), produce a wide variety of surface exposed and secreted proteins (usually described as “surfacome”) used for pathogenesis [4]. These proteins specifically bind host proteins causing bacterial adhesion, invasion and immune system deregulation and evasion. The *S. aureus* CWA (Cell Wall Anchored) protein family are surface exposed proteins covalently attached to peptidoglycan and are responsible for promoting adhesion to the extracellular matrix (ECM). Part of this class of proteins are the subclasses MSCRAMMs (Microbial Surface Component Recognizing Adhesive Matrix Molecules), that mediate the attachment to components of the host ECM, and NEAT (Near iron Transporter) proteins, involved in haem capture from haemoglobin. Examples of MSCRAMMs are the two fibrinogen-binding proteins ClfA (clumping factor A) and ClfB (clumping factor B) and other proteins involved in ECM binding such as SdrB, SdrC and SdrD, while IsdA and IsdB are typical NEAT proteins. Additional non-covalently associated surface proteins are represented by autolysin and adhesins which re-associate with the surface by ionic or hydrophobic interactions and have both enzymatic and adhesive functions [5]. In addition, *S. aureus* is remarkably able to control and subvert human innate immune response, especially the complement system, using a plethora of surface-exposed and secreted proteins [6]. Among the most studied there are the Staphylococcal Protein A (Spa) and Staphylococcal Binder of Immunoglobulins (Sbi) proteins, which bind IgG interfering with the complement classical pathway activation [7]. SCIN protein is instead able to bind the C3 convertase (C3bBb) complex, responsible for C3 cleavage and complement amplification [8]. SSl7 binds to C5 and inhibits the conversion of C5 into C5a57 [9].

The large number of interactions discovered so far, sometimes promiscuous and involving multiple components of the human defence apparatus, seems to suggest a very complex picture where additional interactions may take place at the host-pathogen interface. For this reason, a large-scale screening of human and staphylococcal proteins by protein microarray may represent an ideal tool for the identification of novel protein-protein interactions [10, 11].

In this study, protein microarray containing a large panel of *S. aureus* surface proteins was used to simultaneously investigate interactions with a library of human proteins potentially relevant during bacterial pathogenesis. Using this approach, several novel interactions between *S. aureus* and human proteins were identified. Among all, the interaction between FLIPr (FPRL1 inhibitory protein) and human C1q (complement component 1q) emerged as one of the most statistically relevant hit and it has been further characterized using biophysical and functional assays.

Experimental Procedures

Selection, Cloning and Purification of SA Surface Proteins

Genomic DNA coding for the mature portion of the *Staphylococcus aureus* proteins were cloned into the pET21b expression vector (Novagen) after PCR amplification using *Escherichia coli* BL21(DE3) (Novagen) as competent strain. Recombinant proteins (108 as single constructs and 10 as multiple constructs for a total of 159), obtained as C-terminal His or GST tag fusions, were expressed in HTMC (3% yeast extract, 40mM KH2PO4, 90mM K2HPO4, 2mM MgSO4, 1.5% glycerol, pH7.4) auto-inducing medium for 30h at 27°C and cell were then harvested at 6500xg for 1h. Bacterial lysis was obtained using B-PER buffer (Thermo Scientific) and the lysate was clarified by centrifugation at 30.000xg for 30min. The soluble fraction was loaded onto a His Multitrap HP 96-well plate system (GE Healthcare), washed with phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole pH 8.0) and the protein was finally eluted with the same buffer containing 250 mM imidazole as previously described in [12]. GST tagged proteins were purified using a GST Multitrap HP 96-well plate system (GE Healthcare), washed with phosphate buffer (50 mM Tris, 300 mM NaC pH 8.0). The proteins were eluted with the same buffer containing 10 mM reduced glutathione. Proteins purified in a tagless form were obtained as described by Klock et al. [13]. Briefly, the PCR product of the portion of the gene coding for the mature protein was cloned using the Polymerase Incomplete Primer Extension (PIPE) method into plasmid pSpeedET, which encodes an expression and purification tag followed by a tobacco etch virus protease site (MGSDKIHHHHHHENLYFQG) at the N terminus of the protein. E. coli strain HK100 was transformed with the plasmid coding for the protein and was grown in Luria-Bertani (LB) arabinose-containing broth until OD_{600}= 0,4 - 0,6. Protein expression was achieved using IPTG (Isopropyl-β-D-thiogalactopyranosid) 1mM for 3h at 25°C. Cells were harvested at 6500xg for 1h and lysed in B-PER buffer (Thermo Scientific). Lysate was clarified by centrifugation at 30.000xg for 30min and the protein contained in the soluble fraction was purified using an automated AKTA x-PRESS system on a nickel affinity column, followed by a desalting step using 3 x 5 ml HiTrap Desalting columns equilibrated in 50 mm HEPES pH 8.0, 1 mm tris(2-carboxyethyl)phosphine (TCEP). Purified protein was digested overnight at 4°C with 1 mg of tobacco etch virus (TEV) protease/10 mg of eluted protein and was passed over a nickel affinity column collecting the flow-through. Purified proteins were stored at -20°C and analysed by SDS-PAGE and Matrix-Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF) to assess their integrity, identity, and purity level (range of purity 60–90%).

Construction of the SA Surface Protein Microarray

The SA protein microarray was generated by spotting purified recombinant proteins (0.5mg/ml in a final glycerol concentration of 40%) in 16 replicates on nitrocellulose coated slides (FAST slides; Whatman) using the ink-jet spotter Marathon (Arrayjet) resulting in spots of ~110 µm in diameter. Printing was performed in a cabinet with controlled humidity and temperature (55-60% and 12°C respectively). A standard curve made of eight concentrations (twofold dilution from 0.5 to 0.004 mg/ml) of an amino terminal FLAG-tagged protein (P7582 Sigma), fluorescent BSA Cy3/Cy5 conjugated at 0,5 mg/ml and a standard curve of mouse IgGs were used to obtain eigh replicates of a fluorescence standard curve to assess array coordinates and comparison of replicate experiments.

Preliminary slide validation experiments, in which the slides were probed with mouse anti-GST and anti-6xHis monoclonal antibodies followed by detection with a Cy5-conjugated anti-mouse IgG secondary antibody, were performed to confirm the efficiency and reproducibility of the protein deposition on the chips. Nonspecific binding was minimized by preincubating arrays with a blocking solution containing NAP blocker (G-Bioscences) diluted 1:3 in PBS (Nap-PBS) for 1 hour. Human tagged proteins were diluted in Nap-PBS at final concentration of 10µg/ml and overlaid on the arrays (1 µg protein per slide) at RT for 1 h. After washing with 0.1% Tween 20 in PBS buffer (TPBS), arrays were incubated with mouse anti-FLAG (1:200– Sigma Aldrich) at RT for 1 h. Slides were washed again as before and interactions were detected by incubating with a Cy5 labeled anti-mouse 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. Signals were considered as positive when their MFI value was higher than 3,000, corresponding to the MFI of protein spots after detection with mouse anti-FLAG-Cy5 followed by an Cy5 labeled anti-mouse antibody, plus 3 standard deviation values. Negative control experiments were represented by slides incubated with anti-FLAG and anti-mouse antibodies only; the measured signals for each spot of the negative control slide was subtracted from those obtained for the same spot in the slides probed with human proteins. The average of the 16 spots replicates constitutes the mean fluorescent intensity (MFI) value taken in consideration for the validation experiments.

FLIPr expression and purification

To obtain an amount of protein in a mg range, FLIPr (25-133) was expressed in BL21 E. *coli* (DE3) cells in HTMC autoinducing media (3% yeast extract, 40mM KH2PO4, 90mM K2HPO4, 2mM MgSO4, 1.5% glycerol, pH7.4). Bacteria were grown for 30h at 27°C and cell were then harvested at 10000xg for 1h. Bacterial pellets were suspended in lysis buffer (50mM NaH2PO4 pH 8,2, 300mM NaCl supplemented with Roche EDTA-free protease inhibitors) lysate was clarified by centrifugation at 30.000xg for 30min. The histidine-tagged protein was purified using a nickel column (His Trap FF, 5 ml - GE Healthcare) following the manufacturer's instructions. An additional step on Superdex 75 26/60 (Ge Healthcare) was performed to remove possible aggregates. Sample purity was checked by 4-12% SDS-PAGE and SE-UPLC.

BioLayer Interferometry (BLI)-based OCTET analysis

The interaction between C1q and FLIPr was characterized using an Octet QKe instrument (ForteBio, Pall Science). AR2G (amine reactive 2nd generation) tips were obtained from ForteBio and were used to covalently attach ligand protein using amine based chemistry. Immediately before analysis, AR2G tips were prewet in kinetic buffer (10mM HEPES, 300mM NaCl, 0,02% P-20 pH 7.4). During the entire kinetic assay the sample plate was kept shaking 1000rpm. After 180 sec baseline, the biosensor tips were activated for 300 sec using a freshly mixed solution of 200 mM EDC in 50 mM NHS further diluted 1:20 in dH_2O and the tips were then immersed in ligand solution for 600 sec loading (C1q 12.5 ug/ml in 10 mM NaH_2PO_4 pH 6.5, FLIPr 50ug/ml in 10 mM Na acetate pH 5). Excess reactive groups were blocked with 1 M ethanolamine pH 8.5 for 300 sec and a second 300 sec baseline in kinetic buffer was performed. A column of biosensors where no ligand protein was loaded, was activated and quenched to be used as parallel reference control in association with the analyte titration; a ligand-loaded biosensor was also used in association with buffer as baseline.

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. Data were globally fit in a 1:1 model using the Data Analysis Software v7.1 (Forte Bio)

Assessment of in vitro complement activity

The assessment of in vitro complement activity was analysed using a complement screening kit (Wieslab COMP300). This enzyme based immunoassay was developed for the *in vitro* determination of complement dysfunctions in patients. The system relies on the specific

activation of the three distinct complement pathways (CP, MBL pathway and AP) through activators immobilized on the microtiter wells, using the deposition of the terminal MAC (C5-9) complex as readout. Buffers containing specific blockers to ensure the activation of the respective pathway were used to dilute the samples. For our purposes, serial concentrations (5uM, 1uM and 0,2uM) of FLIPr in HBS buffer (20mM HEPES, 150mM NaCl pH 7,3) were added to human serum previously diluted as suggested by supplier. Positive control was constituted by diluted serum and HBS buffer only, while negative control was constituted by heat inactivated serum. Additional control is represented by HBS buffer pre-incubated with serum. The amount of complement activation correlates with the colour intensity measured as absorbance (OD) at 405 nm. The value for the positive control was defined as 100%; the value for negative control as 0%. All measured values were expressed as relative % of complement activation using the following formula: (sample value – negative control value) / (positive control value – negative control value) x 100. Student's t test was used for comparison of paired means of two groups. P values of 0.05 were considered significant.

Whole blood phagocytosis assay

S. aureus survival in presence of FLIPr was assessed through whole blood phagocytosis assay. For such purpose an overnight culture of *S.aureus* cells from USA 300 LAC strain in BHI, was diluted 1:40 and grown at 37°C with shaking 180 RPM to OD_{600}=0.300. The bacteria were then diluted in BHI and 50 □l of the bacterial suspension ($2.5x10^5$CFU) were added to 1 ml of fresh blood from healthy donors supplemented with anticoagulant lepirudin 50 mg/L (20µl/ml of blood) and previously preincubated for 30 min at 37°C with different amount of FLIPr (0.4-3µM) or with PBS. A portion of this culture was plated on BHI-agar to determine the input CFU. The blood samples were incubated with bacteria at 37°C for 2h with shaking 180 RPM. Aliquots were then incubated for 3 min on ice with a final concentration of 0.5% of saponin-PBS to lyse neutrophils. The number of viable bacteria was determined by serial tenfold dilutions in BHI and plating on BHI- agar plates. Colonies were counted after incubation of the plates at 37°C for 18 h. Control was the blood sample preincubated with PBS.

Results

Microarray design and validation

To shed light on the interactions between the *Staphylococcus aureus* surface proteome and the human innate immune system, a protein microarray screening was performed spotting 159 *S. aureus* purified recombinant proteins (**figure 2.1A** and **supplementary table 1**) onto nitrocellulose coated slides. This microarray chip was used to be tested against a library of 75 human recombinant purified proteins (**figure 2.1B** and **supplementary table 2**) resulting from the selection of a larger library previously described [14]. All the *S. aureus* proteins printed on the arrays were selected using a combined bioinformatics and proteomic approach. The bacterial proteins were first selected by reverse vaccinology approach through bioinformatics analysis on S. *aureus* genome for the presence of a signal peptide, lipoprotein motifs, transmembrane regions and cell wall anchoring sequences and then integrated with surfome and secretome data resulting from the so called "protectome" [15]. The selected proteins all belong to the *S. aureus* strain NTCT 8325 genome except for some proteins coming from the Newman strain.

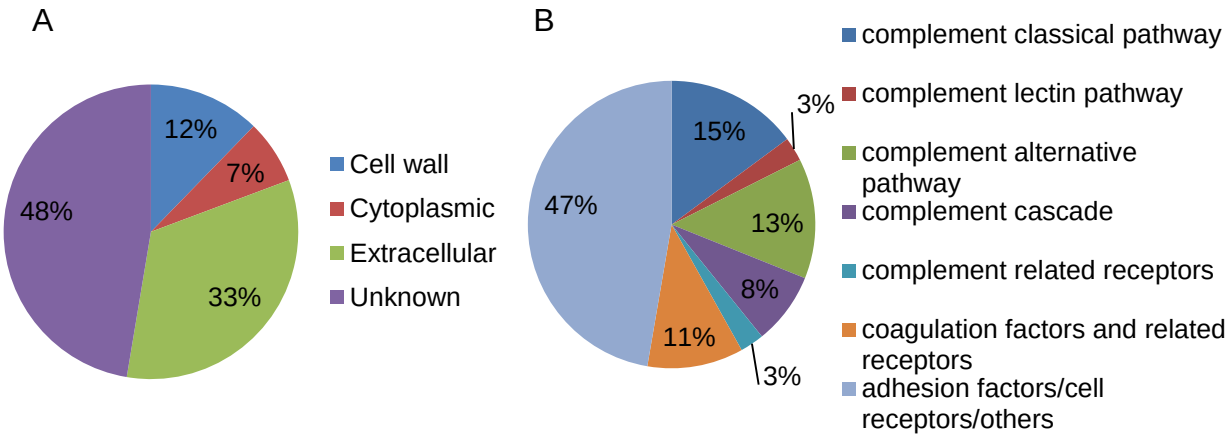


Figure 2.1 Protein microarray design. (A) Classification of the *Staphylococcus aureus* proteins spotted on the chip based on their predicted localization and **(B)** of the 75 human proteins tested in the microarray screening on the base of their biological function.

Among all the predicted proteins, 118 unique proteins (108 as single constructs and 10 as multiple constructs for a total of 159) were chosen for spotting based on their purity level determined through SDS-PAGE and Peptide Mass Fingerprinting coupled to MALDI-TOF analysis (**figure 2.2A**). Among them, many are *S. aureus* toxins known to be relevant for bacterial pathogenesis such as the pore forming toxin HLA (Hemo-Lysin Alpha) [16], the fibrinogen-binding ClfA and ClfB [17], the extracellular adherence proteins Eap and Emp, and other virulence factors like EsxA, EsxB, FnBA and Ebps. Many of the spotted proteins were,

on the other hand, of unknown function. Each protein was printed in 16 replicates all over the slide in a random fashion to prevent possible contamination between adjacent spots. Preliminary validation experiments in which the slides were probed with mouse anti-GST and anti-His6 tagged monoclonal antibodies followed by detection with a Cy5-conjugated anti-mouse IgG secondary antibody confirmed that all *S. aureus* proteins were efficiently and reproducibly deposited and immobilized on the chips.

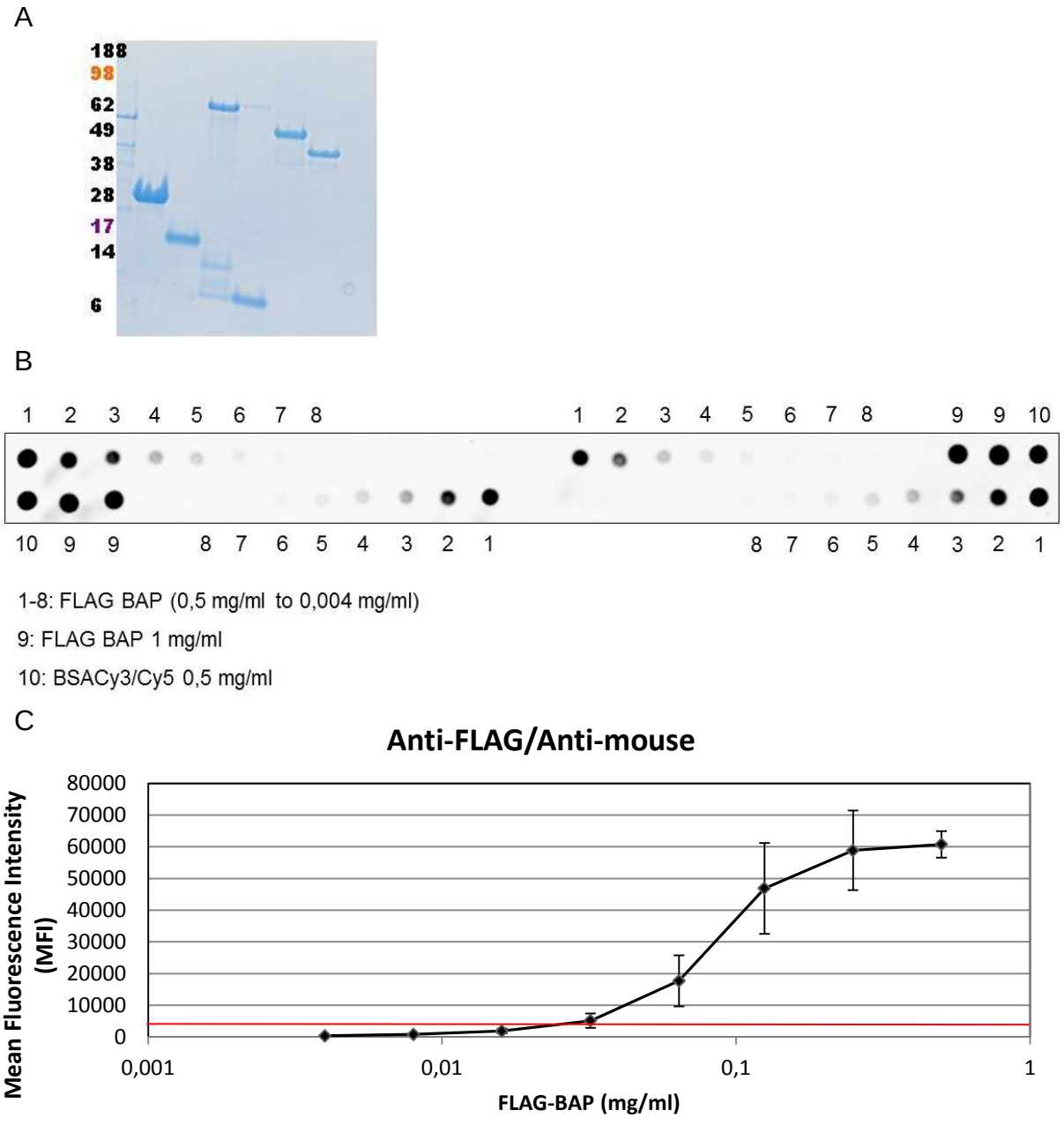


Figure 2.2 Protein microarray validation. (A) SDS-PAGE purity check of a set of *S. aureus* proteins purified with His Multitrap HP 96-well plate system. The purity was also determined by densitometry and MS analysis. **(B)** Fluorescence imaging detected using anti-FLAG and anti mouse antibodies of the spotted amino terminal FLAG-tagged protein used as control. Dilutions and replicates of the curve are shown. BSA Cy3/Cy5 was used for coordinates referencing. **(C)** Plot of the FLAG-tagged protein dilutions MFI values over their concentration. Curves best fit in a sigmoid curve. Standard deviation for each concentration is shown and refers to MFI of 30 different slides.

The interaction with human extracellular proteins was then studied by overlaying 75 human recombinant proteins selected from a larger library previously described [14]. Briefly, starting from previous large scale efforts and additional bioinformatic analysis, the gene coding for the ectodomain of 529 human proteins were cloned in plasmid for transient transfection, expressed with affinity tags (6xHis and FLAG) in HEK293 cells and purified in high-throughput way. During the selection particular priority was given to those proteins known to be relevant during bacterial pathogenesis. In particular, adhesion factors, complement proteins, complement-related receptors and coagulation factors were used for this screening. The binding between two proteins was detected with anti-FLAG antibodies and subsequently with Cy5-conjugated anti-mouse IgG antibody. An amino terminal FLAG-tagged protein was also spotted on the array, in twofold dilutions, as detection control and for slide-to-slide normalization (**figure 2.2B**). The Mean Fluorescence Intensities (MFI) values of the FLAG-tagged protein spots obtained after detection with anti-FLAG and Cy5-conjugated antibodies were fitted best by sigmoid curves (**figure 2.2C**), showing a signal dynamic range of about 2 logs of fluorescence intensity values and a lower detection limit corresponding to ~0.03 ng. Mouse IgGs and PBS buffer were printed on the array as negative controls for spotting and detection while the *Neisseria meningitidis* fHbp (factor H binding protein) was printed as a positive control for interaction with human factor H that is part of the human library.

Microarray screening and data analysis

On the basis of the Mean Fluorescence Intensity (MFI) value of the controls, an MFI value greater than 3000 (equal to the mean signal of the controls spots after detection with anti-Flag and anti-mouse alone, plus ten times standard deviation values) was determined as positive interaction. Three arbitrary MFI thresholds were also assigned for low (3000 to 15000 MFI), medium (15000 to 30000 MFI) and high (30000 to saturation) reactivity between two proteins. Over a total of 11766 possible combinations, 11168 (94,92%) resulted below the background threshold and were thereby considered negative, while 598 (5,08%) resulted positive. Among these, 518 (4,40%) were established to be low, 61 (0,52%) medium and 19 (0,16%) highly reactive (**figure 2.3** upper left panel). If the data were analysed considering each human protein class (as defined in figure 2.1B and supplementary table 2) the global picture does not change (**figure 2.3** and **table 2.1**). The adhesion factors and cell receptors classes are the most representative in the library being constituted by 35 proteins; over a total of 5565 combinations, 5287 were negative (95%), 241 (4,33%) low reactive, 29 medium (0,52%) and 8 high (0,14%). Within the proteins of the complement alternative pathway class, the negative combinations were 1506 (94,72%), the low reactive 73 (4,59%), the medium 8 (0,50%) and the high 3 (0,19%) for a total of 1590 combinations. In the coagulation factors

and related receptors families, on a total of 1272 combinations, 1240 (97,48%) were negative, 28 (2,20%) were low reactive, 3 medium (0,24%) and only 1 (0,08%) high. In the classical complement pathway class of proteins, interestingly, 6 combinations (0,34%) were highly reactive while 10 (0,57%) medium and 104 low (5.95%).

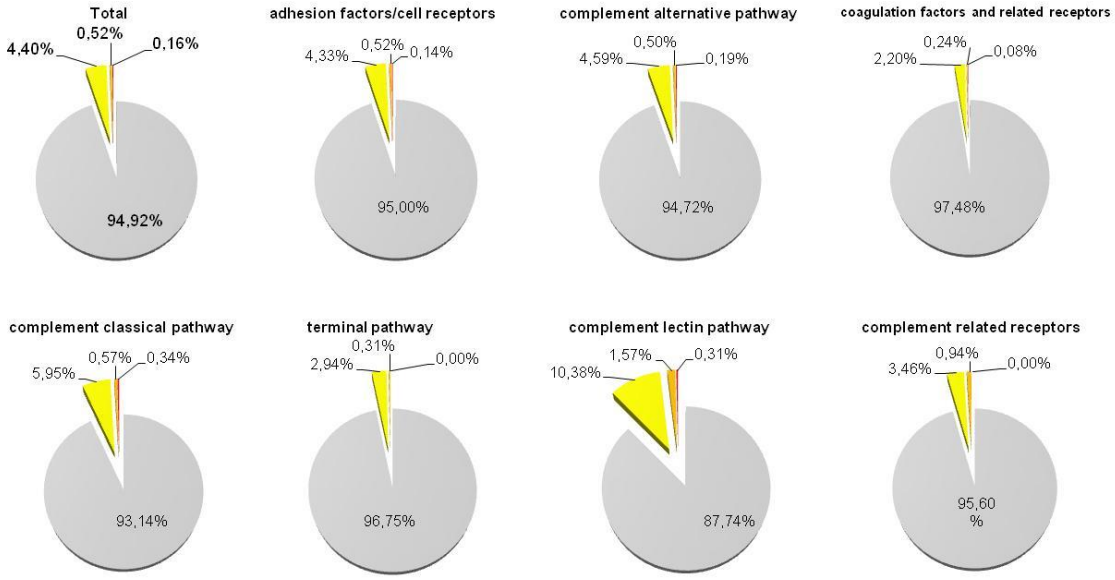


Figure 2.3 Screening results. Classification (differentiated for each human protein class) of the MFI values based on background and arbitrary thresholds. The 96% of the combinations between a *S. aureus* and human protein resulted below the background threshold (grey). The hits above 3000 MFI were classified in low (yellow - 4%), medium (orange - 0.36%) and highly reactive (red - 0.16%). Ficolin 2 was discarded from this analysis.

	adhesion factors/cell receptors/ others	complement alternative pathway	coagulation factors and related receptors	complement classical pathway	complement cascade	complement lectin pathway	complement related receptors	total
Proteins	35	10	8	11	6	2	2	74
total n of combinations	5565	1590	1272	1749	954	318	318	11766
negative combinations	5287	1506	1240	1629	923	279	304	11168
combinations between 3K 15K MFI	241	73	28	104	28	33	11	518
combinations between 15K 30K MFI	29	8	3	10	3	5	3	61
combinations between 30K 65K MFI	8	3	1	6	0	1	0	19

Table 2.1 Detailed microarray analysis divided based on human protein classes. Protein classes are shown in columns; colors used are the same as in figure 1B and supplementary table 2.

The proteins from complement terminal pathway, did not show any highly reactive combination with the bacterial protein, while the medium and the low reactive were respectively 3 (2,94%) and 28 (0,31%). The Complement lectin pathway and the

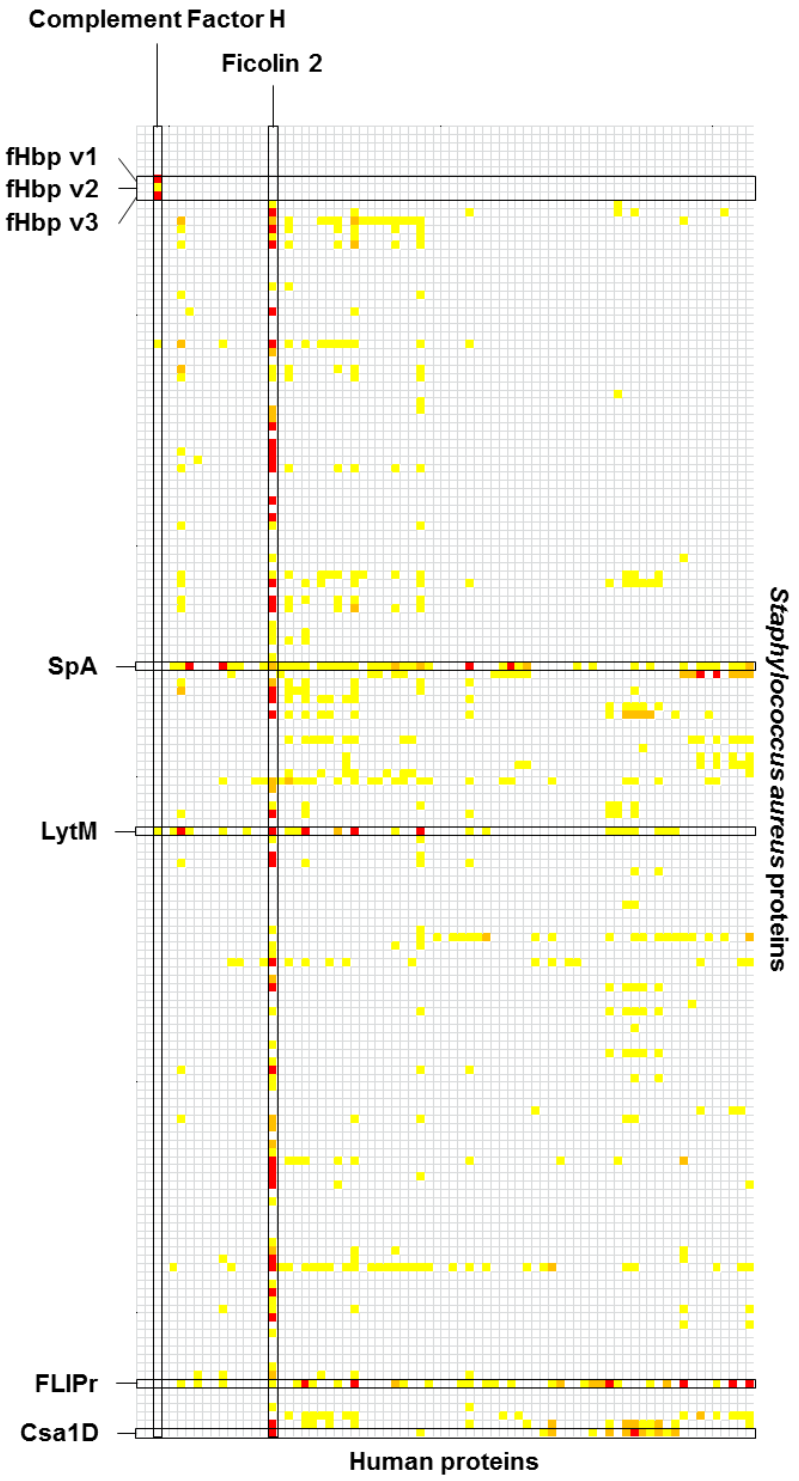


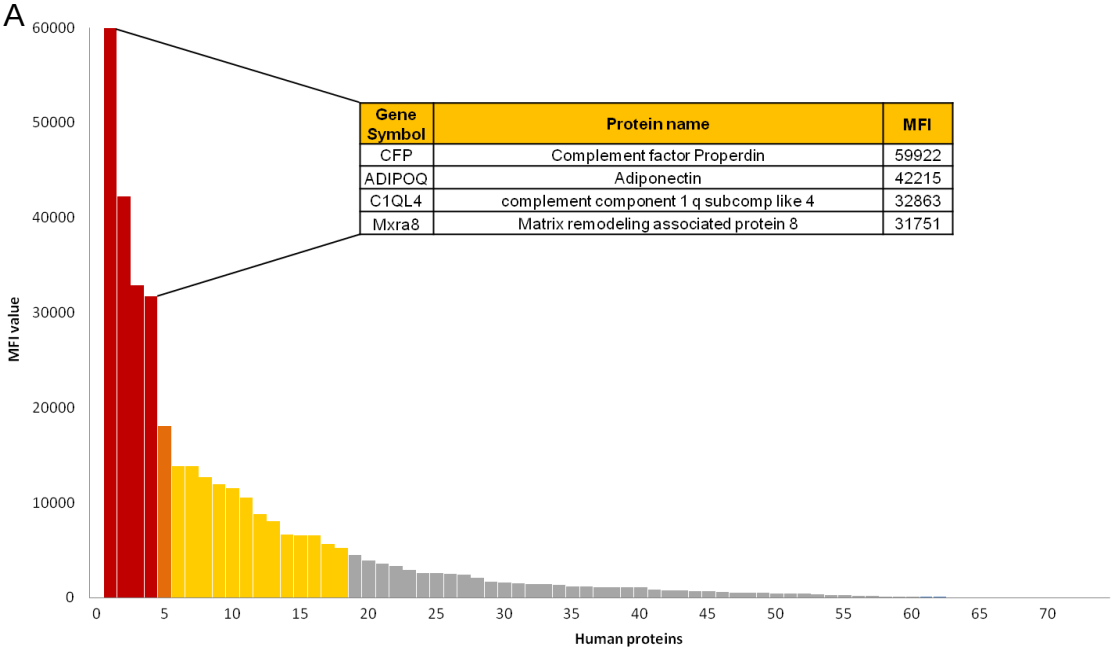
Figure 2.4 Schematic overview of the grid resulting from the screening. *S. aureus* proteins (lines) spotted on the chip were plotted against human proteins (columns) tested in overlay. Cells contain MFI value for each pair. The same colour code (grey - yellow - orange - red) is used for visual information and recognition of a putative interaction. Human complement factor H and ficolin 2 and the bacterial fHbp v 1, 2 and 3, spA, FLIPr and Csa1D are highlighted

complement-related receptors classes were constituted by only 2 proteins each with a total of 318 possible combinations. Regarding the proteins from lectin pathway, only 1 combination (0,31%) is highly reactive, while 5 (1,57%) were medium and 33 (10,38%) low. Considering the Complement related receptors class, none of the combination resulted highly reactive, while 3 (0,94%) were found to be medium and 11 (3,46%) low.

Within the 19 interactions showing high MFI signals two of the three spotted fHbp variants (variant 1 and 3), which constituted the positive control, were found when tested with human complement factor H present in the library.Among the tested human proteins, the Ficolin-2 showed high non-specific signals with several bacterial proteins (**figure 2.4**), and was thereby not considered in the analysis. In the same way, since the

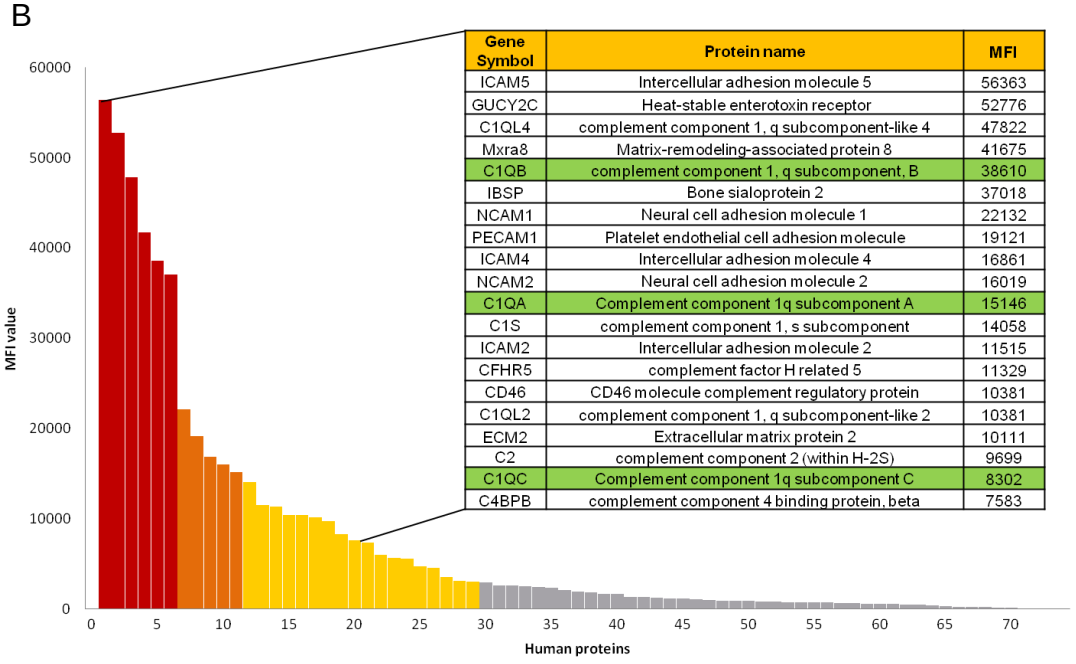
detection system relies on the use of antibodies and several human proteins were fused to an Fc-tag, the putative hits of the staphylococcal protein A (spA) were not considered for further validation knowing its ability to bind immunoglobulins.

A



Gene Symbol	Protein name	MFI
CFP	Complement factor Properdin	59922
ADIPOQ	Adiponectin	42215
C1QL4	complement component 1 q subcomp like 4	32863
Mxra8	Matrix remodeling associated protein 8	31751

B



Gene Symbol	Protein name	MFI
ICAM5	Intercellular adhesion molecule 5	56363
GUCY2C	Heat-stable enterotoxin receptor	52776
C1QL4	complement component 1, q subcomponent-like 4	47822
Mxra8	Matrix-remodeling-associated protein 8	41675
C1QB	complement component 1, q subcomponent, B	38610
IBSP	Bone sialoprotein 2	37018
NCAM1	Neural cell adhesion molecule 1	22132
PECAM1	Platelet endothelial cell adhesion molecule	19121
ICAM4	Intercellular adhesion molecule 4	16861
NCAM2	Neural cell adhesion molecule 2	16019
C1QA	Complement component 1q subcomponent A	15146
C1S	complement component 1, s subcomponent	14058
ICAM2	Intercellular adhesion molecule 2	11515
CFHR5	complement factor H related 5	11329
CD46	CD46 molecule complement regulatory protein	10381
C1QL2	complement component 1, q subcomponent-like 2	10381
ECM2	Extracellular matrix protein 2	10111
C2	complement component 2 (within H-2S)	9699
C1QC	Complement component 1q subcomponent C	8302
C4BPB	complement component 4 binding protein, beta	7583

Figure 2.5 MFI signal distribution of human proteins on spotted LytM and FLIPr proteins. Coloured columns identify the three MFI cut-off thresholds. Grey columns indicate proteins below the background threshold. **(A)** Plotting of MFIs of the 74 human proteins on the spotted LytM. Inset table shows MFI values, protein name and gene symbol of the 4 human proteins displaying fluorescence intensities above 30000 MFI. **(B)** Plotting of MFIs of the 74 human proteins on the spotted FLIPr. Inset table shows MFI values, protein name and gene symbol of the first 20 human proteins. C1qA, C1qB and C1qC subcomponents are highlighted in green.

The rest of the highly reactive hits were shared between the staphylococcal Glycyl-glycine endopeptidase LytM (4 hits), the FPRL1 (formyl peptide receptor-like 1) inhibitory protein

(FLIPr) (6 hits), and the conserved staphylococcal antigen 1D (Csa1D) (1 hit). LytM (**figure 2.5A**) showed high reactivity signals with the complement factor P (CFP - MFI 59922), the adiponectin protein (ADIPOQ - MFI 42215), the complement component 1q subcomponent like 4 (C1QL4 - MFI 32863) and the matrix remodelling associated protein 8 (MXRA8 - MFI 31751). FLIPr, as well, showed several interesting putative interactors (**figure 2.5B**): the intercellular adhesion molecule 5 (ICAM5 - MFI 56363), the heat stable enterotoxin receptor (GUCY2C - MFI 52776), the complement component 1q subcomponent like 4 (C1QL4 - 47822), the matrix remodelling associated protein 8 (MXRA8 - MFI 41675), the complement component 1q subcomponent B (C1QB - MFI 38610) and the bone sialoprotein 2 (IBSP - MFI 37018). Interestingly FLIPr showed weaker but still reliable signals also with the complement component 1q subcomponent A (C1QA - MFI 15146), the complement component 1q subcomponent like 2 (C1QL2 - MFI 10381) and the complement component 1q subcomponent C (C1QC - MFI 8302) among the others. Csa1D was instead highly reactive with ficolin 1 (FCN1 - MFI 34016). These and almost all the identified interactions are novel and would require further validation. Among them, the interaction between the staphylococcal protein FLIPr and the human C1q subcomponents seized our attention, since it involved key players of the offense/defense fighting taking place at the host-pathogen interface and it was never described before in literature.

FLIPr binds to human C1q subcomponents and C1q complex

To validate and characterize the binding between FLIPr and the C1q subcomponents, i*n vitro* binding experiments were performed using BioLayer Interferometry (BLI). FLIPr protein was immobilized onto the BLI biosensor using amine reactive chemistry and C1qA, C1qB and C1qC subunits, previously used in the screening, were tested in titration curve as analytes. The three subcomponents showed low nanomolar affinity for FLIPr (3.2nM, 2.1nM and 9.9nM respectively – **Figure 2.6A-B-C**). These data are in good agreement with MFI values obtained with protein array confirming a direct correlation between binding affinity and MFI values as also previously described [11, 18].

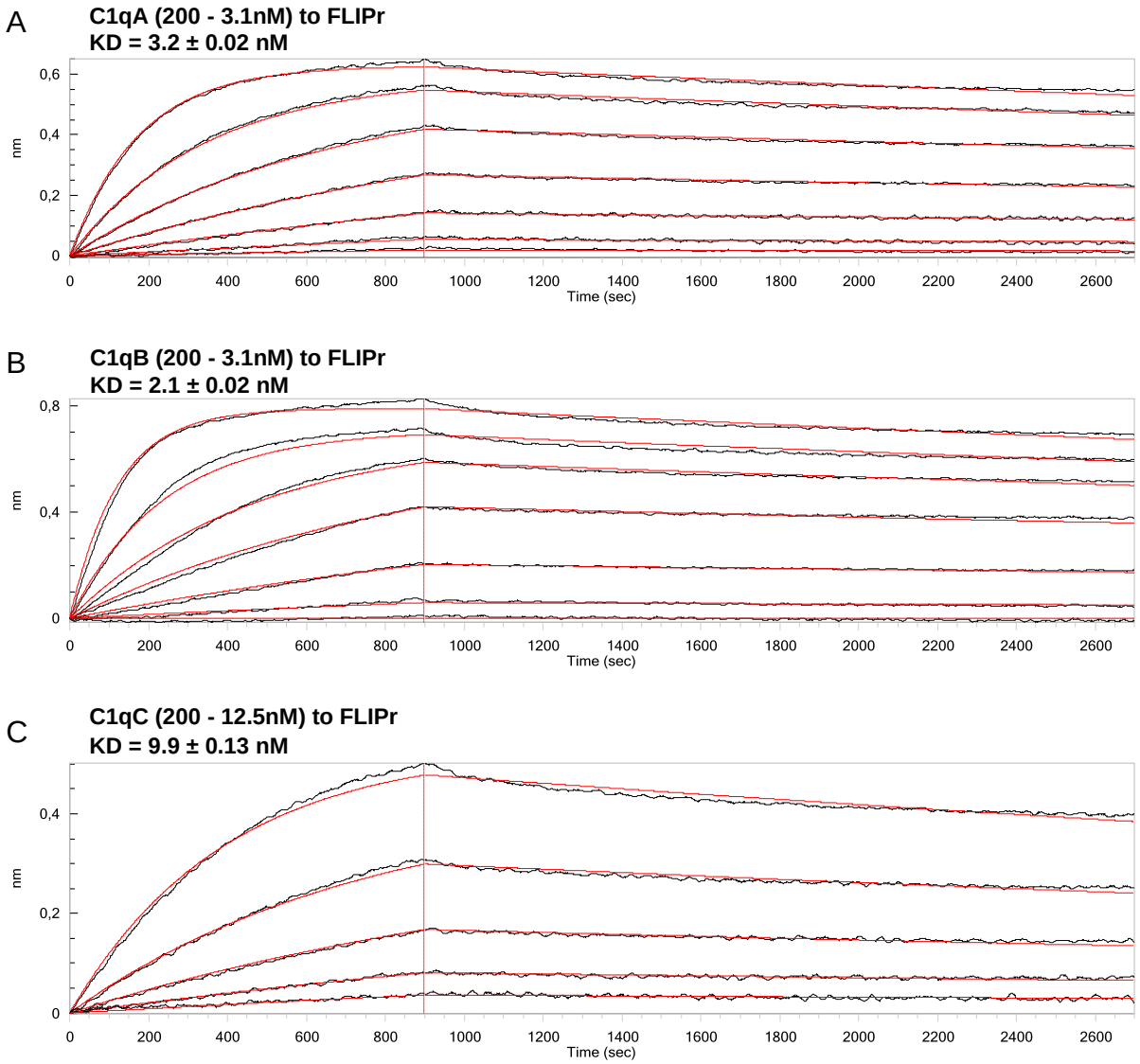


Figure 2.6 Binding of FLIPr to C1q subcomponents and C1q complex validated through BioLayer Interferometry. The amount of ligand associating with the analyte was measured in nanometres (nm). Blank subtracted sensograms of **(A)** C1qA (200-3.1nM), **(B)** C1qB (200-3.1nM) and **(C)** C1qC (200-12.5nM) subcomponents tested on covalently immobilized FLIPr. Association and dissociation curves were fitted in a 1:1 model.

In biological conditions, however, the three C1qA, C1qB and C1qC subunits are super-organized to constitute the C1q complex, the triggering point of the classical complement pathway. For this reason, binding experiments between FLIPr and the human C1q complex affinity purified from human serum were performed. When immobilized, FLIPr showed picomolar affinity for the C1q complex (62 pM – **Figure 2.7A**). Conversely, when the C1q complex was immobilized, the measured affinity was in the high nanomolar range (679 nM – **Figure 2.7B**).

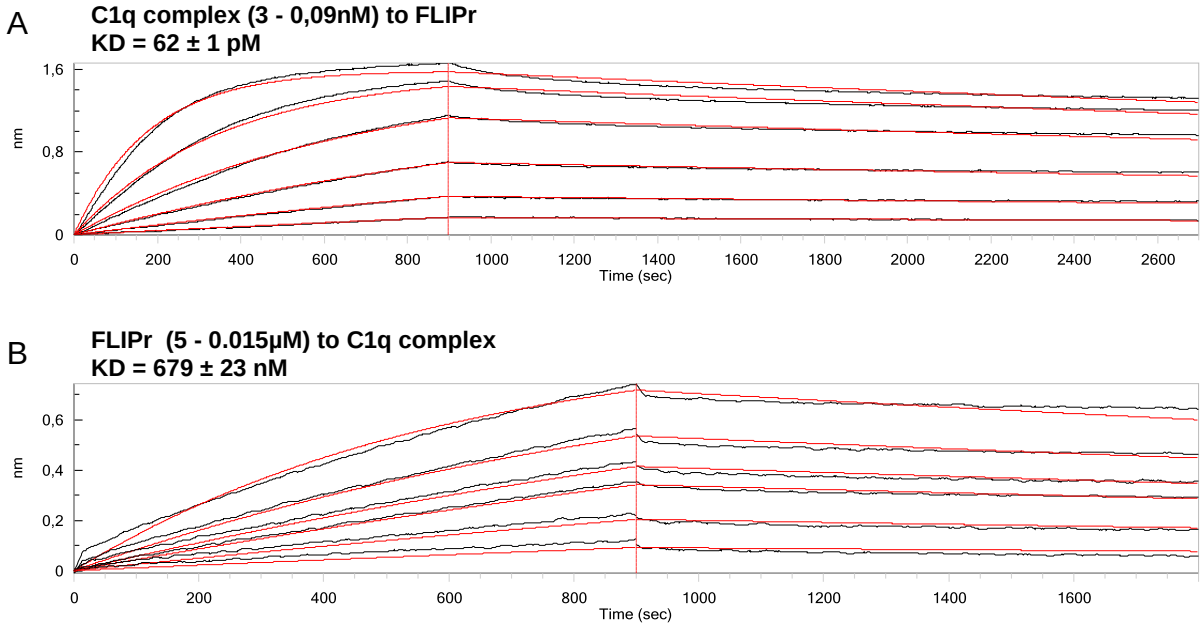


Figure 2. 7 Binding of FLIPr to C1q complex (A) Blank subtracted sensograms of FLIPr (5-0.15 µM) on immobilized C1q and **(B)** of C1q (3-0.09 nM) on immobilized FLIPr

A summary of the affinity constants and kinetic properties of the FLIPr-C1q complex is given in **table3**

Ligand	Analyte	Kon (1/Ms)	Koff (1/s)	KD (M)
FLIPr	C1qA	2,84E+04	9,11E-05	3,21E-09
FLIPr	C1qB	4,10E+04	8,84E-05	2,16E-09
FLIPr	C1qC	1,22E+04	1,21E-04	9,97E-09
FLIPr	C1q complex	1,84E+06	1,14E-04	6,23E-11
C1q	FLIPr	2,93E+02	1,99E-04	6,80E-07

FLIPr inhibits classical complement pathway in vitro

In order to clarify whether the binding of FLIPr to C1q could influence complement classical pathway activation, a WiELISA (Wieslab) complement system screening kit was used. The system is based on ELISA plates pre-coated with specific activators of each complement pathway. Briefly, human serum was first diluted in specific buffers containing inhibitors for the pathways not under investigation, and then was applied to the WiELISA plate resulting in complement activation, monitored through the use of specific antibodies raised against a neo-epitope of the Membrane Attack Complex (MAC). Positive control was constituted by serum and negative control by heat inactivated serum. For our purposes, the complement classical pathway was investigated and the experimental design implied a preincubation of different amount of FLIPr with human serum. When pre-incubated with serum, FLIPr at 1µM

significantly reduced to ~56% the complement classical pathway activation, while when at 5µM the activation was reduced to ~26% (**Figure 2.8A**).

FLIPr increases S. aureus survival in whole blood assay

Since FLIPr was demonstrated to reduce the classical complement pathway activation, to improve the understanding in FLIPr contribution to bacterial pathogenesis, *S. aureus* survival in whole human blood (WHB) was monitored through CFU count in presence and absence of FLIPr. WHB pre-incubation with FLIPr significantly increased *S. aureus* survival (**Figure 2.8B**). When FLIPr was incubated at 1.4 µM an increase of 50% in the survival rate in comparison with control was observed, while when used at 2.8 µM, a 100% *S. aureus* survival was observed, in good agreement with Stemerding et al. 2013 [19].

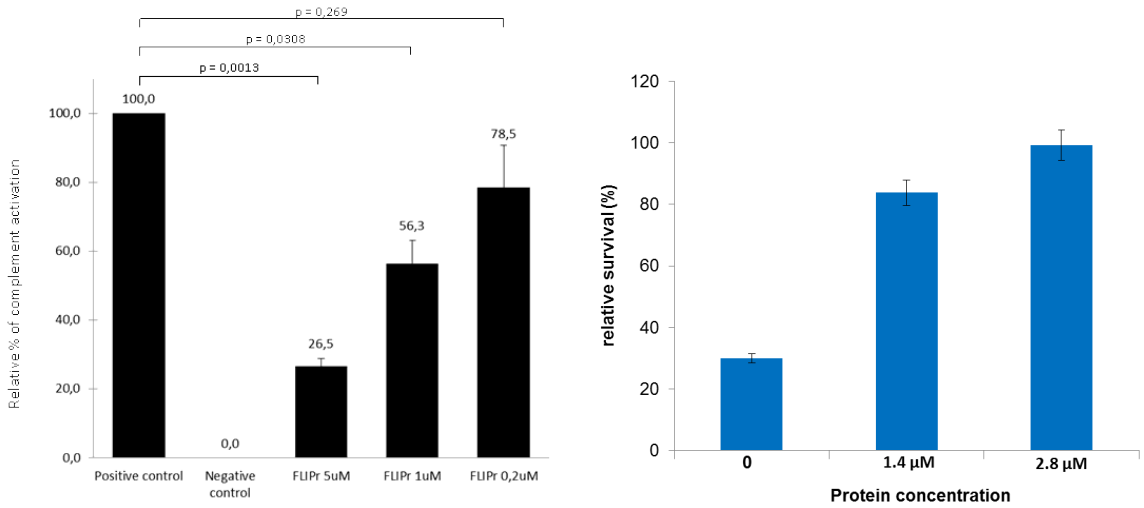


Figure 2. 8 Functional characterization of the FLIPr C1q interaction. **(A)** Complement classical pathway inhibition by FLIPr in WiELISA assay. Relative % of complement activation is shown for each sample. Results are mean of three replicates. P values of 0.05 were considered significant. **(B)** FLIPr-mediated dose dependent increase of *S. aureus* survival in whole blood assay. Relative survival was monitored through CFU count.

Discussion

Staphylococcus aureus like many other opportunistic pathogens secretes and exposes on the surface a wide variety of proteins that specifically target the host immune system leading to bacterial adhesion, invasion and immune system evasion [20]. Several *S. aureus* molecules are known to interact with human immune system effectors. Plenty of examples are available: several proteins like the SCIN family were shown to act directly on complement cascade, directly blocking the innate immune response [8]; others like Cna (Collagen adhesin) and most of the MSCRAMM protein were shown to promote bacterial adhesion through collagen binding [21]; others, instead, acts to block phagocytosis and bacterial opsonisation by direct binding of receptors on phagocytes [22]. Even if a large number of human targets for staphylococcal proteins are already known and described, a lot of information on the surface interactome between *S. aureus* and human are still missing. Several studies utilized protein microarray as a key technique for the identification of novel host pathogen interaction at the protein level [11, 18]. Nevertheless, the limitation caused by the limited number of the human proteins available for the screening, led only to partial findings and results.

In the protein array screening here presented, 118 unique *S. aureus* proteins, selected via a bioformatic approach, were efficiently cloned, expressed, purified and spotted on microarray slides. The chips were used to carry on, for the first time, an unbiased systematic large-scale screening using 75 human recombinant proteins obtained from a larger library [14]. The screening resulted in a total of 11766 combinations between pairs of human and *S. aureus* proteins. Among these, as expected, only a small proportion (<5%) gave a positive signal for interactions and, in particular, 457 (4%) putative interactions have been detected with a low signal, 42 (0,36%) with a medium and 19 (0,16%) with a highly reactive one, on the basis of their normalized MFI values. Within the 19 interactions showing high MFI signals two fHbp (fHbp variant 1 and variant 3, the positive controls) were present when incubated with complement factor H. This proves both the appropriate incubation conditions developed for the screening, the correct thresholds set for the analysis, and the integrity of the bacterial and human proteins used. With the use of such a large scale approach it was also possible to recognize and exclude from the analysis and validation those human proteins highly reactive with the great majority of bacterial proteins and *vice versa*. The human ficolin 2 and staphylococcal protein A, for example, were non-specifically reactive with several proteins and could be easily recognized and discarded from analysis and further validation. These two examples also constitutes a good proof of specificity for the other interaction found. Interestingly, the 17 hits (19 excluding the two fHbp) found in the array were shared between LytM, a staphylococcal autolysin previously shown to be an important regulator of *S. aureus*

cell cycle and cell wall turnover [23], Csa1D, member of the Conserved Staphylococcal Antigen family described as a promising vaccine antigen in Schluepen et al 2013 [24], and FLIPr, previously described as potent inhibitor of formyl-peptide receptors and FcγR antagonist [19, 25]. This last protein was found to interact, potentially strongly based on the detected signals, with six different human proteins: the intercellular adhesion molecule 5 (ICAM5), the heat stable enterotoxin receptor (GUCY2C), the complement component 1q subcomponent like 4 (C1QL4), the matrix remodelling associated protein 8 (MXRA8), the complement component 1q subcomponent B (C1QB) and the bone sialoprotein 2 (IBSP). All the potential interactions found might be interesting for their relevance in the different steps of pathogenesis, but further work is currently needed to prove the functional and biological relevance of these interactions.

On the other hand, a closer analysis of the human proteins reactive with FLIPr, to a lesser, but still significant extent, revealed additional potential interactors among which the complement component 1q subcomponent A (C1QA), the complement component 1q subcomponent like 2 (C1QL2) and the complement component 1q subcomponent C (C1QC - MFI 8302) caught our attention. The interaction between FLIPr and the components of the C1q complex was indeed never described before.

Through the use of BioLayer Interferometry (BLI) the binding between FLIPr and the recombinant C1q A, C1qB and C1qC components was validated. FLIPr was immobilized on the surface and the C1q components were tested as analyte. Using a 1:1 binding model, a nanomolar affinity was calculated for the C1qA and C1qC proteins while a high picomolar affinity was measured for the C1qb component. Binding experiments were also performed with the C1q complex purified from human serum. In this case, when FLIPr was immobilized and the C1q used as analyte, the calculated affinity was in low picomolar range. However when the system was inverted and the C1q was immobilized, the calculated affinity was in the high nanomolar range. The reason of this discrepancy is still unclear and was not deeply investigated, but in our hypothesis, since the C1q complex possess possibly multiple binding sites for FLIPr, it is probably caused by an avidity effect of the C1q complex when FLIPr is immobilized, as also previously shown for other bacterial C1q-binding protein [26].

Together with the biophysical characterization of the interaction between FLIPr and the C1q complement component, functional studies were carried over as well to clarify the biological consequence of this protein complex. To this purpose WiELISA experiments and whole blood assays have been performed and confirmed the importance of FLIPr in bacterial pathogenesis. FLIPr was able to significantly reduce the complement classical pathway activation *in vitro* in WiELISA experiment. Despite this result, it is not yet clear if FLIPr directly prevent complement activation or whether it activates complement, leading to consumption of the complement proteins and thus resulting in a lower complement activation

in the assay, as also previously described for others bacterial proteins [27]. Moreover the whole blood assay, in which the addition of FLIPr resulted in dramatically higher *S. aureus in vitro* survival, confirmed the FLIPr importance and contribution in immune evasion.

Since now FLIPr has always been described as potent inhibitor of the formyl-peptide and Fcγ receptors exposed on neutrophil surface. These data, which describes for the first time FLIPr as a C1q-binder and complement inhibitor protein, might explain why Stemerding et al. [19] did not see an inhibition of phagocitosis at high serum concentration. In their experiments fluorescently labelled *S. aureus* where opsonized with IgG, complement-inactivated human serum, or untreated human serum with intact complement activity and phagocytosis tests using human neutrophyls were performed. Staphylococci opsonized with IgG and complement-inactivated serum were fully protected from phagocytosis, but in the presence of complement, the inhibitory effects of FLIPr on phagocytosis was only observed at lower serum concentrations. A summary of the results obtained is shown in **table 4.**

Work	Assay	Components	FcγR	C1q	Phagocytosis	Survival	Discussion
Stemerding 2013	Phagocytosis	IgG	yes	absent	inhibited	nd	FLIPr inhibits phagocytosys through FcγR
	Phagocytosis	complement inactivated serum	yes	present but inactive	inhibited	nd	FLIPr inhibits phagocytosys through FcγR
	Phagocytosis	active serum	**yes**	**present**	**inhibited only at low serum concentration**	**nd**	**FLIPr preferentially binds C1q instead of Fcg leading phagocytosis to occur**
this work	whole blood assay	blood	**yes**	**present**	**nd**	**yes**	

As the authors correctly argue, this effect might be explained with a complement receptor-dependent phagocytosis. Combining the data present in literature and the functional data obtained in this work an "affinity driven model" was presented (**figure 2.9**). In this model when *S. aureus* faces low serum environment (e.g. interstitial space), FLIPr preferentially binds FcγR to inhibit neutrophil mediated phagocytosis. When *S. aureus* is instead exposed to high serum environment (e.g. blood) preferentially binds C1q. This can lead to both complement inhibition, resulting in FcγR mediated phagocytosis, or to complement activation, resulting as well in complement mediated phagocytosis. In this model *S. aureus* might selectively promote phagocytosis when it deals with unfavorable environment conditions

(complement pressure), preferring an intracellular lifestyle in neutrophils instead of bloodstream. This model can also represent a good proof of concept for the Trojan horse theorized by Thwaites et al. [28] for which neutrophils represent a privileged site for *S. aureus* in the bloodstream, offers protection from most antibiotics and provides a mechanism by which the bacterium can travel to and infect distant sites.

In summary it was demonstrated that protein microarray constitutes a powerful large scale tool for the screening and discovery of novel host-pathogen interactions. This approach lead to the identification of several novel protein-protein interactions between the human and the *S. aureus* extracellular proteomes and represents a proof-of-concept for future large scale screening on different pathogens.

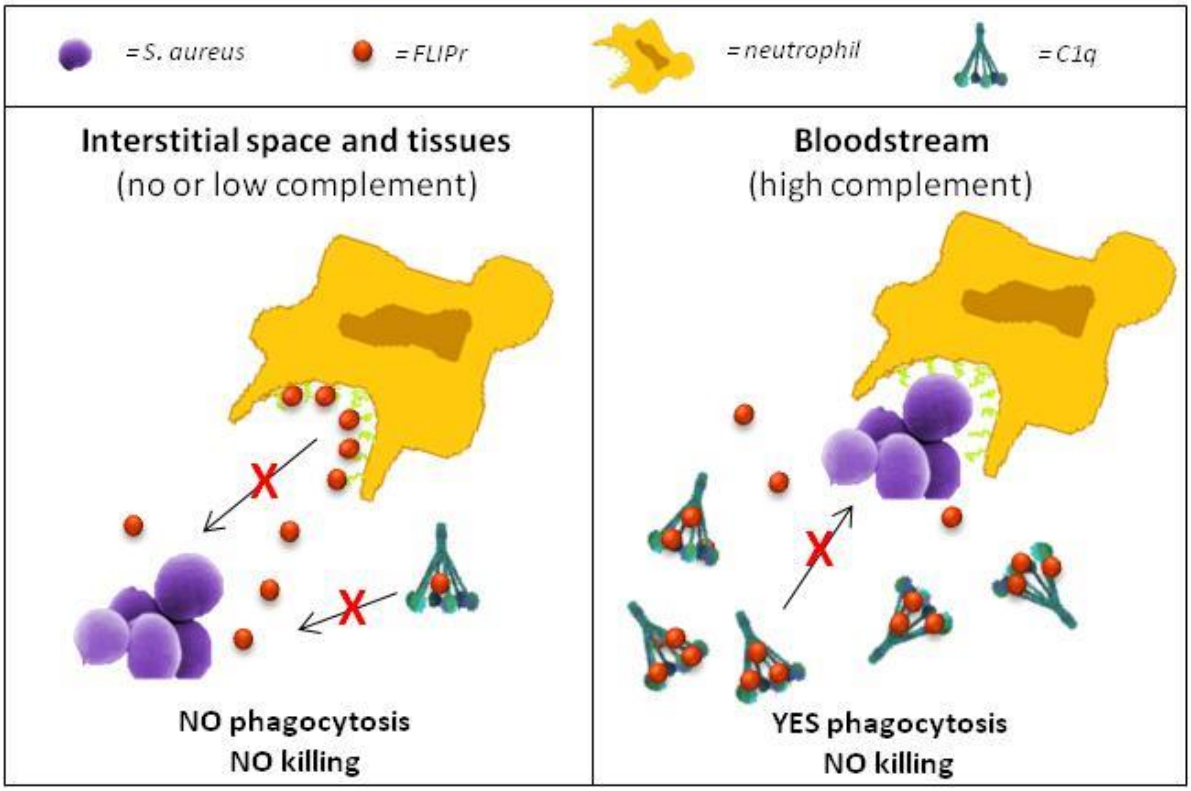


Figure 2.9 "Affinity driven" model. Schematic representation of the model for FLIPr selectivity for FcγR or C1q. Left panel shows a condition resembling interstitial space in which few C1q molecules are present. Some FLIPr molecules saturates the few C1q molecules and the rest can bind FcγR. This results in inhibition of both phagocytosis and killing. Right panel shows a situation similar to bloodstream, in which a lot of C1q molecules are present. FLIPr preferentially binds C1q molecules leaving FcγR free to operate. This results in phagocytosis as previously shown by Stemerding et al (2013), but does not result in killing (this work - whole blood assay). Staphylococci can indeed survive within neutrophils both preventing complement clearance and antibiotic treatment, and leading to bacterial spread in the body.

References

1. Peacock, S.J., I. de Silva, and F.D. Lowy, *What determines nasal carriage of Staphylococcus aureus?* Trends Microbiol, 2001. **9**(12): p. 605-10.
2. Lowy, F.D., *Staphylococcus aureus infections.* N Engl J Med, 1998. **339**(8): p. 520-32.
3. Neu, H.C., *The crisis in antibiotic resistance.* Science, 1992. **257**(5073): p. 1064-73.
4. Bardoel, B.W. and J.A. Strijp, *Molecular battle between host and bacterium: recognition in innate immunity.* J Mol Recognit, 2011. **24**(6): p. 1077-86.
5. Hirschhausen, N., et al., *A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor.* Cell Microbiol, 2010. **12**(12): p. 1746-64.
6. Serruto, D., et al., *Molecular mechanisms of complement evasion: learning from staphylococci and meningococci.* Nat Rev Microbiol, 2010. **8**(6): p. 393-9.
7. Burman, J.D., et al., *Interaction of human complement with Sbi, a staphylococcal immunoglobulin-binding protein: indications of a novel mechanism of complement evasion by Staphylococcus aureus.* J Biol Chem, 2008. **283**(25): p. 17579-93.
8. Rooijakkers, S.H., et al., *Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor.* Nat Immunol, 2009. **10**(7): p. 721-7.
9. Langley, R., et al., *The staphylococcal superantigen-like protein 7 binds IgA and complement C5 and inhibits IgA-Fc alpha RI binding and serum killing of bacteria.* J Immunol, 2005. **174**(5): p. 2926-33.
10. Korf, U. and S. Wiemann, *Protein microarrays as a discovery tool for studying protein-protein interactions.* Expert Rev Proteomics, 2005. **2**(1): p. 13-26.
11. Margarit, I., et al., *Capturing host-pathogen interactions by protein microarrays: identification of novel streptococcal proteins binding to human fibronectin, fibrinogen, and C4BP.* FASEB J, 2009. **23**(9): p. 3100-12.
12. Palumbo, E., et al., *Antigen identification starting from the genome: a "Reverse Vaccinology" approach applied to MenB.* Methods Mol Biol, 2012. **799**: p. 361-403.
13. Klock, H.E., et al., *Combining the polymerase incomplete primer extension method for cloning and mutagenesis with microscreening to accelerate structural genomics efforts.* Proteins, 2008. **71**(2): p. 982-94.
14. Gonzalez, R., et al., *Screening the mammalian extracellular proteome for regulators of embryonic human stem cell pluripotency.* Proc Natl Acad Sci U S A, 2010. **107**(8): p. 3552-7.
15. Altindis, E., et al., *Protectome analysis: a new selective bioinformatics tool for bacterial vaccine candidate discovery.* Mol Cell Proteomics, 2014.
16. Tavares, A., et al., *Insights into alpha-hemolysin (Hla) evolution and expression among Staphylococcus aureus clones with hospital and community origin.* PLoS One, 2014. **9**(7): p. e98634.
17. Kwiecinski, J., T. Jin, and E. Josefsson, *Surface proteins of Staphylococcus aureus play an important role in experimental skin infection.* APMIS, 2014. **122**(12): p. 1240-50.
18. Galeotti, C.L., et al., *Surface interactome in Streptococcus pyogenes.* Mol Cell Proteomics, 2012. **11**(4): p. M111 015206.
19. Stemerding, A.M., et al., *Staphylococcus aureus formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcgammaR antagonists that inhibit IgG-mediated effector functions.* J Immunol, 2013. **191**(1): p. 353-62.
20. Foster, T.J., et al., *Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus.* Nat Rev Microbiol, 2014. **12**(1): p. 49-62.
21. Zong, Y., et al., *A 'Collagen Hug' model for Staphylococcus aureus CNA binding to collagen.* EMBO J, 2005. **24**(24): p. 4224-36.

22. Prat, C., et al., *A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from Staphylococcus aureus (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR.* J Immunol, 2009. **183**(10): p. 6569-78.
23. Chu, X., et al., *Role of Rot in bacterial autolysis regulation of Staphylococcus aureus NCTC8325.* Res Microbiol, 2013. **164**(7): p. 695-700.
24. Schluepen, C., et al., *Mining the bacterial unknown proteome: identification and characterization of a novel family of highly conserved protective antigens in Staphylococcus aureus.* Biochem J, 2013. **455**(3): p. 273-84.
25. Prat, C., et al., *A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1.* J Immunol, 2006. **177**(11): p. 8017-26.
26. Kang, M., et al., *Collagen-binding microbial surface components recognizing adhesive matrix molecule (MSCRAMM) of Gram-positive bacteria inhibit complement activation via the classical pathway.* J Biol Chem, 2013. **288**(28): p. 20520-31.
27. Agarwal, V., et al., *Binding of Streptococcus pneumoniae endopeptidase O (PepO) to complement component C1q modulates the complement attack and promotes host cell adherence.* J Biol Chem, 2014. **289**(22): p. 15833-44.
28. Thwaites, G.E. and V. Gant, *Are bloodstream leukocytes Trojan Horses for the metastasis of Staphylococcus aureus?* Nat Rev Microbiol, 2011. **9**(3): p. 215-22.

Supplementary table 1

Common name, locus tag, gene product and lenght and localization of the *S. aureus* recombinant proteins printed on the microarray slides.

Common name	Locus tag	gene product	gene length	localization
	gna1870	factor h binding protein	819	Outer membrane
	gna1870	factor h binding protein	819	Outer membrane
	gna1870	factor h binding protein	819	Outer membrane
	SAOUHSC_00051	1-phosphatidylinositol phosphodiesterase precursor, putative	328	Extracellular
	SAOUHSC_00052	putative lipoprotein	256	Unknown
	SAOUHSC_00052	putative lipoprotein	256	Unknown
	SAOUHSC_00053	putative lipoprotein	256	Unknown
	SAOUHSC_00054	staphylococcal tandem lipoprotein	256	Unknown
	SAOUHSC_00055	staphylococcal tandem lipoprotein	255	Unknown
spa	SAOUHSC_00069	protein A spA	516	Cell wall
sasD	SAOUHSC_00094	SasD protein SAOUHSC_00094	199	Cell wall
	SAOUHSC_00106	hypothetical protein , leader	514	Unknown
	SAOUHSC_00107	5' nucleotidase family protein	511	Extracellular
isdI	SAOUHSC_00130	heme-degrading monooxygenase IsdI	108	Unknown
	SAOUHSC_00170	extracellular solute-binding protein, RGD containing lipoprotein	591	Unknown
	SAOUHSC_00171	gamma-glutamyltranspeptidase, putative	668	Extracellular
	SAOUHSC_00172	hypothetical protein SAOUHSC_00172	257	Unknown
	SAOUHSC_00174	M23/M37 peptidase domain protein	192	Extracellular
	SAOUHSC_00176	bacterial extracellular solute-binding protein, putative	423	Unknown
	SAOUHSC_00186	lipoprotein, putative	322	Unknown
coA	SAOUHSC_00192	coagulase Coa	636	Extracellular
	SAOUHSC_00201	putative extracellular solute-binding protein	470	Cell wall
	SAOUHSC_00248	peptidoglycan hydrolase, putative	316	Extracellular
	SAOUHSC_00256	secretory antigen SsaA-like protein	297	Unknown
esxA	SAOUHSC_00257	hypothetical protein SAOUHSC_00257	97	Unknown

esxB	SAOUHSC_00265	hypothetical protein SAOUHSC_00265	104	Cytoplasmic
	SAOUHSC_00279	putative lipoprotein	124	Cytoplasmic
	SAOUHSC_00300	lipase precursor	690	Extracellular
	SAOUHSC_00354	staphylococcal enterotoxix, putative	203	Unknown
	SAOUHSC_00356	putative lipoprotein	190	Unknown
	SAOUHSC_00362	putative lipoprotein	208	Unknown
	SAOUHSC_00365	alkyl hydroperoxide reductase	189	Cytoplasmic
	SAOUHSC_00383	superantigen-like protein	226	Unknown
	SAOUHSC_00384	superantigen-like protein	231	Unknown
	SAOUHSC_00386	superantigen-like protein	356	Extracellular
	SAOUHSC_00389	superantigen-like protein	308	Extracellular
	SAOUHSC_00390	superantigen-like protein 5	234	Extracellular
	SAOUHSC_00391	superantigen-like protein	231	Extracellular
	SAOUHSC_00392	superantigen-like protein 7	231	Extracellular
	SAOUHSC_00393	superantigen-like protein	232	Extracellular
	SAOUHSC_00394	superantigen-like protein	232	Extracellular
	SAOUHSC_00395	superantigen-like protein	227	Extracellular
	SAOUHSC_00399	superantigen-like protein	225	Extracellular
	SAOUHSC_00400	putative surface protein	502	Unknown
	SAOUHSC_00404	putative lipoprotein	261	Unknown
	SAOUHSC_00427	autolysin precursor, putative	334	Extracellular
sdrC	SAOUHSC_00544	sdrC protein, putative	995	Cell wall
sdrD	SAOUHSC_00545	sdrD protein, putative	1349	Cell wall
	SAOUHSC_00634	ABC transporter, substrate-binding protein, putative \lipoprotein	312	Unknown
	SAOUHSC_00661	probable lipase	347	Unknown
	SAOUHSC_00671	secretory antigen SsaA-like protein	265	Extracellular
	SAOUHSC_00685	putative lipoprotein	131	Unknown
	SAOUHSC_00717	lipoprotein	146	Unknown
	SAOUHSC_00728	Predicted membrane-associated, metal-dependent hydrolase	646	Cytoplasmic/Membrane

	SAOUHSC_00749	Siderophore binding protein FatB	342	Unknown
	SAOUHSC_00754	ferrichrome binding protein	292	Unknown
	SAOUHSC_00773	immunogenic secreted precursor-like protein (truncated)	279	Unknown
	SAOUHSC_00808	putative lipoprotein	242	Unknown
	SAOUHSC_00814	truncated secreted von Willebrand factor-binding protein (coagulase) VWbp, putative	450	Extracellular
Emp	SAOUHSC_00816	extracellular matrix and plasma binding protein, putative	340	Unknown
	SAOUHSC_00817	hypothetical protein SAOUHSC_00817 \ von willebrand truncated	156	Unknown
	SAOUHSC_00860	5-nucleotidase family protein	439	Extracellular
	SAOUHSC_00872	extramembranal protein	391	Cytoplasmic
	SAOUHSC_00987	cysteine protease precursor, putative	393	Extracellular
	SAOUHSC_00988	glutamyl endopeptidase precursor, putative	336	Extracellular
	SAOUHSC_00998	fmt protein, putative	397	Extracellular
	SAOUHSC_01005	chitinase	105	Unknown
	SAOUHSC_01039	putative lipoprotein	208	Unknown
isdB	SAOUHSC_01079	neurofilament protein isdB	645	Cell wall
isdA	SAOUHSC_01081	IsdA protein	350	Cell wall
IsdC	SAOUHSC_01082	hypothetical protein SAOUHSC_01082 isdC	227	Cellwall
	SAOUHSC_01084	hypothetical iron-regulated protein, leader isdD	358	Unknown
	SAOUHSC_01085	iron ABC transporter, iron -binding protein IsdE	282	Cytoplasmic
	SAOUHSC_01088	NPQTN specific sortase B	244	Unknown
isdG	SAOUHSC_01089	heme-degrading monooxygenase IsdG	107	Cytoplasmic
	SAOUHSC_01110	fibrinogen-binding protein-related	109	Unknown
FLIPr	SAOUHSC_01112	formyl peptide receptor-like 1 inhibitory protein	133	Unknown
efb	SAOUHSC_01114	fibrinogen-binding protein	165	Extracellular
	SAOUHSC_01115	scin paralogue	116	Unknown
hla	SAOUHSC_01121	alpha-hemolysin precursor	319	Extracellular
	SAOUHSC_01124	superantigen-like protein	238	Extracellular
	SAOUHSC_01125	superantigen-like protein	241	Extracellular
	SAOUHSC_01150	cell division protein FtsZ	390	Cytoplasmic

	SAOUHSC_01180	putative lipoprotein	317	Unknown
	SAOUHSC_01256	insulysin, peptidase family M16	428	Unknown
nuc	SAOUHSC_01316	thermonuclease precursor	177	Extracellular
	SAOUHSC_01317	hypothetical protein leader?	284	Unknown
ebpS	SAOUHSC_01501	elastin binding protein EbpS	486	Cell wall
	SAOUHSC_01508	putative lipoprotein	304	Unknown
	SAOUHSC_01627	putative lipoprotein	193	Unknown
	SAOUHSC_01920	putative lipoprotein	208	Unknown
	SAOUHSC_01941	serine protease SplB	240	Extracellular
	SAOUHSC_01949	intracellular serine protease, putative	457	Extracellular
	SAOUHSC_01972	protein export protein PrsA, putative	320	Unknown
	SAOUHSC_02127	staphopain thiol proteinase	388	Extracellular
	SAOUHSC_02147	hypothetical protein, putative leader	280	Unknown
eap	SAOUHSC_02161	MHC class II analog protein	584	Unknown
	SAOUHSC_02167	hypothetical protein SAOUHSC_02167	116	Unknown
	SAOUHSC_02169	chemotaxis-inhibiting protein CHIPS	149	Unknown
	SAOUHSC_02240	truncated beta-hemolysin	274	Extracellular
lukF	SAOUHSC_02241	Leukocidin/Hemolysin toxin family LukF	338	Extracellular
	SAOUHSC_02246	ferric hydroxamate receptor 1	303	Unknown
	SAOUHSC_02257	srdH family protein	419	Cell wall
	SAOUHSC_02333	Probable transglycosylase isaA precursor	231	Extracellular
	SAOUHSC_02463	hyaluronate lyase	807	Extracellular
	SAOUHSC_02554	ferrichrome-binding protein TroA-like -FhuD2?	302	Unknown
	SAOUHSC_02576	secretory antigen precursor SsaA, putative	166	Unknown
	SAOUHSC_02706	immunoglobulin G-binding protein Sbi, putative	436	Unknown
	SAOUHSC_02708	gamma-hemolysin h-gamma-ii subunit, putative	309	Extracellular
	SAOUHSC_02767	peptide ABC transporter, peptide-binding protein, putative	532	Cell wall
	SAOUHSC_02783	hypothetical protein, putative leader	264	Unknown
FnBA	SAOUHSC_02803	fibronectin-binding protein A precursor FnBPA	990	Cell wall

	SAOUHSC_02887	immunodominant antigen A, putative	233	Extracellular
clfB	SAOUHSC_02963	clumping factor B, putative	877	Cell wall
	SAOUHSC_02979	N-acetylmuramoyl-L-alanine amidase	619	Extracellular
sasF	SAOUHSC_02982	sasF protein	635	Cell wall
	SAOUHSC_03006	lipase	680	Extracellular
NW_1				Unknown
NW_2				Unknown
NW_sdrE	NWMN_0525	Serine-aspartate repeat-containing protein E	3498	Unknown

Supplementary table 2

Name, function and UniProt description of the 75 human recombinant proteins used in the microarray screening. The proteins are divided by colours for function, based on the biological process they are involved in. Colours are the same reported in figure 1A. N.D indicates that no uniprot description was available.

Function	Name	Gene name	Description
adhesion / cell receptors / various	Adiponectin	ADIPOQ	Important adipokine involved in the control of fat metabolism and insulin sensitivity, with direct anti-diabetic, anti-atherogenic and anti-inflammatory activities. Stimulates AMPK phosphorylation and activation in the liver and the skeletal muscle, enhancing glucose utilization and fatty-acid combustion. Antagonizes TNF-alpha by negatively regulating its expression in various tissues such as liver and macrophages, and also by counteracting its effects. Inhibits endothelial NF-kappa-B signaling through a cAMP-dependent pathway. May play a role in cell growth, angiogenesis and tissue remodeling by binding and sequestering various growth factors with distinct binding affinities, depending on the type of complex, LMW, MMW or HMW
	Anthrax toxin receptor 1	Antxr1	Plays a role in cell attachment and migration. Interacts with extracellular matrix proteins and with the actin cytoskeleton. Mediates adhesion of cells to type 1 collagen and gelatin, reorganization of the actin cytoskeleton and promotes cell spreading. Plays a role in the angiogenic response of cultured umbilical vein endothelial cells.
	Anthrax toxin receptor 2	Antxr2	n.d.
	Basal cell adhesion molecule	BCAM	Laminin alpha-5 receptor. May mediate intracellular signaling
	Bone sialoprotein 2	IBSP	Binds tightly to hydroxyapatite. Appears to form an integral part of the mineralized matrix. Probably important to cell-matrix interaction. Promotes Arg-Gly-Asp-dependent cell attachment.
	Carcinoembryonic antigen related cell adhesion molecule 1	CEACAM1	n.d.
	Cartilage oligomeric matrix protein	COMP	May play a role in the structural integrity of cartilage via its interaction with other extracellular matrix proteins such as the collagens and fibronectin. Can mediate the interaction of chondrocytes with the cartilage extracellular matrix through interaction with cell surface integrin receptors. Could play a role in the pathogenesis of osteoarthritis. Potent suppressor of apoptosis in both primary chondrocytes and transformed cells. Suppresses apoptosis by blocking the activation of caspase-3 and by inducing the IAP family of survival proteins (BIRC3, BIRC2, BIRC5 and XIAP). Essential for maintaining a vascular smooth muscle cells (VSMCs) contractile/differentiated phenotype under physiological and pathological stimuli. Maintains this phenotype of VSMCs by interacting with ITGA7 (By similarity).
	Cell adhesion molecule 1	Cadm1	n.d.
	Cell adhesion molecule 3	CADM3	Involved in the cell-cell adhesion. Has both calcium-independent homophilic cell-cell adhesion activity and calcium-independent heterophilic cell-cell adhesion activity with IGSF4, PVRL1 and PVRL3. Interaction with EPB41L1 may regulate structure or function of cell-cell junctions (By similarity).
	Cell surface glycoprotein MUC18	MCAM	Plays a role in cell adhesion, and in cohesion of the endothelial monolayer at intercellular junctions in vascular tissue. Its expression may allow melanoma cells to interact with cellular elements of the vascular system, thereby enhancing hematogeneous tumor spread. Could be an adhesion molecule active in neural crest cells during embryonic development. Acts as surface receptor that triggers tyrosine phosphorylation of FYN and PTK2/FAK1, and a transient increase in the intracellular calcium concentration.
	Endothelial cell selective adhesion molecule	ESAM	Can mediate aggregation most likely through a homophilic molecular interaction

	Epithelial cell adhesion molecule	EPCAM	May act as a physical homophilic interaction molecule between intestinal epithelial cells (IECs) and intraepithelial lymphocytes (IELs) at the mucosal epithelium for providing immunological barrier as a first line of defense against mucosal infection. Plays a role in embryonic stem cells proliferation and differentiation. Up-regulates the expression of FABP5, MYC and cyclins A and E
	Epithelial cell adhesion molecule precursor	EPCAMP	n.d.
	Extracellular matrix protein 1	ECM1	Involved in endochondral bone formation as negative regulator of bone mineralization. Stimulates the proliferation of endothelial cells and promotes angiogenesis. Inhibits MMP9 proteolytic activity
	Extracellular matrix protein 2	ECM2	Promotes matrix assembly and cell adhesiveness
	Fibroleukin	FGL2	May play a role in physiologic lymphocyte functions at mucosal sites
	Glucagon preproprotein		n.d.
	Heat stable enterotoxin receptor	GUCY2C	Receptor for the E.coli heat-stable enterotoxin (E.coli enterotoxin markedly stimulates the accumulation of cGMP in mammalian cells expressing GC-C). Also activated by the endogenous peptides guanylin and uroguanylin.
	Integrin alpha L	ITGAL	Integrin alpha-L/beta-2 is a receptor for ICAM1, ICAM2, ICAM3 and ICAM4. It is involved in a variety of immune phenomena including leukocyte-endothelial cell interaction, cytotoxic T-cell mediated killing, and antibody dependent killing by granulocytes and monocytes.
	Integrin alpha M	ITGAM	Integrin alpha-M/beta-2 is implicated in various adhesive interactions of monocytes, macrophages and granulocytes as well as in mediating the uptake of complement-coated particles. It is identical with CR-3, the receptor for the iC3b fragment of the third complement component. It probably recognizes the R-G-D peptide in C3b. Integrin alpha-M/beta-2 is also a receptor for fibrinogen, factor X and ICAM1. It recognizes P1 and P2 peptides of fibrinogen gamma chain.
	Integrin beta 2	ITGB2	Integrin alpha-L/beta-2 is a receptor for ICAM1, ICAM2, ICAM3 and ICAM4. Integrins alpha-M/beta-2 and alpha-X/beta-2 are receptors for the iC3b fragment of the third complement component and for fibrinogen. Integrin alpha-X/beta-2 recognizes the sequence G-P-R in fibrinogen alpha-chain. Integrin alpha-M/beta-2 recognizes P1 and P2 peptides of fibrinogen gamma chain. Integrin alpha-M/beta-2 is also a receptor for factor X. Integrin alpha-D/beta-2 is a receptor for ICAM3 and VCAM1. Triggers neutrophil transmigration during lung injury through PTK2B/PYK2-mediated activation
	Intercellular adhesion molecule 1	ICAM1	ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2). During leukocyte trans-endothelial migration, ICAM1 engagement promotes the assembly of endothelial apical cups through ARHGEF26/SGEF and RHOG activation. In case of rhinovirus infection acts as a cellular receptor for the virus
	Intercellular adhesion molecule 2	ICAM2	ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2). ICAM2 may play a role in lymphocyte recirculation by blocking LFA-1-dependent cell adhesion. It mediates adhesive interactions important for antigen-specific immune response, NK-cell mediated clearance, lymphocyte recirculation, and other cellular interactions important for immune response and surveillance.
	Intercellular adhesion molecule 3	ICAM3	ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2). ICAM3 is also a ligand for integrin alpha-D/beta-2.
	Intercellular adhesion molecule 4	ICAM4	ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2). ICAM4 is also a ligand for alpha-4/beta-1 and alpha-V integrins.
	Intercellular adhesion molecule 5	ICAM5	ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2).
	Matrix Gla protein	MGP	Associates with the organic matrix of bone and cartilage. Thought to act as an inhibitor of bone formation.
	Matrix remodeling associated	Mxra8	May play a role in the maturation and maintenance of blood-brain barrier.

	protein 8		
	Mucosal addressin cell adhesion molecule 1	Madcam1	n.d.
	Neural cell adhesion molecule 1	NCAM1	This protein is a cell adhesion molecule involved in neuron-neuron adhesion, neurite fasciculation, outgrowth of neurites, etc
	Neural cell adhesion molecule 2	NCAM2	May play important roles in selective fasciculation and zone-to-zone projection of the primary olfactory axons.
	Single immunoglob tollinterleu 1 receptor (TIR)	SIGIRR	Acts as a negative regulator of the Toll-like and IL-1R receptor signaling pathways. Attenuates the recruitment of receptor-proximal signaling components to the TLR4 receptor, probably through an TIR-TIR domain interaction with TLR4. Through its extracellular domain interferes with the heterodimerization of Il1R1 and IL1RAP
	Spondin 1	SPON1	Cell adhesion protein that promotes the attachment of spinal cord and sensory neuron cells and the outgrowth of neurites in vitro. May contribute to the growth and guidance of axons in both the spinal cord and the PNS (By similarity). Major factor for vascular smooth muscle cell.
	Toll like receptor 2	TLR2	Cooperates with LY96 to mediate the innate immune response to bacterial lipoproteins and other microbial cell wall components. Cooperates with TLR1 or TLR6 to mediate the innate immune response to bacterial lipoproteins or lipopeptides. Acts via MYD88 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response. May also promote apoptosis in response to lipoproteins. Recognizes mycoplasmal macrophage-activating lipopeptide-2kD (MALP-2), soluble tuberculosis factor (STF), phenol-soluble modulin (PSM) and B.burgdorferi outer surface protein A lipoprotein (OspA-L) cooperatively with TLR6.
	Vitronectin	VTN	Vitronectin is a cell adhesion and spreading factor found in serum and tissues. Vitronectin interact with glycosaminoglycans and proteoglycans. Is recognized by certain members of the integrin family and serves as a cell-to-substrate adhesion molecule. Inhibitor of the membrane-damaging effect of the terminal cytolytic complement pathway.
complement alternative pathway	CD46 molecule complement regulatory protein	CD46	Acts as a cofactor for complement factor I, a serine protease which protects autologous cells against complement-mediated injury by cleaving C3b and C4b deposited on host tissue. May be involved in the fusion of the spermatozoa with the oocyte during fertilization. Also acts as a costimulatory factor for T-cells which induces the differentiation of CD4+ into T-regulatory 1 cells. T-regulatory 1 cells suppress immune responses by secreting interleukin-10, and therefore are thought to prevent autoimmunity. A number of viral and bacterial pathogens seem to exploit this property and directly induce an immunosuppressive phenotype in T-cells by binding to CD46
	Complement factor B	CFB	Factor B which is part of the alternate pathway of the complement system is cleaved by factor D into 2 fragments: Ba and Bb. Bb, a serine protease, then combines with complement factor 3b to generate the C3 or C5 convertase. It has also been implicated in proliferation and differentiation of preactivated B-lymphocytes, rapid spreading of peripheral blood monocytes, stimulation of lymphocyte blastogenesis and lysis of erythrocytes. Ba inhibits the proliferation of preactivated B-lymphocytes.
	Complement factor D (adipsin)	CFD	Factor D cleaves factor B when the latter is complexed with factor C3b, activating the C3bbb complex, which then becomes the C3 convertase of the alternate pathway. Its function is homologous to that of C1s in the classical pathway.
	Complement Factor H	CFH	Factor H functions as a cofactor in the inactivation of C3b by factor I and also increases the rate of dissociation of the C3bBb complex (C3 convertase) and the (C3b)NBB complex (C5 convertase) in the alternative complement pathway.
	Complement factor H related 1	CFHR1	Involved in complement regulation. The dimerized forms have avidity for tissue-bound complement fragments and efficiently compete with the physiological complement inhibitor CFH. Can associate with lipoproteins and may play a role in lipid metabolism
	Complement factor H related 2	CFHR2	Involved in complement regulation. The dimerized forms have avidity for tissue-bound complement fragments and efficiently compete with the physiological complement inhibitor CFH. Can associate with lipoproteins and may play a role in lipid metabolism
	Complement factor H related 4	CFHR4	Involved in complement regulation. Can associate with lipoproteins and may play a role in lipid metabolism.
	Complement factor H related 5	CFHR5	Involved in complement regulation. The dimerized forms have avidity for tissue-bound complement fragments and efficiently compete with the physiological complement inhibitor CFH

	Complement factor I	CFI	Responsible for cleaving the alpha-chains of C4b and C3b in the presence of the cofactors C4-binding protein and factor H respectively
	Complement factor properdin	CFP	A positive regulator of the alternate pathway of complement. It binds to and stabilizes the C3- and C5-convertase enzyme complexes
coagulation factors and related receptors	Fibrinogen beta chain	FGB	Cleaved by the protease thrombin to yield monomers which, together with fibrinogen alpha (FGA) and fibrinogen gamma (FGG), polymerize to form an insoluble fibrin matrix. Fibrin has a major function in hemostasis as one of the primary components of blood clots. In addition, functions during the early stages of wound repair to stabilize the lesion and guide cell migration during re-epithelialization. Was originally thought to be essential for platelet aggregation, based on in vitro studies using anticoagulated blood. However subsequent studies have shown that it is not absolutely required for thrombus formation in vivo. Enhances expression of SELP in activated platelets. Maternal fibrinogen is essential for successful pregnancy. Fibrin deposition is also associated with infection, where it protects against IFNG-mediated hemorrhage. May also facilitate the antibacterial immune response via both innate and T-cell mediated pathways.
	Fibrinogen gamma chain	FGG	Together with fibrinogen alpha (FGA) and fibrinogen beta (FGB), polymerizes to form an insoluble fibrin matrix. Has a major function in hemostasis as one of the primary components of blood clots. In addition, functions during the early stages of wound repair to stabilize the lesion and guide cell migration during re-epithelialization. Was originally thought to be essential for platelet aggregation, based on in vitro studies using anticoagulated blood. However, subsequent studies have shown that it is not absolutely required for thrombus formation in vivo. Enhances expression of SELP in activated platelets via an ITGB3-dependent pathway. Maternal fibrinogen is essential for successful pregnancy. Fibrin deposition is also associated with infection, where it protects against IFNG-mediated hemorrhage. May also facilitate the antibacterial immune response via both innate and T-cell mediated pathways
	Fibrinogen like protein 1	FGL1	Has hepatocyte mitogenic activity.
	Platelet endot cell adhesion molecule precursor	PECAM1	Induces susceptibility to atherosclerosis (By similarity). Cell adhesion molecule which is required for leukocyte transendothelial migration (TEM) under most inflammatory conditions. Tyr-690 plays a critical role in TEM and is required for efficient trafficking of PECAM1 to and from the lateral border recycling compartment (LBRC) and is also essential for the LBRC membrane to be targeted around migrating leukocytes. Prevents phagocyte ingestion of closely apposed viable cells by transmitting 'detachment' signals, and changes function on apoptosis, promoting tethering of dying cells to phagocytes (the encounter of a viable cell with a phagocyte via the homophilic interaction of PECAM1 on both cell surfaces leads to the viable cell's active repulsion from the phagocyte. During apoptosis, the inside-out signaling of PECAM1 is somehow disabled so that the apoptotic cell does not actively reject the phagocyte anymore. The lack of this repulsion signal together with the interaction of the eat-me signals and their respective receptors causes the attachment of the apoptotic cell to the phagocyte, thus triggering the process of engulfment). Isoform Delta15 is unable to protect against apoptosis. Modulates BDKRB2 activation. Regulates bradykinin- and hyperosmotic shock-induced ERK1/2 activation in human umbilical cord vein cells (HUVEC).
	Platelet endothelial cell adhesion molecule	PECAM1	
	Von W factor C domain protein like 2	VWA2	n.d.
	Von Will factor A domain containing protein 2	VWCE	May be a regulatory element in the beta-catenin signaling pathway and a target for chemoprevention of hapatocellular carcinoma
	Von Willebrand factor C and EGF domain containing protein	VWC2L	May play a role in neurogenesis. May play a role in bone differentiation and matrix mineralization
complement classical pathway	Complement component 1 q subcomponent A chain	C1QA	C1q associates with the proenzymes C1r and C1s to yield C1, the first component of the serum complement system. The collagen-like regions of C1q interact with the Ca2+-dependent C1r2C1s2 proenzyme complex, and efficient activation of C1 takes place on interaction of the globular heads of C1q with the Fc regions of IgG or IgM antibody present in immune complexes

	Complement component 1 q subcomponent B chain	C1QB	
	Complement component 1 q subcomponent C chain	C1QC	
	Complement component 1 q subcomponent like 1	C1QL1	
	Complement component 1 q subcomponent like 2	C1QL2	May regulate the number of excitatory synapses that are formed on hippocampus neurons. Has no effect on inhibitory synapses (By similarity).
	Complement component 1 q subcomponent like 4	C1QL4	
	Complement component 1 r subcomponent	C1R	C1r B chain is a serine protease that combines with C1q and C1s to form C1, the first component of the classical pathway of the complement system.
	Complement component 1 r subcomponent like	C1RL	Mediates the proteolytic cleavage of HP/haptoglobin in the endoplasmic reticulum
	Complement component 1 s subcomponent	C1S	C1s B chain is a serine protease that combines with C1q and C1r to form C1, the first component of the classical pathway of the complement system. C1r activates C1s so that it can, in turn, activate C2 and C4.
	Complement component 2 (within H 2S)	C2	C3 plays a central role in the activation of the complement system. Its processing by C3 convertase is the central reaction in both classical and alternative complement pathways. After activation C3b can bind covalently, via its reactive thioester, to cell surface carbohydrates or immune aggregates. Derived from proteolytic degradation of complement C3, C3a anaphylatoxin is a mediator of local inflammatory process. In chronic inflammation, acts as a chemoattractant for neutrophils (By similarity). It induces the contraction of smooth muscle, increases vascular permeability and causes histamine release from mast cells and basophilic leukocytes. C3-beta-c: Acts as a chemoattractant for neutrophils in chronic inflammation
	Complement component 4 binding protein, beta	C4BPB	Controls the classical pathway of complement activation. It binds as a cofactor to C3b/C4b inactivator (C3bINA), which then hydrolyzes the complement fragment C4b. It also accelerates the degradation of the C4bC2a complex (C3 convertase) by dissociating the complement fragment C2a. It also interacts with anticoagulant protein S and with serum amyloid P component. The beta chain binds protein S.
complement terminal pathway	Complement component 6	C6	Constituent of the membrane attack complex (MAC) that plays a key role in the innate and adaptive immune response by forming pores in the plasma membrane of target cells.
	Complement component 7	C7	Constituent of the membrane attack complex (MAC) that plays a key role in the innate and adaptive immune response by forming pores in the plasma membrane of target cells. C7 serves as a membrane anchor
	Complement component 8 alpha polypeptide	C8A	Constituent of the membrane attack complex (MAC) that plays a key role in the innate and adaptive immune response by forming pores in the plasma membrane of target cells. C8A inserts into the target membrane, but does not form pores by itself

	Complement component 8 beta polypeptide	C8B	Constituent of the membrane attack complex (MAC) that plays a key role in the innate and adaptive immune response by forming pores in the plasma membrane of target cells
	Complement component 8 gamma polypeptide	C8G	C8 is a constituent of the membrane attack complex. C8 binds to the C5B-7 complex, forming the C5B-8 complex. C5-B8 binds C9 and acts as a catalyst in the polymerization of C9. The gamma subunit seems to be able to bind retinol.
	Complement factor 9	C9	Constituent of the membrane attack complex (MAC) that plays a key role in the innate and adaptive immune response by forming pores in the plasma membrane of target cells. C9 is the pore-forming subunit of the MAC.
complement lectin pathway	Ficolin 1	FCN1	Extracellular lectin functioning as a pattern-recognition receptor in innate immunity. Binds the sugar moieties of pathogen-associated molecular patterns (PAMPs) displayed on microbes and activates the lectin pathway of the complement system. May also activate monocytes through a G protein-coupled receptor, FFAR2, inducing the secretion of interleukin-8/IL-8 (PubMed:21037097). Binds preferentially to 9-O-acetylated 2-6-linked sialic acid derivatives and to various glycans containing sialic acid engaged in a 2-3 linkage
	Ficolin 2	FCN2	May function in innate immunity through activation of the lectin complement pathway. Calcium-dependent and GlcNAc-binding lectin. Enhances phagocytosis of S.typhimurium by neutrophils, suggesting an opsonic effect via the collagen region
	Ficolin 3	FCN3	May function in innate immunity through activation of the lectin complement pathway. Calcium-dependent and GlcNAc-binding lectin. Has affinity with GalNAc, GlcNAc, D-fucose, as mono/oligosaccharide and lipopolysaccharides from S.typhimurium and S.minnesota
complement related receptors	Compl comp (3d Epstein Barr virus) receptor 2	CR1L	n.d.
	Complement component (3b4b) receptor 1 like	CR2	Receptor for complement C3Dd, for the Epstein-Barr virus on human B-cells and T-cells and for HNRPU. Participates in B lymphocytes activation

Chapter THREE

High-throughput protein microarray screening revealed human interactors for the *Neisseria meningitidis* adhesin A (NadA)

Introduction

Neisseria meningitidis is a gram negative encapsulated bacterium and commensal of human nasopharynx. For still not completely understood reasons, it may become an aggressive pathogen leading to fulminant sepsis and meningitides. Invasive meningococcal disease (IMD) is calculated to cause 5–15% mortality; in addition, overwhelming events affecting quality of life such as amputations and neurodevelopmental disabilities are shown in 11–19% of IMD survivors [1]. Twelve serogroups were described on the base of their capsular polysaccaride, but the majority of invasive meningococcal infections are caused by serogroups A, B, C, X, W-135 and Y. The annual number of disease caused worldwide is calculated to be at least 1.2 million, with 135,000 deaths related to IMD [2]. Since the early 1990s, effective vaccines against *N. meningitidis* serogroups A, C, W, and Y based on mono- or polyvalent-conjugated polysaccharide were available [3]. However, the structural similarity of the serogroup B meningococcus (MenB) capsular polysaccaride with the neuraminic acid present on the surface of human fetal neural tissues [4], prevented the development of an efficient capsular polysaccharide-based vaccine against MenB, responsible for the majority of endemic and epidemic meningococcal disease in developed countries [5, 6]. The effort to develop an effective vaccine against MenB resulted in the recent release of the Bexsero vaccine licensed by Novartis Vaccines and Diagnostics. Bexsero is a four component protein based vaccine (4CMenB) composed of three surface-exposed meningococcal proteins identified by the reverse vaccinology approach plus outer membrane vesicles (OMV) from the New Zealand epidemic clone [7, 8]. The three protein antigens are factor H-binding protein (fHbp), neisserial heparin-binding antigen (NHBA) and neisserial adhesin A (NadA).

NadA is a trimeric coiled-coil outer membrane protein whose gene is present in three out of four known hypervirulent lineages of serogroup B strains [9]. NadA is usually classified in the trimeric autotransporter adhesion (TAA) family, a class of outer membrane adhesin present in Gram-negative bacteria. Nevertheless, the recently solved structure showed features of a novel trimeric autotransporter adhesin that has no close homologs among other TAAs proteins present in the Protein Data Bank (PDB) [10]. The protein is constituted by a trimeric coiled-coil structure which includes both the apical N-terminal region and the main stalk, with a peculiar sequence insertion which gives rise to wing-like structures without altering the coiled-coil geometry.

Several studies, carried out on different cell types including epithelial cells, monocytes, macrophages, and monocyte-derived dendritic cells, showed NadA's importance in bacterial adhesion. NadA have been shown to be involved in bacterial uptake in epithelial cells. *E coli* bacteria expressing NadA were indeed shown to be internalized in Chang cells in a NadA-

dependent fashion. In addition a deletion of the N-terminal globular domain of recombinant NadA abrogated the adhesive phenotype [11]. Additional studies revealed the regions between aminoacids 94 to 110 and 109 to 121 on NadA head as the one involved in NadA-mediated cell-bacterium adhesion [12]. Further studies have shown that recombinant NadA binds to hsp90 *in vitro* and on the surface of monocytes [13]. In the proposed model, NadA binds to an unknown receptor, encounters hsp90 probably by lateral diffusion and then it is recruited into a complex also comprising hsp70 and TLR4. This complex is inhibited by polymixin B, which interferes whith NadA-hsp90 binding but not with NadA cell binding. The complex is also necessary for full monocyte stimulation and may be important to modulate or enhance the vaccine immune response. Furthermore NadA has been shown to interact with β-integrins on epithelial-like GE-11 and fibroblast-like 2-4-8 cells using flow cytometry. The direct binding data have been corroborated by blocking experiments with anti-human integrin β1 monoclonal antibodies which have been described to compete with NadA for β-integrin binding [14]. Based on these studies, NadA appears to be a key determinant of meningococcal interactions with the human host at different stages of meningococcal infection. However, specific molecular interactions taking place at the host-pathogen interface are still not completely understood. In order to identify novel human receptors for NadA, an un-biased systematic large scale protein microarray-based approach for the identification of novel interactions was applied. In particular, NadA variant 3 (NadA3) was screened for interactions against ~2700 recombinant proteins representing the “human extracellular proteome” [15] and label-free technologies and FACS analysis were used for hit validation.

Materials and methods

Protein microarray construction and processing

The NOV-GNF protein microarrays were generated by using 2354 human recombinant proteins from the GNF library, which were produced as described in Gonzalez et al. (2009). Each protein of the library was spotted in duplicates in 24 blocks per array onto ultra-thin nitrocellulose coated glass slides (PATH slides; Grace Bio Labs) using a contact printer (MicroGrid II; Digilab). Printing was performed at room temperature and ambient relative humidity. Proteins were diluted ½ in arraying buffer (100 mM NaCl, 0.05% TritonX and 5% Glycerol in PBS) and printed between 10 amol to 75 fmol per 150 µm diameter spot. A Chrompure human IgG (Jackson IR 009-000-003) was printed at 0.4 fmol per spot several times on each single block and served as a printing control and to define the blocks. Eight empty blocks were available to print additional antigens and relevant controls such as biotinylated BSA. Since all secretomics proteins have a 6-His tag and approximatively 1/3 have an additional mouse Fc-tag, the printing efficiency of the proteins and microarray quality was evaluated by probing the microarrays with a mouse anti-His (Sigma H1029) antibody followed by a detection using a mix of a Cy5 conjugated $F(ab')_2$ goat a-mIgG Fc specific (Jackson IR 115-176-071) and a Cy5 conjugated goat a-hIgG $F(ab')_2$ specific secondary antibody (Jackson IR 109-175-097). The last secondary antibody (Jackson IR 109-175-097) was systematically used in the assays to detect the human IgG control spots printed on the arrays in order to define the blocks. 4CMenB vaccine antigen NadA3, was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) following manufacturer's instruction. The mass of the proteins and degree of biotinylation was checked with Liquid chromatography coupled to mass *spectrometry (LC-MS)*. The incubation of protein microarrays with NadA3 was performed using an automated hybridization station (HS400; Tecan), programmed with an internal protocol. First, the protein microarrays were incubated with a PBS blocking solution containing 1% BSA and 0.1% Tween20 for 1 hour at 23 °C to reduce non-specific binding. Then, NadA3, diluted in probing buffer (2.5% glycerol, 1% BSA, and 0.05% TritonX in PBS) at final concentrations of 150 nM and 1 µM, were incubated for 1 h at 23 °C. After a washing step with probing buffer, the microarrays were probed with Cy5-Streptavidin (Invitrogen SA1011) combined with Cy5-goat a-hIgG $F(ab')_2$ specific (Jackson IR 109-175-097) diluted in probing buffer at 7.5 µg/ml final concentration. Finally, after two washing steps using probing buffer and Milli-Q water, the microarrays were dried under a flow of nitrogen at 30 °C for 4 min. In parallel a control microarray slide probed with buffer and the mix of the detection antibodies was performed.

Microarray imaging and data analysis

Microarray image acquisition was done using a fluorescence microarray scanner (GenePix Professional 4200A; Molecular Devices) equipped with a red laser (635 nm) at a resolution of 10 µm/pixel and images were analyzed using the GenePix Pro v6.1 software (Molecular Devices). 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. The MFI obtained for each protein was normalized by setting the MFI of the control biotinylated BSA to 100 %. Proteins with a high coefficient variation (CV) between the two replicates were discarded. All signals which were equal or greater than 25% were considered as positive hits, excluding the positive hits found in the control microarray where the Cy5-Streptavidin alone was probed to determine its unspecific reactivity to the printed proteins.

NadA3 and NadA3 constructs cloning, expression, purification and characterization

The nadA gene fragments from N. meningitidis strain 2996 were cloned by PCR, using the PIPE (polymerase incomplete primer extension) method, as described previously [10]. The cloned fragments lacked the first 23 residues of NadA which encode a signal peptide for protein export. The fragments were inserted into a pET-21 vector (Novagen), enabling cytoplasmic expression of the NadA proteins with a C-terminal 6-His tag. The residue numbering employed refers to the full-length NadA protein, Uniprot code Q8KH85. All NadA proteins were produced in Escherichia coli BL21D3 (T1r) cells (Invitrogen) and were purified at room temperature (RT, 18-26°C) using an AKTA purifier 10 system (GE Healthcare) by Ni-affinity chromatography (5mL HiTrap Ni-NTA column) and by size-exclusion chromatography on a HiLoad (16/60) Superdex 75 column equilibrated in 20 mM Tris–HCl, 150 mM NaCl pH8.0. The quality of the final NadA samples was checked using 4–12% SDS–PAGE gradient gels in MES buffer and also by size-exclusion high-performance liquid chromatography (SE-HPLC), revealing a high level of purity (estimated to be >97% for all proteins) and a lack of any aggregated species. SE-HPLC was performed at RT on an analytical size exclusion TSK Super SW3000 column by loading 20µl of each sample at a concentration of ~ 40µM. Samples were eluted isocratically in 0.1M NaH2PO4, 0.4M (NH4)2SO4 buffer at pH 6.0. Coupling SE-HPLC with Multi-angle laser light scattering (SE-HPLC/MALLS) NadA samples were analyzed for molecular size. Data analyses were carried out using Astra V software (Wyatt) to determine the weight-average molecular mass (MW) in Daltons and the polydispersity index (MW/Mn) for each oligomer present in solution.

BioLayer Interferometry (BLI)-based OCTET assay

Octet QKe (ForteBio, Pall Science) was used for binding studies. SA (streptavidin) and AR2G (amine reactive 2nd generation) tips were obtained from ForteBio and were used respectively to immobilize biotinylated proteins and to covalently attach ligand protein using amine based chemistry. Immediately before analysis, tips were pre-wet in kinetic buffer (10mM HEPES, 150mM NaCl, 0,02% P-20 pH 7.4). After 180 sec baseline, AR2G tips were activated for 300 sec using a freshly mixed solution of 200 mM EDC in 50 mM NHS further diluted 1:20 in dH2O. The tips were then immersed in ligand solution (LOX-1 at 12.5µg/ml in acetate pH 5.5) for 600 sec. Excess reactive groups were blocked with 1 M ethanolamine pH 8.5 for 300 sec and a second 300 sec baseline in kinetic buffer was performed. Association and dissociation were monitored. For SA biosensor, after 180 sec baseline, tips were directly immersed in ligand solution for 600 sec loading (biotinylated NadA3, $NadA3_{24-170}$ and $NadA3_{91-342}$ at 25µg/ml in kinetic buffer) and a second 300 sec baseline in kinetic buffer was performed. Kinetic was then monitored. During the entire kinetic assay the sample plate was kept shaking 1000rpm. A column of biosensors where no ligand protein was loaded, was activated and quenched to be used as parallel reference control in association with the analyte titration; a ligand-loaded biosensor was also used in association with buffer as baseline.

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. Data were globally fit in a 1:1 model using the Data Analysis Software v7.1 (Forte Bio).

Dynamic Light Scattering (DLS) measurements

For DLS analysis DynaPro® Plate Reader II (WYATT Technology) with 96 Well Plates was used. NadA3, LOX-1 and the complex midex in a 1:1 ratio were diluted in HBS-P buffer in a final concentration of 5 µM. A cut off from 0,1 to 100nm was applied. results are mean of 30 measurements per sample.

mAbs and Fabs selection and binding competiton

For competition experiments, a preliminary screening to select high-affinity anti-NadA3 mAbs and Fabs was performed. Protein G biosensors tips were pre-wet in kinetic buffer (10mM HEPES, 150mM NaCl, 0,02% P-20 pH 7.4). After 180 sec baseline, tips were directly immersed in each mAb (diluted at 50 µg/ml in kinetic buffer) for 600 sec. A second 300 sec baseline in kinetic buffer was performed. NadA3 was tested as analyte at 200 nM. Competition experiments were performed immobilizing LOX-1 as above. NadA3 was pre-

incubated with an excess of mAb (1:3) overnight at 4°C and the mix was used as analyte in the same assay conditions as above.

FACS binding experiments

To perform binding assay, LOX-1 transfected and non-transfected CHO cells were detached using trypsin (CDS, Sigma), harvested and suspended in F12-K medium supplemented with 10% FBS. Approximately 1 x 106 cells were incubated with 200 µg/ml NadA or PBS alone for 30 minutes at 4°C. Cells were then incubated with 5 µg/ml of anti-NadA 9F11 mouse monoclonal antibody for 1 hour at 4°C, and with Allophycocyanin (APC)-conjugated goat F(ab)2 anti-mouse Ig (diluted 1:100; Jackson ImmunoResearch Laboratories) for 30 minutes at 4°C. Cells were stained with Live/Dead Aqua (Invitrogen) diluted 1:400 for 20 minutes in the dark and analyzed with a Canto II analyzer (Becton Dickinson, Pharmingen, San Diego, CA). Flow-cytometric analysis was performed using FlowJo software (Treestar Inc.).

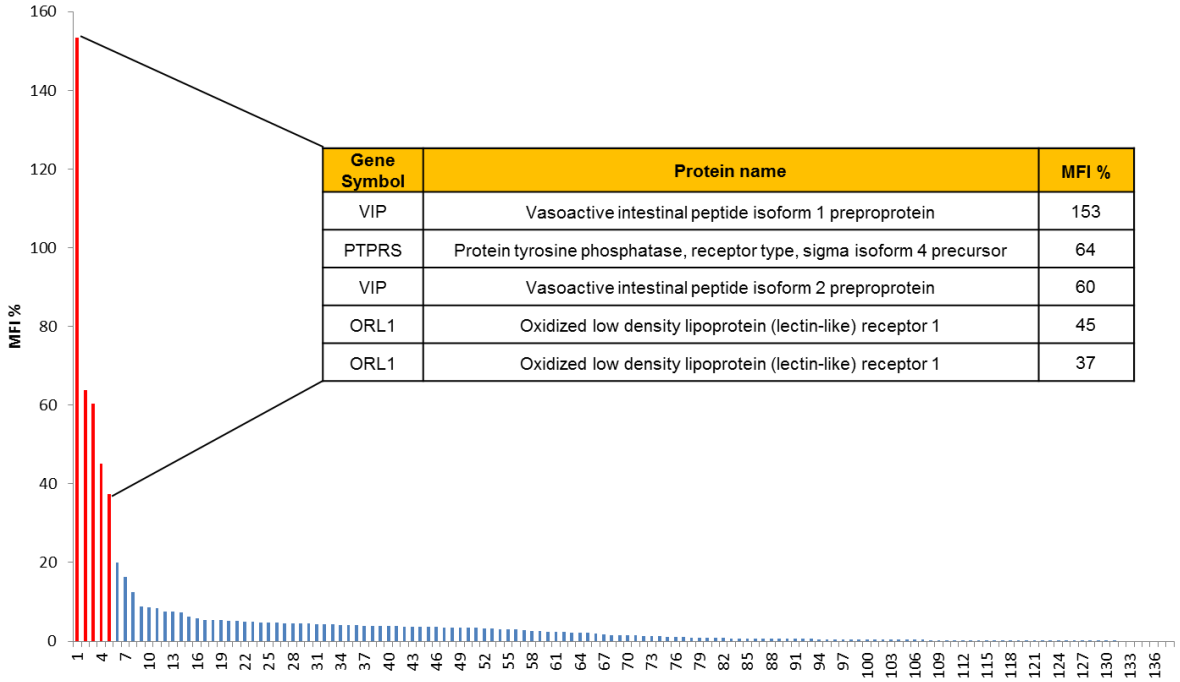
Results

Microarray Screening for NadA3 interactors

In order to identify human proteins interacting with the *Neisseria meningitidis* adhesin A (NadA), an important component of the Bexsero® vaccine [16], a large scale protein microarray screening was performed. The protein array was built spotting 2354 human recombinant proteins selected and produced as previously described [15]. Briefly, starting from previous large scale efforts and additional bioinformatic analysis, the gene coding for the ectodomain of 2687 human proteins were cloned in plasmid for transient transfection, expressed with affinity tags (6xHis and FLAG) in HEK293 cells and purified in high-throughput way. The NOV-GNF microarrays were validated using anti-His and anti-mouse antibodies showing that 71% of the proteins were detected, thus correctly printed and immobilized onto the slides. The interaction of the biotinylated NadA3 to printed proteins was detected using fluorescently labeled streptavidin. Internal control biotinylated BSA spots were taken as reference signal (100% signal) and the mean fluorescence intensities (MFI) of all duplicate spots were normalized and indicated as percentage of the reference. An arbitrary threshold of 25% of the reference signal was defined in order to select relevant interactions. Biotinylated NadA3FL (NadA3 Full length protein) was first used in overlay at 150 nM. The resulting screening showed several putative human interactors (**figure 3.1A**). Isoform 1 and 2 of the Vasoactive Intestinal Peptides (VIP) with an MFI score of 153% and 60% respectively and the Protein Tyrosine Phosphatase Receptor type Sigma isoform 4 (PTPRS) with an MFI of 64% were highlighted as strong hits. Two replicates of the low-density oxidized lipoprotein (lectin-like) receptor 1 (LOX-1) with a MFI score of 45% and 37% represent potentially relevant hits. These data were substantiated when NadA3 was tested at 1 µM. All five hits identified in the 150 nM screen could be confirmed (**figure 3.1B**). In addition to these proteins, further putative hits were identified: the Stromal interaction molecule 1 precursor (STIM1, MFI 54%), the Natriuretic Peptide Precursor C (NPPC, MFI 30%), the hypothetical protein LOC122258 precursor (MFI, 27%) and the Interleukin 6 precursor (IL-6, MFI 26%).

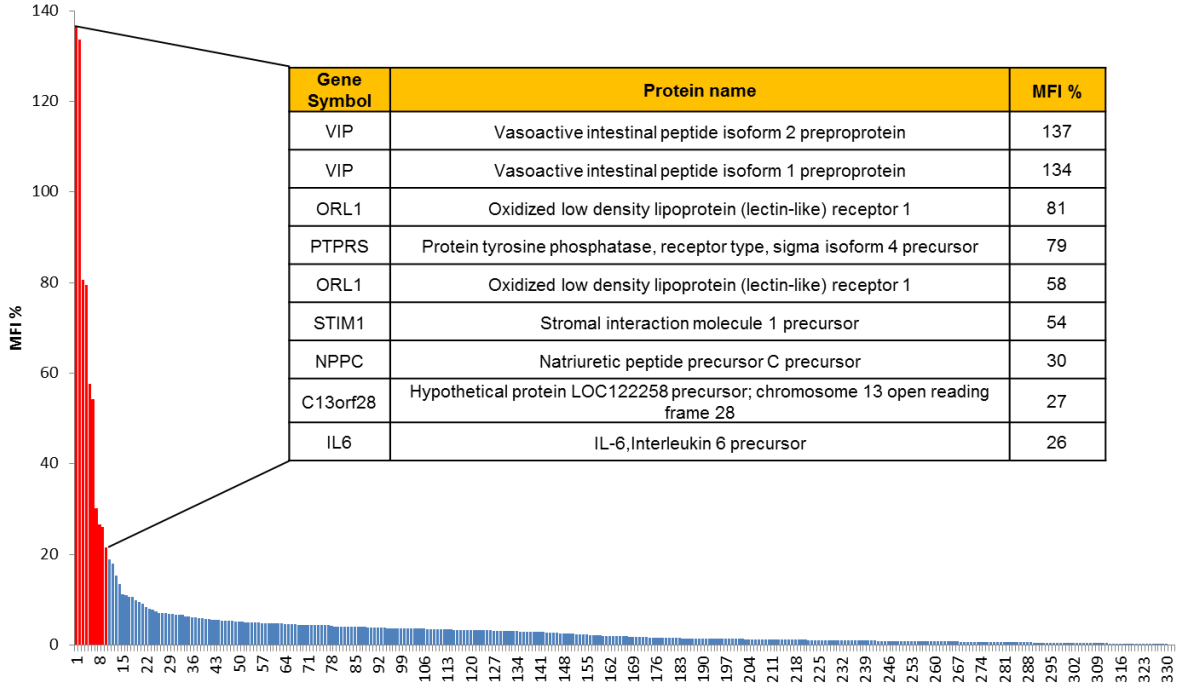
Among all the identified hits, LOX-1 represented an interesting candidate for further validation processes since it is known to be involved in bacterial adhesion and invasion [17, 18], endotoxin-induced inflammation [19] and its deletion enhances bacterial clearance in a murine polymicrobial sepsis model [20].

A



Gene Symbol	Protein name	MFI %
VIP	Vasoactive intestinal peptide isoform 1 preproprotein	153
PTPRS	Protein tyrosine phosphatase, receptor type, sigma isoform 4 precursor	64
VIP	Vasoactive intestinal peptide isoform 2 preproprotein	60
ORL1	Oxidized low density lipoprotein (lectin-like) receptor 1	45
ORL1	Oxidized low density lipoprotein (lectin-like) receptor 1	37

B



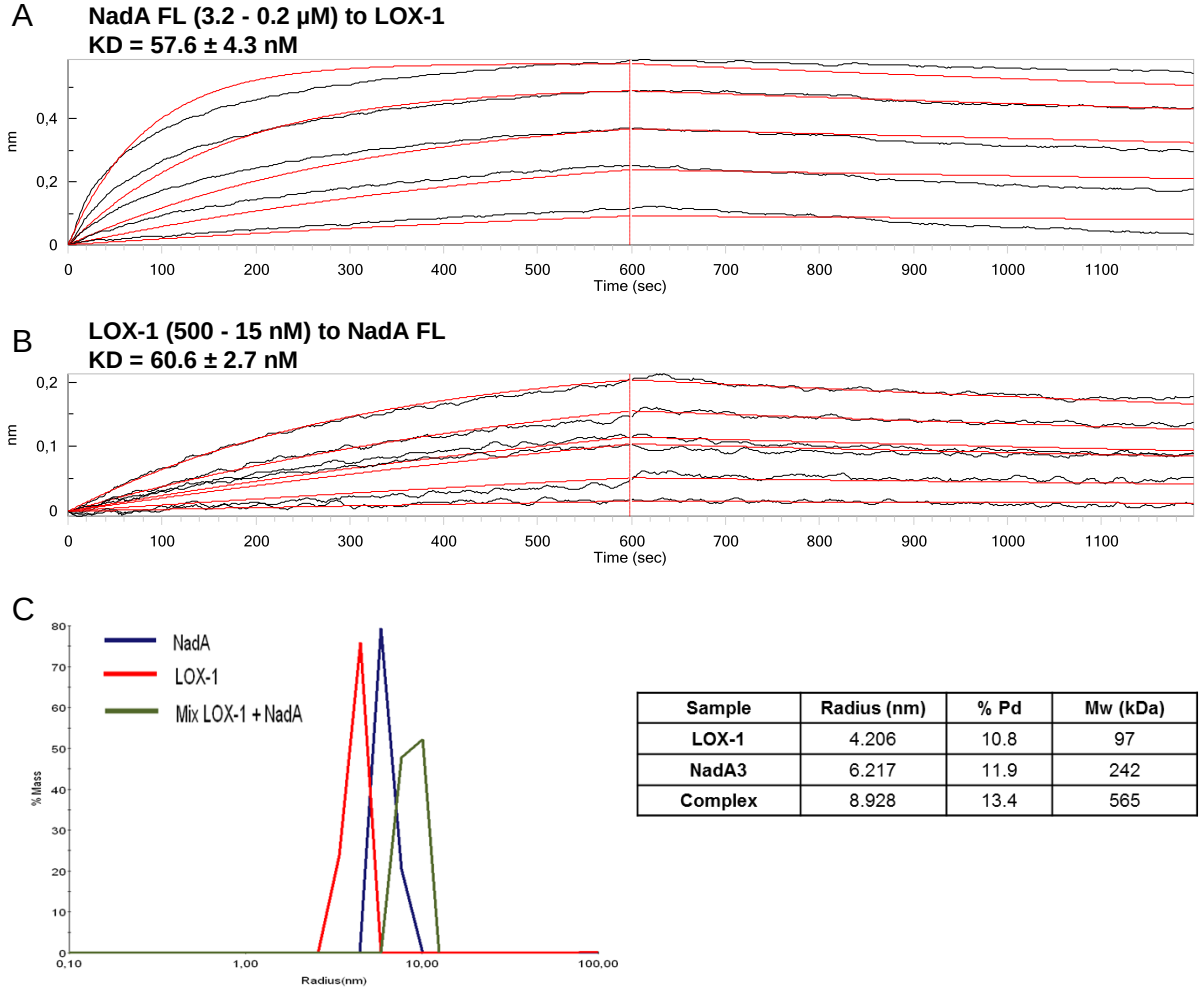
Gene Symbol	Protein name	MFI %
VIP	Vasoactive intestinal peptide isoform 2 preproprotein	137
VIP	Vasoactive intestinal peptide isoform 1 preproprotein	134
ORL1	Oxidized low density lipoprotein (lectin-like) receptor 1	81
PTPRS	Protein tyrosine phosphatase, receptor type, sigma isoform 4 precursor	79
ORL1	Oxidized low density lipoprotein (lectin-like) receptor 1	58
STIM1	Stromal interaction molecule 1 precursor	54
NPPC	Natriuretic peptide precursor C precursor	30
C13orf28	Hypothetical protein LOC122258 precursor; chromosome 13 open reading frame 28	27
IL6	IL-6,Interleukin 6 precursor	26

Figure 3.1 Screening for NadA3 interactors. (A) Plotting of MFIs relative percentage of spotted human proteins tested against NadA3FL at 150nM and **(B)** 1µM. Red bars represent human proteins above the 25% cut-off threshold defining relevant interactions. Blue bars represent human proteins above the 4% cut-off threshold. Inset table shows gene symbol, protein name and MFI values of the relevant interactions

NadA3 binds LOX-1 through its head&neck region

Based on the protein array results, the NadA3–LOX-1 interaction was validated and characterized by label-free methods. *In vitro* binding experiments were performed using

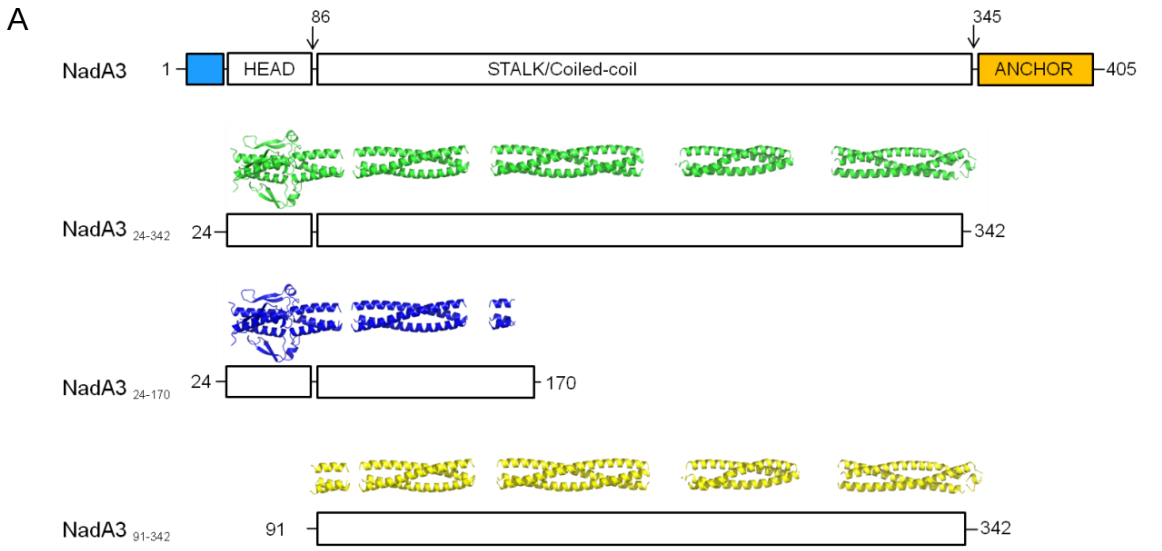
BioLayer Interferometry (BLI). LOX-1 recombinant protein was first immobilized onto the BLI biosensor using amine reactive chemistry and NadA3FL was tested in serial dilutions as analyte showing dose dependency (**figure 3.2A**). The calculated affinity using a 1:1 fitting model was 57nM. This value was also confirmed when the system was inverted and LOX-1 was used as analyte on immobilized NadA3FL. An affinity constant of 60nM was indeed calculated (**figure 3.2B**). DLS analysis was carried out to determine the interaction in solution (**figure 3.2C**): the proteins were first analyzed alone, resulting in a hydrodynamic radius of 4.2 nm and 6.2 nm for LOX-1 and NadA3FL, respectively, and then analyzed as mix in a 1:1 ratio, resulting in a single peak with hydrodynamic radius of 8.9 nm and low polydispersity index, *i.e.* 13.4%. The peaks corresponding to the single proteins were not detected, indicating s complete involvement of the proteins in complex formation.

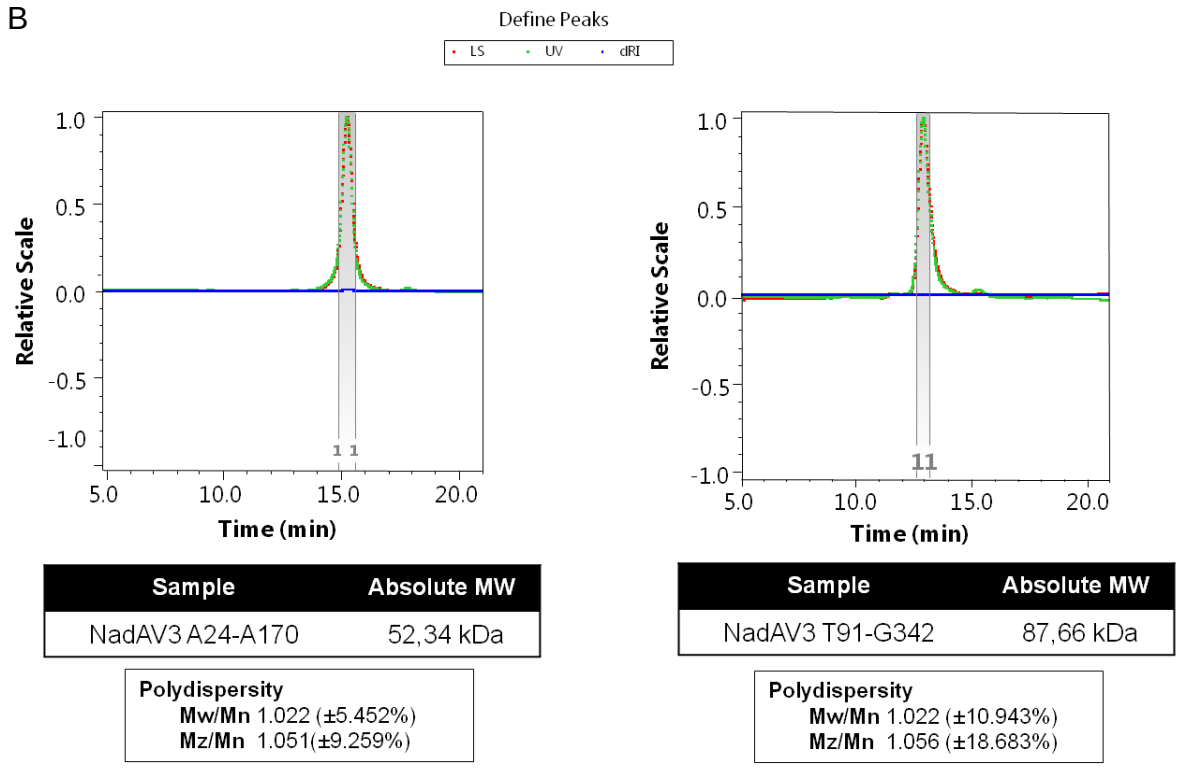


Sample	Radius (nm)	% Pd	Mw (kDa)
LOX-1	4.206	10.8	97
NadA3	6.217	11.9	242
Complex	8.928	13.4	565

Figure 3.2 Binding of NadAFL to LOX-1 was validated through BioLayer Interferometry and DLS. (A) Blank subtracted sensograms of NadA3FL (3.2 – 0.2µM) tested on covalently immobilized LOX-1 and **(B)** of LOX-1 (500 - 15 nM) tested on biotinylated NadA3FL immobilized on SA biosensors. Association and dissociation curves were fitted in a 1:1 model. **(C)** DLS analysis of NadA3FL, LOX-1 and the complex. Results are mean of 30 measurements. The hydrodynamic radius of LOX-1, NadA3 and their complex were 4.2 nm, 6.2 nm and 8.9 nm, while the calculated MW were 97KDa, 242KDa and 565KDa respectively. Sample polydispersity is also shown in table.

To identify the binding region for LOX-1, the NadA3$_{24-170}$ (head&neck) and NadA3$_{91-342}$ (stalk) constructs were designed based on the recently published crystal structure [10] and produced as his-tagged recombinant proteins (**figure 3.3A**). SE-HPLC/MALLS showed a measured MW of 52KDa and 87KDa for NadA3$_{24-170}$ and NadA3$_{91-342}$ respectively, confirming the expected MW of the trimeric constructs (**figure 3.3B**). Octet BLI binding experiments performed using biotinylated NadA3 constructs as ligand and LOX-1 as analyte, revealed the retained capability of the NadA3$_{24-170}$ construct to bind LOX-1. The binding was instead abolished in the NadA3$_{91-342}$ construct (**figure 3.3C**). The calculated affinity between NadA3$_{24-170}$ and LOX-1 was in the pM range (**figure 3.3D**).





Sample	Absolute MW
NadAV3 A24-A170	52,34 kDa

Polydispersity
Mw/Mn 1.022 (±5.452%)
Mz/Mn 1.051(±9.259%)

Sample	Absolute MW
NadAV3 T91-G342	87,66 kDa

Polydispersity
Mw/Mn 1.022 (±10.943%)
Mz/Mn 1.056 (±18.683%)

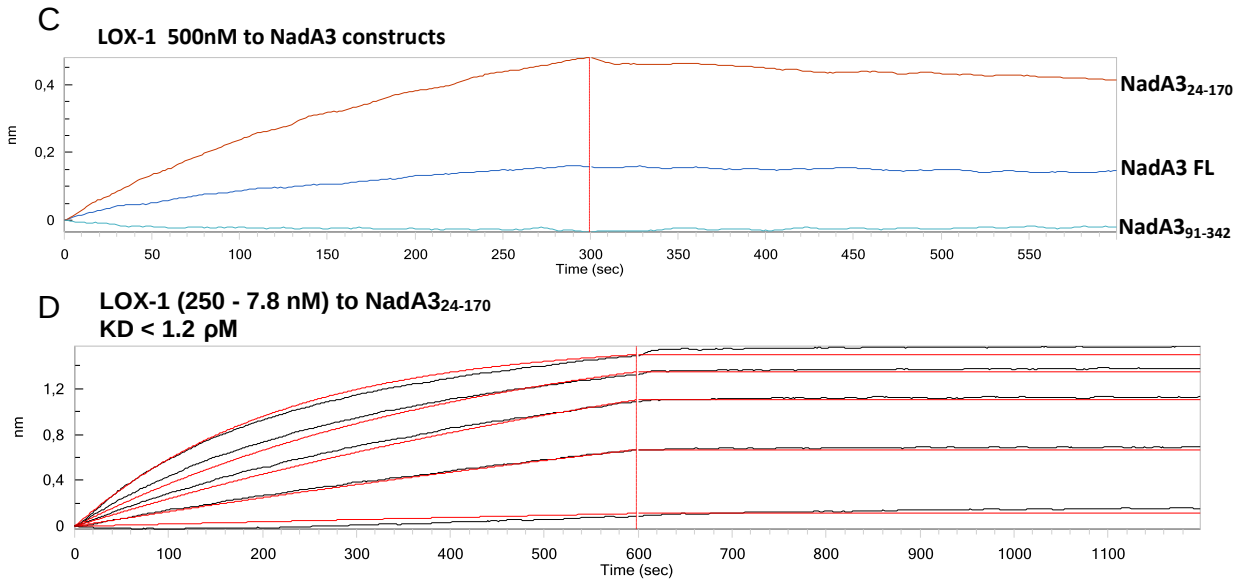


Figure 3.3 Characterization and binding experiments of the NadA3 24-170 and NadA3 91-342 constructs. (A) Schematic representation of the NadA3 constructs used to determine NadA3 binding site on LOX-1. NadA3 gene, NadA3$_{24-342}$ (green), NadA3$_{24-170}$ (blue) and NadA3$_{91-342}$ (yellow) structure are shown. Signal peptide (light blue) and membrane anchor (orange) are shown in the cartoon. **(B)** SEC-HPLC/MALLS measurements of the NadA3$_{24-170}$ (left) and NadA3$_{91-342}$ (right). Light Scattering (LS - red), UV (green), and refractive index (RI - blue) signals are shown. Inset table and box shows respectively the calculated MW and the sample polydispersity. **(C)** Blank subtracted sensograms of LOX-1 (200nM) on biotinylated NadA3$_{24-342}$ (blue), NadA3$_{24-170}$ (red) and NadA3$_{91-342}$ (light blue). **(D)** Blank subtracted sensograms of LOX-1 (125 – 7.8 nM) tested on biotinylated NadA3$_{24-170}$. Binding curves were fitted in a 1:1 model.

Anti-head mAbs inhibits NadA-LOX-1 binding

In order to understand the potential ability of anti-head monoclonal antibodies (mAbs) to inhibit the NadA-LOX-1 binding, competition experiments using Octet BLI were performed. Anti-NadA3 mAbs were first selected based on their binding site on NadA3 using protein microarray and Biacore binding experiments (in house data - not shown). Additional KD ranking experiments were used to select high affinity anti-NadA3 mAb (**table 1**).

Loading Sample ID	Binding site on NadA3	KD (M)
9F11	stalk (269-316)	1,02E-09
1C9/A9	head (24-107)	1,61E-10
8D9/A9	head (24-107)	2,51E-10
3C11/H7	head (1-235)	1,63E-09

Table 3.1 **mAbs KD ranking.** Name, binding site and KD of the antibodies used in the competition assay are reported.

For competition tests, LOX-1 was covalently immobilized on biosensor surface and NadA3 pre-incubated with mAb was used as analyte. BLI binding assay (**figure 3.4**) showed a marked ability of mAb mapping the NadA3 head region (24-170) to inhibit the binging with LOX-1. By contrast mAb 9F11, mapped on NadA3 stalk region (269-316), did not inhibit the binding confirming the data obtained with NadA3 constructs.

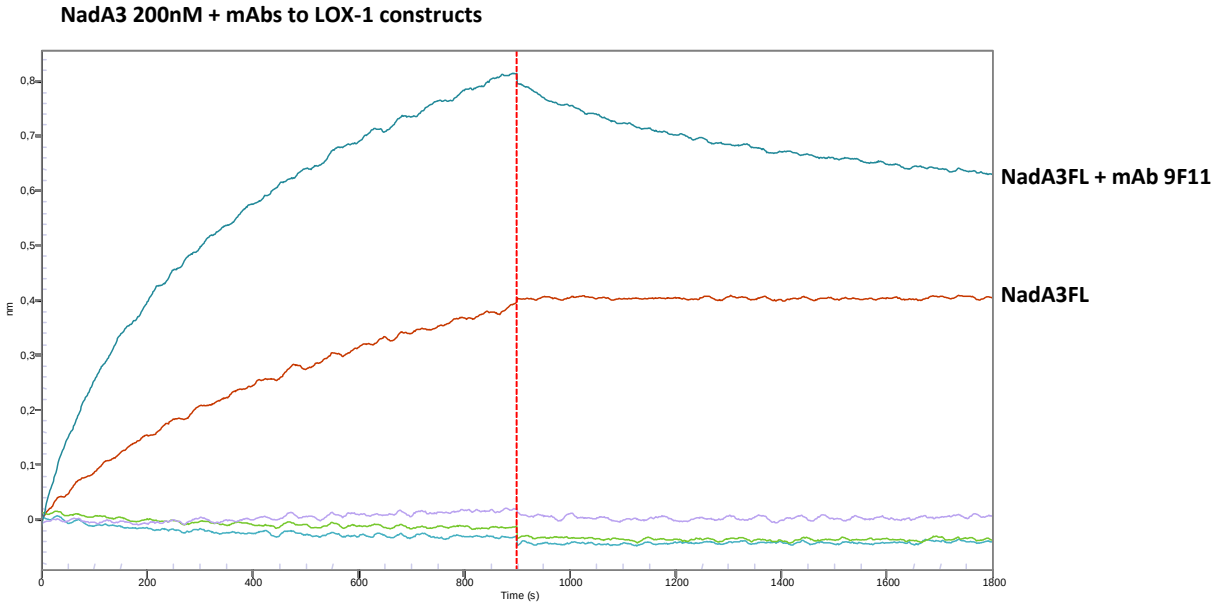


Figure 3.4 Blank subtracted sensograms of NadA incubated with mAbs or Fabs on covalenty immobilized LOX-1. Binding between NadA3 and LOX-1 is shown (red). mAb 9F11 mapping NadA3 stalk does not inhibit the binding with NadA3 (blue). mAb 3C11/H7 (violet), mAb 1C9/A9 (light blue) and mAb 8D9/A9 (green) mapping on NadA3 head abrogated NadA3 binding on LOX-1.

NadA binds CHO cells expressing hLOX-1

To further validate the interaction between LOX-1 and NadA3, binding assays in eukaryotic cell lines were performed. CHO (Chinese Hamster Ovary) cell line was chosen since does not express human LOX-1 constitutively (data not shown). CHO cells were transfected with a pEYFP-N1 vector containing LOX-1 cDNA and the expression was tested by FACS analysis. When transfected, the LOX-1 expressing cell population is the 32.9% of the total, while, as expected, no protein expression was detected on non-transfected cells. The binding with NadA3 was monitored after incubation of the protein with the transfected and non-transfected cells (negative control). NadA3 can bind only the LOX-1 transfected cell population, while non-transfected cells did not show any binding, confirming the results obtained with label-free technologies (**figure 3.5**).

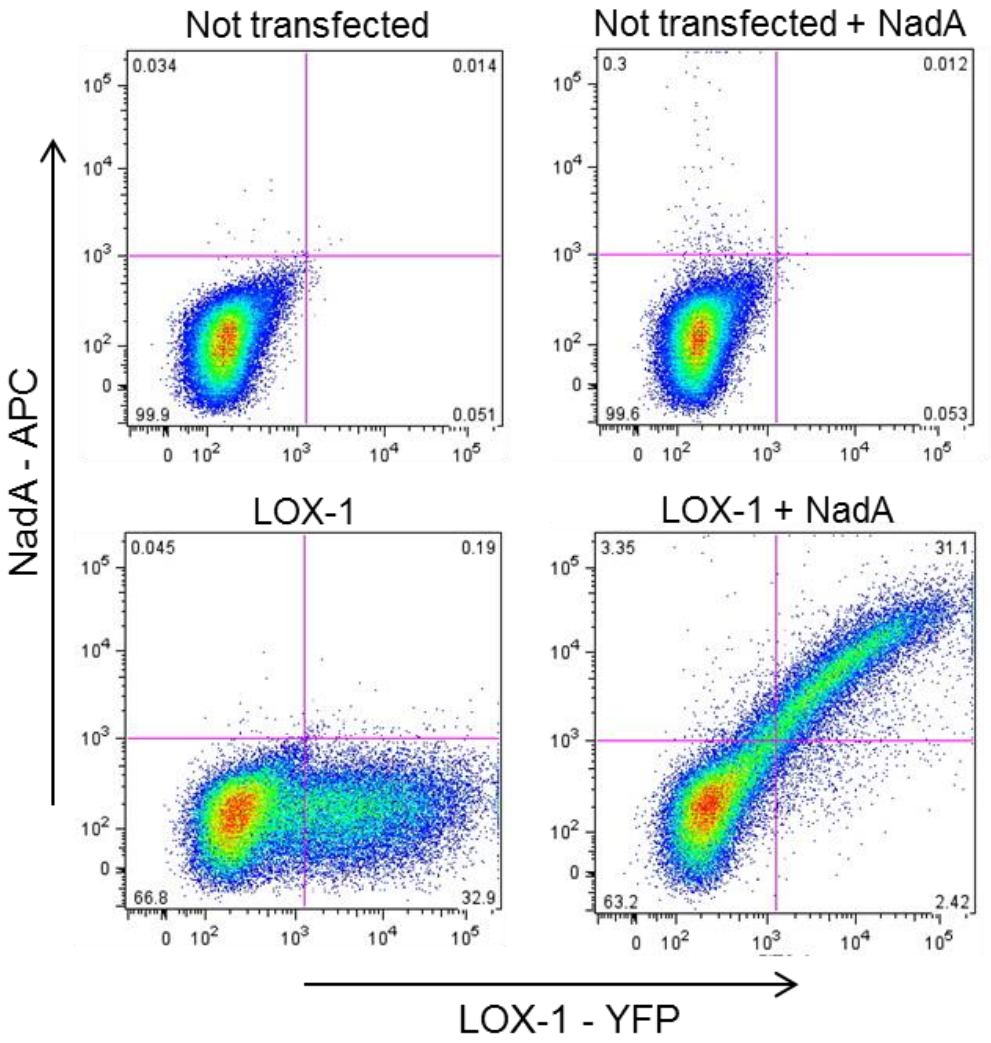


Figure 3.5 NadA binds CHO cells expressing hLOX-1. FACS plots representative of LOX-1 (YFP) expression in CHO cells and cells able to bind NadA (APC). The gating strategy was designed to separate the cells of interest from large aggregates and debris [initial gate on forward scatter (FSC) versus side scatter (SSC) plot], deplete dead cells (Live/Dead Aqua staining) and doublets/aggregates (standard gates on both FSC-width and SSC-width) (data not shown). Simple gating by quadrants allowed defining the absolute percentages of cells positive for LOX-1 only (32%, bottom left) and double positive cells (30%, bottom right) representing the cells able to bind recombinant NadA. The upper right panel shows that not-transfected cells are not able to bind NadA if incubated with the recombinant protein. The upper left panel shows the non-transfected cells.

Discussion

Neisseria meningitidis is a pathogenic bacteria and a leading cause of meningitis and bacterial sepsis in the world. Twelve strains are known but the most aggressive and infective are caused by serogroups A, B, C, X, W-135 and Y. Carbohydrate based vaccines are already available for the A, C, W, Y strains [3]. Due to structural similarities between the capsular polysaccharides of the type B with the neuraminic acid present on the surface of human fetal neural tissues, the development of a vaccine against the type B needed a protein-based approach. In 2013 the first recombinant 4 component vaccine against MenB (4CMenB) was released [7]. One of the components is the neisserial adhesin A (NadA) protein, a trimeric coiled coil adhesin whose structure was recently solved [10]. Several functional studies indicated NadA as an important adhesion factor, and key effector in pathogenesis [21, 22]. Despite the structural and functional information a global picture of human receptors targeted by NadA has remained unknown.

The microarray format is an ideal high throughput technology for analyzing multiple protein-protein interaction in parallel. The NOV-GNF protein microarray is a unique platform constituted with a large collection of human secretomics proteins. An internal experimental protocol was developed to analyze protein-protein interactions in case of NadA3 where the lack of definitive positive control (i.e known interacting proteins for NadA3) added another level of uncertainty to set the analysis threshold. The fact that positive biotinylated BSA control printed on the slides probed with only Cy5-Streptavidin secondary detection worked well testified that experimental conditions (e.g. buffer, composition, temperature, pH, salts, concentrations, etc.) were favorable. An arbitrary threshold of 25% of the reference signal (biotinylated BSA control signal) was defined in order to select the most significant interactions. However, is it possible that many of the protein-protein interactions are weak in nature yet biologically meaningful. Moreover, there is no guarantee all proteins retain their secondary and tertiary structure when immobilized or their biological activity.

The screening was performed using biotinylated NadA3 at two concentrations and lead to the identification of several human putative interactors. The hits resulted from the screening using NadA3 at 150nM were two isoforms of the VIP (Vasoactive Intestinal Peptides), LOX-1 (low-density oxidized lipoprotein lectin-like receptor 1) and PTPRS (Protein Tyrosine Phosphatase Receptor type Sigma isoform 4). The vasoactive intestinal peptides were shown to be involved in vasodilation, relaxation of the smooth muscle of trachea, stomach and gall bladder [23]. In addition VIP were already described to protect, through the inhibition of protein kinase C (PKC) activity, against epithelial barrier disruption and colitis caused by bacterial pathogen like EPEC (enteropathogenic *Escherichia coli*) [24]. PTPRS was instead

described in neuronal development and as a positive regulator of intestinal epithelial barrier. It modulates epithelial cells adhesion targeting proteins associated with apical junction complex [25]. This might drive to epithelial cell relaxation facilitating bacterial permeation. LOX-1 is the receptor for oxidized low-density lipoproteins and it is implicated in the pathogenesis of different diseases, including atherosclerosis, hypertension, obesity, diabetes mellitus and metabolic syndrome [26-28]. It was identified as a OxLDL receptor primarily in endothelial cells and it was described to be involved in OxLDL internalization [29]. Several studies already demonstrated the role of LOX-1 in bacterial adhesion to endothelial cells. Shimaoka et al. described *Staphylococcus aureus* and *Escherichia coli* to be able to bind CHO cells transfected with LOX-1 [17]. The binding was abrogated with anti-LOX-1 antibodies. Further studies demonstrated that the infection of endothelial cells by *Chlamydia pneumoniae* is inhibited by ligands that bind to LOX-1. Also in this case both the binding of *C. pneumoniae* on endothelial cells and its infectivity were inhibited by anti-LOX-1 antibodies [18]. It was also shown that LOX-1 deletion in mice improves neutrophil responses, enhances bacterial clearance, and reduces lung injury [20]. STIM1 (Stromal interaction molecule 1 precursor), NPPC (Natriuretic Peptide Precursor C), the hypothetical protein LOC122258 precursor and IL-6 (Interleukin 6 precursor) were instead revealed only in the screening performed using NadA3at 1µm, possibly meaning a lower affinity for NadA if compared to the previous hits, which were all confirmed. STIM1 was described to play a role in mediating cellular store-operated Ca2+ entry (SOCE), by acting as Ca2+ sensor in the endoplasmic reticulum via its EF-hand domain. This leads the protein to be involved in several cellular functions, like microtubules remodeling and phagocytes and T-cell activation [30-32]. Moreover this receptor was previously described to be involved in bacterial and viral pathogenesis. The *Clostridium difficile* transferase toxin (CTD) induces microtubule-based protrusions in human coloncarcinoma (Caco-2) cells, and increases pathogen adherence through STIM1. The toxin reroutes Rab11-positive vesicles containing fibronectin, which is involved in bacterial adherence, from basolateral to the apical membrane sides in a microtubule- and STIM1-dependent manner [33]. The rotavirus nonstructural protein 4 (NSP4) was also shown to be involved in changes in cellular calcium homeostasis through STIM1 activation, which was constitutively active in rotavirus-infected cells [34]. In a similar way, Streptolysin O (SLO) from Group A *Streptococcus* induces diverse types of Ca(2+) signaling in host cells including Ca(2+) release from intracellular stores in a STIM1-dependent way [35]. NPPC or CNP (C-type natriuretic peptide) is part of the natriuretic hormones (NH), a class which includes three groups of compounds: the natriuretic peptides (ANP, BNP and CNP), the gastrointestinal peptides (guanylin and uroguanylin), and endogenous cardiac steroids. These substances stimulate the kidney to excrete sodium and therefore have a function in the regulation of sodium and water homeostasis, blood volume,

and blood pressure [36]. In addition they were also described to act as neurotransmitters or neuromodulators in the brain [37, 38]. CNP was also described to be involved in bacterial virulence and toxin production in host environment by *Pseudomonas aeruginosa,* enhancing its virulence [39]. It was shown that *P. aeruginosa* is sensitive to natriuretic peptides through a cyclic nucleotide-dependent sensor system. A pre-treatment of *P. aeruginosa* with CNP was shown to increase the capacity of the bacteria to kill *Caenorhabditis elegans* through diffusive toxin production. In contrast, brain natriuretic peptide (BNP) did not affect the capacity of the bacteria to kill *C. elegans*. The hypothetical protein LOC122258 (c13orf28) also called SPACA7 (Sperm acrosome-associated protein 7) was recently shown to be a novel male germ cell-specific protein localized on the sperm acrosome, but its involvement in bacterial pathogenesis was never described and it's not easy to find a relationship with NadA3 role in adhesion and invasion. IL-6 (Interleukin 6) is a cytokine with a wide variety of biological functions and plays an essential role in the final differentiation of B-cells into Ig-secreting cells. It is also involved in lymphocyte and monocyte differentiation. IL-6 is involved in response to pathogens and it was shown to down-regulate the activation of the cytokine network in the lung and contributes to host defense during pneumococcal pneumonia [40].

Within the identified hits, LOX-1 among the others constituted a potentially remarkable hit for further characterization since it was the only endothelial receptor identified in the screening. In addition, the interaction between NadA3 and an endothelial receptor as never been described before and it may constitute a key feature in the understanding of *N. meningitidis* pathogenesis. Binding experiments using Octet BLI confirmed the interaction between NadA3 and LOX-1 showing a KD of ~60nM in both the binding orientations. This data was also confirmed by DLS analysis which showed the complex is formed also in solution. The hydrodynamic radius and the calculated MW of both NadA3FL, LOX-1 and the complex is actually much larger than the expected based on calculations carried out on globular proteins. This discrepancy can be easily explained by the rod-shaped structure of the proteins as well as, for the NadA3-LOX-1 complex, by the possible head-to-head interaction, as also suggested by the constructs tested in BLI. In fact, experiments carried out using NadA3 stalk (91-342) and head&neck (24-170) constructs identified the latter as the major binding region. A KD measurement of the $NadA3_{24-170}$ construct on LOX-1 revealed an affinity much higher than the full-length protein. This effect was not investigated further, however it seems to suggest that the decreased steric hindrance of $NadA3_{24-170}$ construct compared to NadA3FL leads to a faster accessibility of LOX-1 for the ligand. The identification of NadA3 head as the binding region for LOX-1 was also confirmed by competition experiments using anti-NadA3 mAbs. All the tested mAbs targeting NadA3 head (24-170) showed competition with LOX-1 for NadA binding, while the mAb 9F11 mapping on NadA stalk (269-316) showed

higher binding intensity if compared with NadA3 alone. This effect is probably due to an increased mass of NadA3 complexed with 9F11 which results in higher interference, and thus in higher signal in BLI. The binding between LOX-1 and NadA3 was further corroborated by cellular binding assay. Despite the low level of LOX-1 transfection (32.9%), all the CHO cells transiently expressing LOX-1 showed binding with recombinant NadA3, confirming the binding happens also on cell surface.

The identification and biophysical validation of an endothelial receptor, among the others, for NadA3 constitutes an additional value for the understanding of *N. meningitidis* pathogenesis. Further studies will be necessary to clarify NadA role in endothelium binding, in order to understand if it can contribute in endothelial crossing, being for this reason responsible in crucial mechanisms like blood-brain barrier crossing and meningitis development.

References

1. Pace, D. and A.J. Pollard, *Meningococcal disease: clinical presentation and sequelae.* Vaccine, 2012. **30 Suppl 2**: p. B3-9.
2. Rouphael, N.G. and D.S. Stephens, *Neisseria meningitidis: biology, microbiology, and epidemiology.* Methods Mol Biol, 2012. **799**: p. 1-20.
3. Pace, D., A.J. Pollard, and N.E. Messonier, *Quadrivalent meningococcal conjugate vaccines.* Vaccine, 2009. **27 Suppl 2**: p. B30-41.
4. Finne, J., et al., *An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues.* J Immunol, 1987. **138**(12): p. 4402-7.
5. Black, S., et al., *Toward a meningitis-free world.* Sci Transl Med, 2012. **4**(123): p. 123ps5.
6. Rosenstein, N.E., et al., *Meningococcal disease.* N Engl J Med, 2001. **344**(18): p. 1378-88.
7. Pizza, M., et al., *Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing.* Science, 2000. **287**(5459): p. 1816-20.
8. Serruto, D., et al., *The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens.* Vaccine, 2012. **30 Suppl 2**: p. B87-97.
9. Comanducci, M., et al., *NadA diversity and carriage in Neisseria meningitidis.* Infect Immun, 2004. **72**(7): p. 4217-23.
10. Malito, E., et al., *Structure of the meningococcal vaccine antigen NadA and epitope mapping of a bactericidal antibody.* Proc Natl Acad Sci U S A, 2014. **111**(48): p. 17128-33.
11. Capecchi, B., et al., *Neisseria meningitidis NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells.* Mol Microbiol, 2005. **55**(3): p. 687-98.
12. Tavano, R., et al., *Mapping of the Neisseria meningitidis NadA cell-binding site: relevance of predicted {alpha}-helices in the NH2-terminal and dimeric coiled-coil regions.* J Bacteriol, 2011. **193**(1): p. 107-15.
13. Cecchini, P., et al., *The soluble recombinant Neisseria meningitidis adhesin NadA(Delta351-405) stimulates human monocytes by binding to extracellular Hsp90.* PLoS One, 2011. **6**(9): p. e25089.
14. Nagele, V., et al., *Neisseria meningitidis adhesin NadA targets beta1 integrins: functional similarity to Yersinia invasin.* J Biol Chem, 2011. **286**(23): p. 20536-46.
15. Gonzalez, R., et al., *Screening the mammalian extracellular proteome for regulators of embryonic human stem cell pluripotency.* Proc Natl Acad Sci U S A, 2010. **107**(8): p. 3552-7.
16. Giuliani, M.M., et al., *A universal vaccine for serogroup B meningococcus.* Proc Natl Acad Sci U S A, 2006. **103**(29): p. 10834-9.
17. Shimaoka, T., et al., *LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria.* J Immunol, 2001. **166**(8): p. 5108-14.
18. Campbell, L.A., et al., *Chlamydia pneumoniae binds to the lectin-like oxidized LDL receptor for infection of endothelial cells.* Microbes Infect, 2012. **14**(1): p. 43-9.

19. Honjo, M., et al., *Lectin-like oxidized LDL receptor-1 is a cell-adhesion molecule involved in endotoxin-induced inflammation.* Proc Natl Acad Sci U S A, 2003. **100**(3): p. 1274-9.
20. Wu, Z., et al., *LOX-1 deletion improves neutrophil responses, enhances bacterial clearance, and reduces lung injury in a murine polymicrobial sepsis model.* Infect Immun, 2011. **79**(7): p. 2865-70.
21. Bozza, G., et al., *Role of ARF6, Rab11 and external Hsp90 in the trafficking and recycling of recombinant-soluble Neisseria meningitidis adhesin A (rNadA) in human epithelial cells.* PLoS One, 2014. **9**(10): p. e110047.
22. Montanari, P., et al., *Human heat shock protein (Hsp) 90 interferes with Neisseria meningitidis adhesin A (NadA)-mediated adhesion and invasion.* Cell Microbiol, 2012. **14**(3): p. 368-85.
23. Yiangou, Y., et al., *Isolation, characterization, and pharmacological actions of peptide histidine valine 42, a novel prepro-vasoactive intestinal peptide-derived peptide.* J Biol Chem, 1987. **262**(29): p. 14010-3.
24. Morampudi, V., et al., *Vasoactive Intestinal Peptide Prevents PKCepsilon-Induced Intestinal Epithelial Barrier Disruption During EPEC Infection.* Am J Physiol Gastrointest Liver Physiol, 2014: p. ajpgi 00195 2014.
25. Murchie, R., et al., *Protein tyrosine phosphatase sigma targets apical junction complex proteins in the intestine and regulates epithelial permeability.* Proc Natl Acad Sci U S A, 2014. **111**(2): p. 693-8.
26. Kita, T., et al., *Role of oxidized LDL in atherosclerosis.* Ann N Y Acad Sci, 2001. **947**: p. 199-205; discussion 205-6.
27. Kita, T., et al., *Oxidized-LDL and atherosclerosis. Role of LOX-1.* Ann N Y Acad Sci, 2000. **902**: p. 95-100; discussion 100-2.
28. Mehta, J.L., et al., *Inhibition of LOX-1 by statins may relate to upregulation of eNOS.* Biochem Biophys Res Commun, 2001. **289**(4): p. 857-61.
29. Sawamura, T., et al., *An endothelial receptor for oxidized low-density lipoprotein.* Nature, 1997. **386**(6620): p. 73-7.
30. Honnappa, S., et al., *An EB1-binding motif acts as a microtubule tip localization signal.* Cell, 2009. **138**(2): p. 366-76.
31. Shaw, P.J., et al., *CD4(+) and CD8(+) T cell-dependent antiviral immunity requires STIM1 and STIM2.* J Clin Invest, 2014. **124**(10): p. 4549-63.
32. Zhang, H., et al., *STIM1 calcium sensor is required for activation of the phagocyte oxidase during inflammation and host defense.* Blood, 2014. **123**(14): p. 2238-49.
33. Schwan, C., et al., *Clostridium difficile toxin CDT hijacks microtubule organization and reroutes vesicle traffic to increase pathogen adherence.* Proc Natl Acad Sci U S A, 2014. **111**(6): p. 2313-8.
34. Hyser, J.M., et al., *Activation of the endoplasmic reticulum calcium sensor STIM1 and store-operated calcium entry by rotavirus requires NSP4 viroporin activity.* J Virol, 2013. **87**(24): p. 13579-88.
35. Usmani, S.M., et al., *Molecular basis of early epithelial response to streptococcal exotoxin: role of STIM1 and Orai1 proteins.* Cell Microbiol, 2012. **14**(3): p. 299-315.
36. Marin-Grez, M., J.T. Fleming, and M. Steinhausen, *Atrial natriuretic peptide causes pre-glomerular vasodilatation and post-glomerular vasoconstriction in rat kidney.* Nature, 1986. **324**(6096): p. 473-6.
37. Komatsu, Y., et al., *C-type natriuretic peptide (CNP) in rats and humans.* Endocrinology, 1991. **129**(2): p. 1104-6.

38. Hodes, A. and D. Lichtstein, *Natriuretic hormones in brain function.* Front Endocrinol (Lausanne), 2014. **5**: p. 201.
39. Blier, A.S., et al., *C-type natriuretic peptide modulates quorum sensing molecule and toxin production in Pseudomonas aeruginosa.* Microbiology, 2011. **157**(Pt 7): p. 1929-44.
40. van der Poll, T., et al., *Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia.* J Infect Dis, 1997. **176**(2): p. 439-44.

Chapter FOUR

Protein microarrays and validation technologies for protein-protein interaction studies: pros and cons

Introduction

Cellular processes take place thanks to wide and extended networks of protein-protein interactions (PPIs) [1]. It is therefore not surprising that with the advance of -omics approaches like proteomics, genomics, transcriptomics and secretomics the map of the interactome, constituted by all the protein-protein interactions identified within a cellular process or compartment, is in exponential increase. The complete map of interaction partners of a protein is critical to understand its function and the cellular process in which it takes part within a cell [2]. Among the incredible number of processes based on protein-protein interactions, host-pathogen interactions represent a key research topic that can help to gain a deeper understanding of molecular mechanism underlying pathogenesis and therefore support an easier and faster identification and development of efficient therapies against pathogenic bacteria. The basic idea which lies beneath this statement is the increasing evidence that surface-exposed bacterial proteins known to target human immune components can possibly constitute good vaccine candidates against that pathogen. Indeed, antibodies elicited against such bacterial proteins have been shown to act in two ways: they mediate direct bacterial lysis through complement activation; and they prevent binding of the immune evasion molecule to its human target, therefore increasing bacterial susceptibility within the host. This is the case for fHbp, a constituent of the 4cMenB vaccine (four component meningococcus B vaccine) that is able to elicit bactericidal antibodies [3-5]. Exploring host-pathogen interactions for these reasons is a precious resource for both scientific and medical community.

Usually, the identification of novel host-pathogen interactions involves an initial medium/large-scale screening, which will hopefully results in the identification of several potential partners for the protein(s) under investigation. Plenty of techniques have been developed for this purpose. Among all, protein microarray is well established and it will be described in detail in this chapter. This technique has several advantages and in the same way different disadvantages, which will also be discussed in the following pages.

The hits derived from the initial screening must be validated to increase the reliability and confidence of the data generated. Indeed, a deep and accurate statistical analysis and/or a downstream experimental validation are required. The lab-based validation of such kind of PPI screening is usually performed through the use of label-based or label-free techniques to confirm and better characterize the interaction. In the past years, thanks to technical advances in optics, several label-free instruments have become the gold standard for PPI validation. In particular, SPR (surface plasmon resonance) and more recently BLI (BioLayer interferometry) are widely used for hit validation and characterization. In addition to these

techniques, the increasing need of simultaneous screening and characterization, led to the development of hybrid instruments like SPRi (Surface Plasmon Resonance Imaging) designed in a way that the advantages of label-free techniques are combined with the higher throughput of the screening methodologies. A description of this technique will also be part of this chapter.

Screening technologies

The first step in the identification of novel protein-protein interactions is constituted by methods that allow the identification of potential interactors of a protein under investigation. A lot of techniques have been developed, each one with different throughputs as well as different pros and cons. the Yeast two hybrid system (Y2H), co-immuno-precipitation, affinity chromatography, protein-fragment complementation, phage display, TAP-MS and protein microarrays are the most used and consolidated in literature. Y2H, protein fragment complementation, phage display and protein microarray are usually used for binary PPI identification and require a laborious process of construct and clone preparation (followed by protein purification in the case of microarrays) since large libraries are usually required to obtain satisfactory results. In contrast, co-immuno-precipitation, affinity chromatography and TAP-MS are used for macro-complexes identification and usually require few construct preparations which then are tested against a complex mixture of proteins such as biological fluids, cell culture supernatants, whole cell preparations or cell compartments.

Protein microarrays

Protein microarray is an emerging screening technology that constitutes a flexible tool for large scale PPI screenings. It allows indeed the simultaneous screening of thousands of proteins in a parallel and high-throughput way. Protein microarray usually consists of purified proteins or antibodies (or DNA, RNA, peptides or glycans for other purposes than PPI discovery) immobilized on a small surface to build an array resembling a miniaturized ELISA plate. Despite the wide panel of diverse configurations that can be used, three major classes of protein microarrays are defined: analytical, functional and reverse-phase protein microarrays [6, 7].

Analytical protein microarrays

In the analytical protein microarray, the affinity or capture agent is immobilized onto the solid array surface and incubated with the test sample (**figure 4.1A**). The most representative model of analytical protein microarrays is the antibody array in which a panel of antibodies is immobilized and tested with an antigen and *vice versa*. In this conformation the analyte is

usually labeled with a fluorescent dye for the detection. Several other immobilization formats have been described, among which the "sandwich" format is the most well known. The “sandwich” format exploits two different antibodies to detect the targeted protein [8, 9]. One is the capture antibody, which is immobilized on the microarray chip, while the reporter antibody is usually fluorescent-labeled, generating a signal when it binds to the captured target protein.

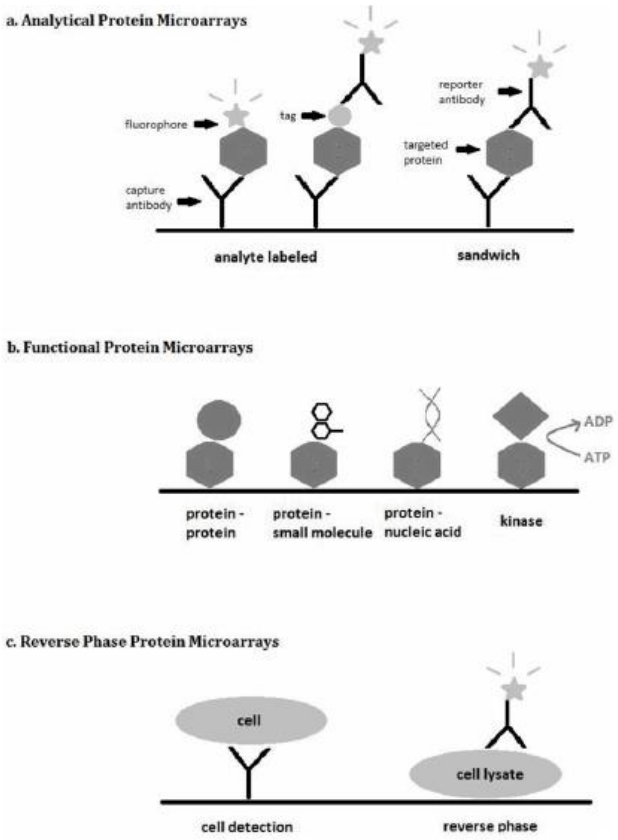


Figure 4.1 The three main categories of protein microarrays. (**A**) Analytical protein microarrays are mainly characterized by antibody arrays. A direct (left) or indirect (center) detection can be used. A reporter antibody is used to create a "sandwich" format (right). (**B**) Functional protein microarrays have broad applications in studying protein interactions, including protein binding and enzyme-substrate reactions. (**C**) Reverse-phase protein microarrays are based on the immobilization of different lysate samples on the same chip. (*adapted from Sutandy et al. 2013*)

Functional protein microarrays

Functional protein microarrays are used to characterize protein functionality at the proteome level (**figure 4.1B**). It has been widely used for the characterization of biochemical properties of proteins, such as binding activities, including protein-protein, protein-DNA, protein-lipid, protein-drug, and protein-peptide interactions.In addition, it has been applied to study enzyme-substrate relationships via different types of biochemical reactions [10] and for profiling protein post-translational modifications, including phosphorylation, ubiquitylation, acetylation, and nitrosylation [11].

Reverse-phase protein microarrays

Reverse-phase microarrays are based on the immobilization of complex samples obtained at different time points or under various experimental conditions (**figure 4.1C**). By direct spotting of tissue, cell lysates or fractionated cell lysates it allows the simultaneous analysis of many different probes. This kind of array is mainly used to monitor histological changes in samples like cancer tissues, allowing a proteomic spatio-temporal profiling of protein expression or post-translational modifications.

Protein microarrays in this thesis: configurations, limitations and future perspectives

The chapters two and three of this thesis describe analytical microarrays in which the target proteins were indirectly detected applying a fluorescently-labeled probe. In particular, in the screening applied to *Staphylococcus aureus*, *S. aureus* recombinant purified proteins were immobilized on the microarray chip, and this chip was tested against the test sample (human proteins fused with a FLAG-tag). Binding was detected using an anti-FLAG antibody and a secondary fluorescently labeled antibody to amplify and increase the signal. In chapter three, instead, human recombinant proteins were immobilized on the chip. A biotinylated N*eisseria meningitidis* protein was used as test sample and the binding was detected using fluorescently labeled streptavidin. The first approach has the major advantage to drastically amplify the signal, with the consequence of an increased detection limit, in particular for low-affinity interactions, which usually are characterized by low MFI (mean fluorescence intensity) values. Despite that, the use of antibodies can constitute a source of non-specific or promiscuous signals requiring an accurate setting of appropriate experimental conditions and a stringent negative control. In the opposite way, in the second approach the use of a biotinylated analyte minimized the non-specific signal, although, also in this case a dedicated negative control was needed. On the other hand, in this experimental setting signal amplification was not obtained and, in addition, direct biotinylation of the protein and the degree of biotinylation could contribute to modify the biological activity of the protein. Since known interactors that could have served as positive controls were not present among the immobilized proteins, it was not possible to understand if the biotinylated protein retained its biological function. This last drawback does not exist if, like in the case of the screening performed in chapter two, an antibody is used for detection, since the protein structure is not modified by any direct coupling procedure.

Furthermore, in both the microarray configurations used, it is not easy to understand whether the immobilized proteins retain their 3D structure and activity [12]. A global check of spotting efficiency is usually performed as validation step, but it typically relies on antibodies directed

against linear epitopes or protein tags, *e.g.* His-tag, GST-tag (Glutathione S-transferase) and Flag-tag. To avoid this issue, in the last years, derivatized surfaces were used to immobilize proteins in an oriented way. Instead of a common adsorption procedure on nitrocellulose or PVDF (Polyvinylidene difluoride) coated chips, the affinity-based capture exploits the use of different tags like His-tag, GST-tag or streptavidin-tag at the N- or C-terminus of a protein to avoid direct immobilization on nitrocellulose and to preserve, at least in principle, its 3D conformation. Since the protein orientation can dramatically influence a given interaction, the part involved in binding may be buried, preventing the protein partner to interact. Thus, both the N- and C-terminal tagged protein versions should be produced to obtain reliable results.

Protein microarray constitutes a very powerful large scale tool for PPI discovery and the amount of data generated with a single experiment is very high, since several hundreds of interactions can be found with a screening. Despite that, the approaches used in this work and the previously described microarrays formats do not allow the identification of very low affinity interactions (*i.e*. micromolar interactions). In nature, indeed, many protein-protein interactions are weak yet biologically meaningful. Recently, an avidity-based approach (AVEXIS - Avidity based extracellular interaction screening) was developed to fill this gap. Through the forced pentamerization of the proteins to be assessed, it has been possible to increase their affinity for the immobilized ones, thus allowing the identification of low affinity interactions [13, 14]. This approach constitutes a great breakthrough in the identification of novel PPIs.

Validation technologies

Downstream of a large scale screening approach that resulted in identification of novel protein-protein interactions, a validation process is necessary to confirm and characterize these interactions. In the case of PPI, validation methods usually are classified by two main categories: label-based and label-free technologies. While the label-based systems rely on the use of chemical modifications such as sequence tags, biotinylation, or fluorescent chromophores, the label-free detection techniques measure a physical property of the protein, such as mass, dielectric or optical properties that changes in the course of the interaction. Whereas label-based techniques provide a relatively simple, rapid and cost effective detection method, label-free approaches are more preferred since they offer higher sensitivity and real-time kinetic data. In addition, the detection without the interference of any labeling agent is an advantage since in principle should ensure proper protein folding and functionality. Label-free approaches indeed rely on the measurement of an intrinsic property of the query molecule without the use of any label. The limitations posed by the label-based

detection strategies have increased the interest in label-free approaches which are overcoming the limitations intrinsic to label-based approaches.

SPR (Surface Plasmon Resonance)

Surface plasmon resonance (SPR) is a label-free technique that allows to measure biomolecular interactions real-time and with very high sensitivity. In addition it allows the direct and rapid determination of association (k_{ass} or k_{on}) and dissociation (k_{dis} or k_{off}) rates of a binding process, the determination of the strength of the binding and the specificity of interactions [15]. It is based on the creation of surface plasmons, which are oscillations of free electrons that propagate parallel to a metal/dielectric interface. The conventional SPR technique requires one binding component (the ligand) to be immobilized on a sensor chip whilst the other binding component in solution (the analyte) is flowed over the sensor surface. Signal obtained from SPR is derived from the measure of changes in refractive index in the proximity of the sensor surface [16]. Any adsorption or desorption of molecules, therefore any binding close to the biosensor surface, is reflected in the sensorgram by a change in the reflection intensity with respect to the incident angle (**Figure 4.2**).

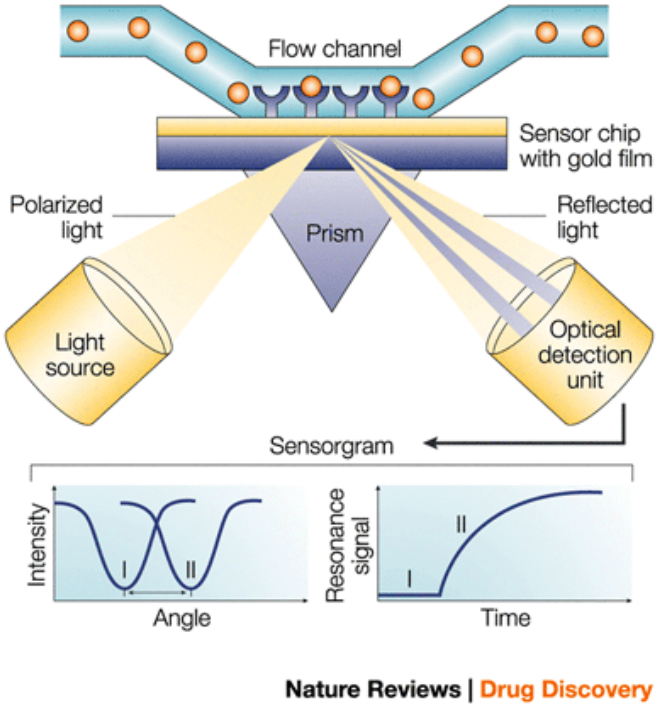


Figure 4.2 Surface plasmon resonance (SPR) principle. SPR detects changes in the refractive index in the immediate vicinity of the surface layer of a sensor chip. The SPR signal is dependent on the mass of material at the surface. (*adapted from Cooper, 2002, Nature Reviews Drug Discovery 1, 515-528*)

The most popular SPR instrument is BiaCore (Ge Healthcare), which is considered the gold standard for binary PPI validation and characterization. It relies on gold layer based sensor

chips, usually derivatized with a wide variety of different chemicals or compounds (*e.g.* carboxymethyl dextran, streptavidin, or functionalized with several different kinds of antibodies) by which the ligand protein is immobilized or captured. Depending on the type of sensor chip, the immobilization procedure may change. The sensor chip is inserted in the instrument and coupled to to a microfluidic-based system where the flow cells (usually four) are formed and onto which the analyte sample is injected. A real-time signal measured in RU (Response Units) is the output for the binding. The great sensitivity allows not only the characterization of very weak interactions but also it permits the study of protein-small molecule interactions. Despite the broad range of applications it requires a deep instrumental knowledge and good experimental settings to achieve the best results, since several parameters like the immobilization threshold and regeneration conditions are critical for correct data generation and interpretation.

BioLayer Interferometry (BLI)

Recently, BLI (BioLayer Interferometry), a novel optical technique, has been developed by ForteBio under the name of the "Octet" system for PPI validation and screening. BLI is an optical analytical method that measures interference patterns formed by light waves. Central component of the device is a biosensor tip, through which white light is passed. Light is reflected from each of the two ends of the tip and, depending on the distance between these ends, the reflected beams interfere constructively or destructively at different wavelengths in the spectrum. The interference pattern is then detected by a CCD array detector.

The binding of molecules to the tip forms a layer that increases in thickness as more and more target molecules bind to the surface. As the thickness at the tip grows, the effective distance between the two reflective layers increases, creating a shift in the interference pattern formed by the reflected light. The spectral-intensity maximum of the reflected light changes as a function of the optical thickness of the molecular layer. Consequently, any change in the number of molecules bound to the biosensor tip causes a shift of the spectral maximum (Δλ expressed in nanometers or nm) that can be measured in real-time and reported as a sensorgram (**figure 4.3**). Thus, a change in optical mass thickness of 1 nm results in a 1 nm shift in the interferometry wave pattern. According to this principle, association is detected as a positive shift caused by the increase in thickness of the layer, while dissociation is correlated with a negative shift, thus providing the basics for binding kinetics calculation [17].

Similarly to SPR, the analysis of biomolecular interactions starts with the immobilization of a ligand on the biosensor surface. Also in this case a wide variety of tips with different surface chemistries are available on the market. The ligand-bearing tip, previously blocked against

non-specific binding (if needed), is then used to monitor direct interactions with an analyte diluted in a buffer solution. The real-time signal in this case is expressed as a nanometer shift. The absence of microfluidics or flow cells permits also the direct use of crude samples like cell extracts or sera. In addition, the possibility to screen in simultaneously 8 or 16 tips (depending on the instrument type) allows for the screening of different samples or working conditions very easily and in short time. This configuration allows also for working with regeneration-sensitive samples. Furthermore, the user friendly software allows for easier experimental design in comparison with BiaCore. Nevertheless, due to its physical principle itself, the sensor tips are characterized by a two-dimensional surface, therefore loosing the advantages provided by dextran-coated matrixes used in SPR and SPRi instruments. In addition, the instrument sensitivity is lower as compared with SPR, resulting in certain limitations for its experimental use.

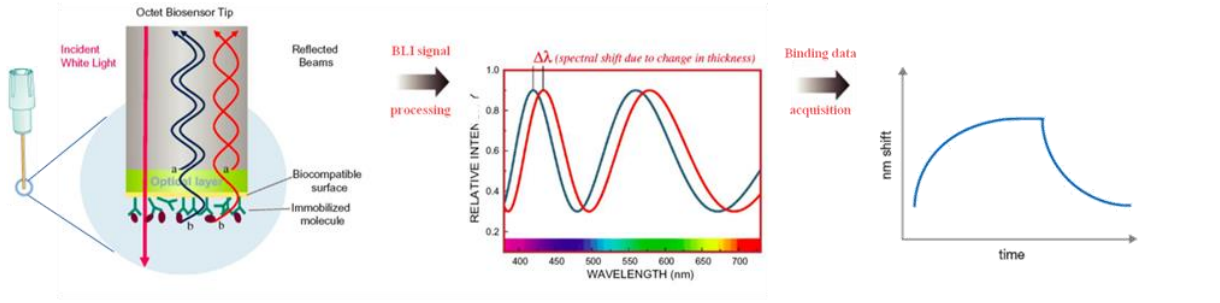


Figure 4.3 BLI working principle. White light is run through the biosensor tip producing constructive and destructive waves (left panel). Unbound molecules and changes in the refractive index of the medium do not affect the interference pattern. The wavelength shift (Δλ) is a direct measure of the change in thickness of the biological layer (central panel). Wavelength shift is plotted against time to have BLI sensograms (right panel) (*adapted from Pall-Fortebio website - http://www.fortebio.com/octet-platform.html*)

SPRi

In the last years, the need of a faster, more sensitive and higher throughput screening technology has driven the development of hybrid instruments that can merge sensitivity and real-time data acquisition of label-free technologies with the high throughput of screening technologies like protein microarrays. This is the case of SPRi (Surface Plasmon Resonance imaging - Horiba), an SPR-based instrument with a medium throughput. The SPRi instrument's working principle is indeed very similar to that of the BiaCore, since it is based on an SPR detection system with a sensor chip on which ligand proteins are immobilized and a flow cell though which the analyte protein is injected. The medium-throughput of this instrument derives from the protein immobilization procedure. While in common SPR instruments the ligand is occupying the entire flow cell, in SPRi the ligand proteins are spotted in a microarray-like manner. The surface chemistry of the SPRi sensor chip is usually characterized by gold surface derivatization using similar reagents as conventional SPR. The

result is a double advantage of identifying and characterizing interactions between an analyte and hundreds of ligand proteins simultaneously. Furthermore, the charge-coupled device (CCD) camera present in the instrument is able to reconstruct an instantaneous image of the flow cell showing the real-time binding between a ligand spot and the analyte protein (**figure 4.4**). Protein binding to the ligand spot increases the refractive index close to the surface and changes the fraction of light reflected at that spot, thus generating the SPR signal.

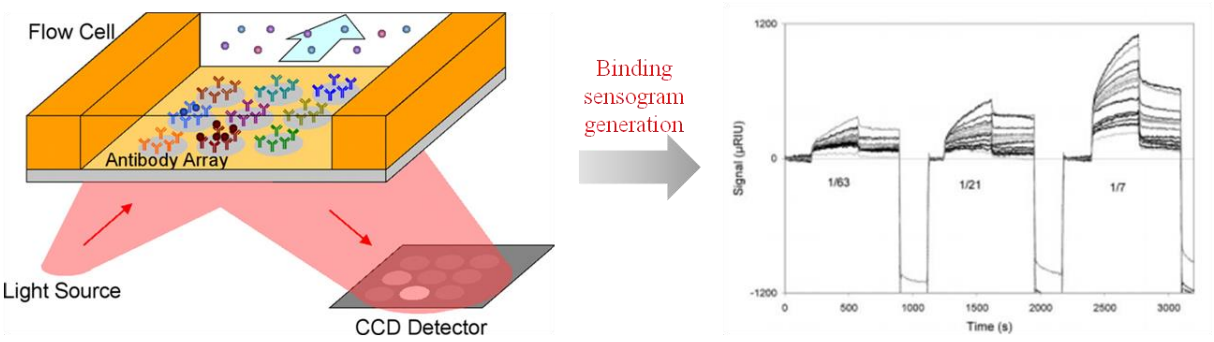


Figure 4.4 The design of an SPRi binding assay. Proteins are spotted in a microarray format on gold-covered- glass slides. Analyte protein is tested simultaneously against all the immobilized proteins. An SPR sensorgram is generated for each protein spot (right panel). In addition, a visual reconstruction of the array is produced. (*adapted from Lausted et al 2008 Mol. Cell. Proteomics)*

Despite the many advantages that this technique provides, several cons need to be considered. First, while protein microarray slides are in mostly disposable, meaning that only one analyte protein is tested on a given slide, SPRi slides can be regenerated. This may constitute an important additional value in terms of time and costs for ligands with similar properties (*e.g.* a panel of antibodies). Nevertheless, since regeneration conditions can vary a lot between different ligand proteins, it may be difficult or impossible to find a common regeneration setting for ligands with differing or unknown behavior, allowing for complete dissociation of the ligand without damaging the immobilized proteins.

Final considerations

Protein-protein interaction discovery and characterization is becoming more and more simplified thanks to a broad range of techniques and instruments specifically developed for this purpose. Of course each method has its own pros and cons, but in a more general view it is possible to summarize some positive features and negative issues that accompany these techniques. When dealing with PPI, in fact it is worth to keep in mind some general aspects that arise during the experimental procedure:

- All the techniques presented in this chapter rely on the immobilization of one of the two putative binding partners; the immobilization procedure whether it is covalent, affinity-based (like in case of biotin streptavidin or antibody-based immobilization) or

diffusion-based (like in case of nitrocellulose for protein array) can contribute to the loss of the native 3D structure of the protein or to its false orientation. This aspect can dramatically and irreversibly influence the binding properties of the ligand, resulting in false negative results. In most cases, especially when validating with label-free technique a binary interaction, both the interaction partners should be used both as ligand and analyte, in particular if the immobilization procedure mask a part of the protein involved in the binding (*e.g.* a protein immobilized though amine coupling in which a patch of lysines are involved in the binding). This is often impossible in large-scale screening like in the case of protein microarray, but it is the price to pay in high-throughput methods. Oriented capture using protein tag can partially solve this problem. However, it brings as a drawback the need for even larger number of protein constructs, since for a complete dataset for each protein to be tested, two versions, tagged either at the N or the C-terminus, would be required.

- Similar considerations need to be made also for regeneration procedures, which are based on the use of low pH, high ionic strength or detergents. These compounds can indeed alter the protein folding, thus modifying the response to the binding partner. For this reason it is important to screen an extended set of possible regeneration conditions, trying to find those that even after repeated exposure to the regenerating agent do not change the original binding properties of the molecule under investigation This aspect is crucial for SPR and SPRi, with the limitations already described, while it can be overcome in protein microarray and BLI.
- Protein folding and retained functionality can also be modified by direct coupling of labels. Label-based methods, which requires direct labelling of one of the two protein partners, have indeed the great disadvantage of relying on a label for the detection. The presence of fluorescent labels or biotin in the binding site, for example, can interfere with the binding between the two proteins. It is therefore not easy to understand if and how labelling procedure would influence the binding. This drawback can be overcome thanks to the use of secondary dyes (like labelled secondary antibodies) in order to prevent direct labelling of one of the binding partners.
- When dealing with PPI screening and validation, is very important to keep in mind the detection limit (in terms of binding affinity) of the technique used for the assay. This consideration is important for both protein microarrays and label-free technologies. Protein microarrays used with purified proteins as both ligand and analyte, have indeed an important detection limit defect. All the usual detection procedures lead to a partial (in most of cases even total) loss of the low affinity interactions, since repeated washes during these procedures usually disrupt such a kind of binding between two proteins. This drawback may result in a large number of false negative

combinations, meaning that also known low-affinity interactions might not be detected under certain conditions in a protein microarray screening. This disadvantage is absent in SPR, BLI and SPRi, since real-time measurements and the absence of washes procedures allow to monitor and characterize also low-affinity interactions.

- An additional important quality to take in consideration is the sensitivity, in terms of mass of analyte protein needed to identify the interaction. In the case of protein microarray, this is strictly related to binding affinity, since the higher the affinity, the higher the mass of analyte protein will be retained on the spot. In the case of label-free techniques, sensitivity is a crucial parameter for the investigation of interactions involving low MW proteins or peptides.
- A final note considering the preparations upstream of screening and PPI characterization, is on sample preparation and purity. It is quite obvious that for a large scale screening, the purer the protein samples are, the less artefacts, background noise or false positives can arise. With the development of cutting-edge instrumentation in high-throughput protein expression and purification, the quality of the protein used in large scale screening is steadily increasing, bringing also a higher quality and confidence for the data generated. Less obvious, instead, are considerations on sample quality used for most label-free techniques. In this case, sample quality is not only referring to possible contaminations from heterologous proteins, but also from different oligomeric or even aggregated forms of the protein itself. For a correct data interpretation and binding analysis, it is indeed fundamental to use the most homogeneous and well characterized samples available in order to minimize misleading results.

In summary, when dealing with PPIs discovery and validation, it is worth to keep in mind the fragile and susceptible structure of the proteins. Thus, it is important, while setting up the experimental conditions, to choose the one that implies a minimum of protein modification and the most native conditions. Furthermore, since each method has its own advantages and disadvantages, it is recommended to validate the interaction with at least two different techniques to obtain the most reliable results.

References

1. Braun, P. and A.C. Gingras, *History of protein-protein interactions: from egg-white to complex networks.* Proteomics, 2012. **12**(10): p. 1478-98.
2. Jaeger, S. and P. Aloy, *From protein interaction networks to novel therapeutic strategies.* IUBMB Life, 2012. **64**(6): p. 529-37.
3. Beernink, P.T. and D.M. Granoff, *Bactericidal antibody responses induced by meningococcal recombinant chimeric factor H-binding protein vaccines.* Infect Immun, 2008. **76**(6): p. 2568-75.
4. Madico, G., et al., *The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance.* J Immunol, 2006. **177**(1): p. 501-10.
5. Seib, K.L., et al., *Factor H-binding protein is important for meningococcal survival in human whole blood and serum and in the presence of the antimicrobial peptide LL-37.* Infect Immun, 2009. **77**(1): p. 292-9.
6. Fuentes, M., *Protein microarrays overview.* Recent Pat Biotechnol, 2013. **7**(2): p. 83.
7. Sutandy, F.X., et al., *Overview of protein microarrays.* Curr Protoc Protein Sci, 2013. **Chapter 27**: p. Unit 27 1.
8. Haab, B.B., *Multiplexed protein analysis using antibody microarrays and label-based detection.* Methods Mol Med, 2005. **114**: p. 183-94.
9. Poetz, O., et al., *Protein microarrays: catching the proteome.* Mech Ageing Dev, 2005. **126**(1): p. 161-70.
10. Poetz, O., et al., *Protein microarrays for antibody profiling: specificity and affinity determination on a chip.* Proteomics, 2005. **5**(9): p. 2402-11.
11. Hu, S., et al., *Functional protein microarray technology.* Wiley Interdiscip Rev Syst Biol Med, 2011. **3**(3): p. 255-68.
12. Zhu, H., et al., *Severe acute respiratory syndrome diagnostics using a coronavirus protein microarray.* Proc Natl Acad Sci U S A, 2006. **103**(11): p. 4011-6.
13. Martin, S., et al., *Construction of a large extracellular protein interaction network and its resolution by spatiotemporal expression profiling.* Mol Cell Proteomics, 2010. **9**(12): p. 2654-65.
14. Bushell, K.M., et al., *Large-scale screening for novel low-affinity extracellular protein interactions.* Genome Res, 2008. **18**(4): p. 622-30.
15. Ramachandran, N., et al., *Emerging tools for real-time label-free detection of interactions on functional protein microarrays.* FEBS J, 2005. **272**(21): p. 5412-25.
16. Ray, S., G. Mehta, and S. Srivastava, *Label-free detection techniques for protein microarrays: prospects, merits and challenges.* Proteomics, 2010. **10**(4): p. 731-48.
17. Wallner, J., et al., *Application of Bio-Layer Interferometry for the analysis of protein/liposome interactions.* J Pharm Biomed Anal, 2013. **72**: p. 150-4.

Discussion and conclusions

Thousands years of host and pathogen co-evolution have driven to the development of fine mechanisms for pathogen clearance and astute effectors for bacterial survival within the host. Every day, within the host field, a real molecular battle between the hosts' and pathogen's molecular arsenals take place and a precarious balance between the two armies lie beneath pathogenesis or health. Thus, a wide variety of host-pathogen interactions occur giving rise to a global cross-talk between the two components. The vast majority of these interactions take place between the host and pathogen extracellular effectors, namely the extracellular proteomes, constituted by all the proteins secreted or surface exposed by the cells. From human side, typical effectors are proteins constituting the innate immune system, like the complement proteins and antimicrobial peptides, the first lines of human defense. Complement system is indeed characterized by a cascade of proteolytic events involving more than 30 proteins which are activated by non-self stimuli [1-3]. Three main pathway have been described, each of it recognizing specific non-self molecules. The complement classical pathway recognizes mainly aggregated antibodies on bacterial/viral surfaces, the complement lectin pathway recognizes carbohydrates on bacterial surface while alternative pathway is self-activating. All the pathways converge in the central cleavage of complement component C3 driving to bacterial opsonization, phagocytosis and direct killing thanks to membrane attack complex (MAC) formation. During cascade activation pro-inflammatory products (anaphylatoxins) are produced leading to cell mediated immune response activation. Pathogenic bacteria and viruses have evolved several mechanisms to adhere to host cells, penetrate, elude the immune system and spread throughout the host body. In the recent years a more detailed but still incomplete understanding of the molecular mechanisms which underlie bacterial pathogenesis has started to grow. Plenty of examples are present in literature and the knowledge is spreading widely through the main pathogenic bacteria [4-8]. *Staphylococcus aureus* and *Neisseria meningitidis* constitutes two peculiar examples. *S.aureus* has widely been described as the master in immune evasion, since it possess a plethora of proteins identified to specifically target host immune components. In particular extensive literature is present on its ability in complement evasion [9, 10]. Several *S. aureus* proteins can indeed bind complement proteins and complement receptors therefore impairing the first line in immune response. *N. meningitidis*, in a similar way, has been described to be able to recruit complement regulators consequently resulting complement down-regulation and inhibition [11-13]. Bacteria take advantage of the common structural features of complement regulators to broaden the number of human protein targeted; it is not uncommon, indeed, that a single bacterial protein targets multiple host proteins with common structural features. All the studies has led to the identification of three main mechanism of

complement activation: the recruitment or mimicking of complement regulators, the inactivation by enzymatic degradation (proteolysis) and the modulation or inhibition of complement proteins by direct interactions [14-20].

Despite the large variety of examples already described, a deeper knowledge in mechanisms that underlie bacterial pathogenesis and immune evasion is essential for therapeutic purposes. Bacterial immune evasion molecules and all the surface-exposed molecules that can be target for complement activating or neutralizing antibodies constitutes indeed excellent vaccine antigens. Capsular polysaccharides from *pneumococcus* and *meningococcus*, for example, are part of effective licensed vaccines [21]. Furthermore, factor H binding protein (fHbp) from *N. meningitidis*, a protein involved in complement factor H binding and deregulation of complement activity within the host, is part of the recently released 4 components vaccine against type B *meningococcus* (4cMenB) [22-24]. Antibodies elicited against fHbp act both mediating direct bacterial lysis through classical complement pathway activation, and preventing binding of fHbp to complement factor H thereby increasing bacterial susceptibility to complement [25-27]. The large number of interactions discovered so far, sometimes promiscuous and involving multiple components of the human defense apparatus, seems to suggest a very complex picture where additional interactions may take place at the host-pathogen interface. In this study an un-biased systematic large scale protein microarray-based approach was used to indentify novel interactions between the human and the *S. aureus* extracellular proteomes. The same approach was used to identify novel potential receptors for the Neisseria adhesin A (NadA), part of the 4CMenB vaccine and key determinant of mengicoccal interactions with the human host at different stages of infection.

In chapter two, *S. aureus* proteins predicted from bioinformatic analysis to be surface exposed or secreted were efficiently cloned, expressed and purified in high-throughput way. The 159 purified proteins (108 as single constructs and 10 as multiple constructs), among which known virulence factors were present, were spotted on nitrocellulose coated slides together with internal and positive controls. The immobilization of the *S. aureus* proteins on the chip was confirmed by preliminary validation experiments. The resulting microarray chips were tested using 75 human recombinant proteins selected from a larger library [28]. Among the human proteins several complement factors, complement receptors and coagulation factors and receptors were present. The screening resulted in a total of 11766 combinations between pairs of human and *S. aureus* proteins. Among these, as expected, only a small proportion (<5%) gave a positive signal for interactions and, in particular, 457 (4%) putative interactions have been detected with a low signal, 42 (0,36%) with a medium and 19 (0,16%) with a highly reactive one, on the basis of their normalized MFI values. The appropriate

incubation conditions developed for the screening, the correct thresholds set for the analysis and the integrity of the bacterial and human proteins were confirmed by the positive controls constituted by fHbp, which showed high MFI values when incubated with complement factor H. This large scale approach led also to the identification and exclusion from analysis of non-specific interactions. Human ficolin 2 and staphylococcal protein A, for example, were non-specifically reactive with several proteins and could be easily recognized. Interestingly, the 17 hits (19 minus the positive controls) found in the array were shared between LytM, a staphylococcal autolysin previously shown to be an important regulator of *S. aureus* cell cycle and cell wall turnover [29], Csa1D, member of the Conserved Staphylococcal Antigen family described as a promising vaccine antigen in Schluepen et al 2013 [30] and FLIPr, previously described as potent inhibitor of formyl-peptide receptors and FcγR antagonist [31, 32]. This last protein was found to interact, potentially strongly based on the detected signals, with six different human proteins: the intercellular adhesion molecule 5 (ICAM5), the heat stable enterotoxin receptor (GUCY2C), the complement component 1q subcomponent like 4 (C1QL4), the matrix remodelling associated protein 8 (MXRA8), the complement component 1q subcomponent B (C1QB) and the bone sialoprotein 2 (IBSP). All the potential interactions found might be interesting for their relevance in the different steps of pathogenesis, but further work is currently needed to prove and underline their functional and biological relevance. On the other hand, a closer analysis of the human proteins reactive with FLIPr, to a lesser, but still significant extent, revealed additional potential interactors among which the complement component 1q subcomponent A (C1QA), the complement component 1q subcomponent like 2 (C1QL2) and the complement component 1q subcomponent C (C1QC - MFI 8302) constituted potentially remarkable hits for further validation and characterization since involved key players of the offense/defence mechanism. The interaction between FLIPr and the C1qA, C1qB and C1qC subcomponents was validated through the use of BLI (BioLayer Interferometry). The analysis showed low nanomolar affinity, meaning of a high affinity interaction, as also expected from MFI (Mean Fluorescent Intensity) signals showed by protein microarray. A direct correlation between MFI signals and interaction affinity was indeed previously described [33, 34]. Since in serum the three C1q subcomponents complex together to form the C1q complex, additional binding experiments were performed using C1q complex purified from human serum. The resulting affinity changed in order to the immobilized protein. A low picomolar affinity was measured when FLIPr was immobilized, while high nanomolar affinity was measured when C1q was immobilized. The reason of this discrepancy is still unclear and was not deeply investigated, but in our hypothesis, since the C1q complex possess possibly multiple binding sites for FLIPr, it is probably caused by an avidity effect of the C1q complex when FLIPr is immobilized, as also previously shown for other bacterial C1q-binding protein [35]. In order to have the biological affinity constant of the

complex, an in-solution technique (e.g ITC) should be used. Nevertheless, this type of technique is avid of protein, and a lot of additional work should be needed to provide the material necessary for the experiment. Additional *in vitro* assays provided functional information on this protein complex and confirmed the importance of FLIPr in bacterial pathogenesis. FLIPr was indeed able to significantly reduce the complement classical pathway activation *in vitro* in WiELISA experiment. Despite this result, it is not yet clear if FLIPr directly prevent complement activation or whether it activates complement, leading to consumption of the complement proteins and thus resulting in a lower complement activation in the assay, as also previously described for others bacterial proteins [36]. Even though complement inhibition and deregulation is the main mechanism in immune evasion, there are increasing evidences that some pathogens (in particular viruses) actively induce complement activity [4, 37]. A fine balance between complement inhibition and activation is indeed used by pathogens to prevent killing on one side, and favour phagocytosis on the other. The FLIPr pro-evasion properties were also confirmed by whole blood assay, in which the addition of FLIPr resulted in dramatically higher *S. aureus in vitro* survival. In this work FLIPr is described for the first time as a C1q-binder and complement inhibitor protein, while in literature several works described FLIPr as an inhibitor of the FcγR, a phagocytosis activator [31, 32]. Combining the data present in literature and the functional data obtained in this work an "affinity driven model" was presented. In this model when *S. aureus* faces low serum environment (e.g. interstitial space), FLIPr preferentially binds FcγR to inhibit neutrophil mediated phagocytosis. When *S. aureus* is instead exposed to high serum environment (e.g. blood) preferentially binds C1q. This can result in both complement inhibition, resulting in FcγR mediated phagocytosis or in complement activation, resulting as well in complement mediated phagocytosis. In this model *S. aureus* might selectively promote phagocytosis when it deals with unfavorable environment conditions (complement pressure), preferring an intracellular lifestyle in neutrophils instead of bloodstream. This model can also represent a good proof of concept for the Trojan horse theorized by Thwaites et al. [38] for which neutrophils represent a privileged site for *S. aureus* in the bloodstream, offers protection from most antibiotics and provides a mechanism by which the bacterium can travel to and infect distant sites.

The same large scale approach, was also used in chapter three to identify potential novel receptor for NadA. In this case, instead, the whole human library constituted by ~2700 human recombinant proteins was spotted on slides. NadA variant 3 (NadA3) was tested in overlay at two concentrations and lead to the identification of several human putative interactors. The hits resulted from the screening using NadA3 at 150nM were two isoforms of the VIP (Vasoactive Intestinal Peptides), LOX-1 (low-density oxidized lipoprotein lectin-like

receptor 1) and PTPRS (Protein Tyrosine Phosphatase Receptor type Sigma isoform 4). All these proteins have been previously described to be involved in bacterial pathogenesis [39-48]. STIM1 (Stromal interaction molecule 1 precursor), NPPC (Natriuretic Peptide Precursor C), the hypothetical protein LOC122258 precursor and IL-6 (Interleukin 6 precursor) were instead revealed only in the screening performed at 1µm, possibly meaning a lower affinity for NadA if compared to the previous hits, which were all confirmed also in this second screening. As well, for all of them except for the hypothetical protein LOC122258, an implication in bacterial pathogenesis has been previously described [49-59]. Despite the identification of such a large number of hits, is it possible that many of the protein-protein interaction are weak in nature yet biologically meaningful. Moreover, there is no guarantee all proteins retain their secondary and tertiary structure when immobilized or their biological activity.

Within the identified hits, LOX-1, the receptor for oxidized low-density lipoproteins, constituted a potentially remarkable hit for further characterization since it was the only endothelial receptor identified in the screening. In addition, the interaction between NadA3 and an endothelial receptor was never described before and it can constitute a key feature in the understanding of *N. meningitidis* pathogenesis. Binding experiments using BLI confirmed the interaction between NadA3 and LOX-1 showing an affinity of ~60nM constant in both the binding orientations. This data was also confirmed by dynamic light scattering (DLS) analysis which showed the complex is formed also in solution. The hydrodynamic radius and the calculated MW of both NadA3, LOX-1 and the complex is actually much larger than the expected based on calculations carried out on globular proteins. This discrepancy can be easily explained by the rod-shaped structure of the proteins as well as, for the NadA3-LOX-1 complex, by the possible head-to-head interaction, as also suggested by the constructs tested in BLI. Indeed further experiments carried out using NadA3 stalk (91-342) and head&neck (24-170) constructs identified the latter as the major binding region. Affinity constant measurement of the $NadA3_{24-170}$ construct on LOX-1 revealed an affinity much higher than the full-length protein. This effect was not investigated further, however it seems to suggest that the decreased steric hindrance of $NadA3_{24-170}$ construct compared to $NadA3_{24-342}$ leads to a faster accessibility of LOX-1 for the ligand. The identification of NadA3 head as the binding region for LOX-1 was also confirmed by competition experiments using anti-NadA3 mAbs. All the tested mAbs targeting NadA3 head (24-170) showed competition with LOX-1 for NadA binding, while the mAb 9F11 mapping on NadA stalk (269-316) showed higher binding intensity if compared with NadA3 alone. This effect is probably due to an increased mass of NadA3 complexed with 9F11 which results in higher interference, and thus in higher signal in BLI. The binding between LOX-1 and NadA3 was further corroborated by cellular binding assay. Despite the low level of LOX-1 transfection (32.9%), all the CHO

cells transiently expressing LOX-1 showed binding with recombinant NadA3, confirming the binding happens also on cell surface.
The identification and biophysical validation of an endothelial receptor, among the others, for NadA3 constitutes an additional value for the understanding of *N. meningitidis* pathogenesis. NadA was indeed always showed to bind receptors on monocytes and epithelial cells *in vitro* [60, 61]. Further studies will be necessary to clarify NadA role in endothelium binding, in order to understand if it can contribute in endothelial crossing, being for this reason responsible in crucial mechanisms like blood-brain barrier crossing and meningitis development.

This work has widely demonstrated that protein microarray constitutes an un-biased powerful large scale tool for discovery of novel host-pathogen interactions. Despite the need of large libraries to be expensively (in terms of money, resources and time) produced for the screening, the amount of data generated over time with such approach can easily compensate for material preparation. In addition, being a "non-hypothesis-driven" approach, it prevents hypothesis-based bias in the investigation of an interaction mechanism. Of course, as a drawback, a deep statistical analysis or, like in the case of this work and as well as for other screening techniques, a downstream lab-based validation is needed to confirm the validity of the identified hits. Despite that, in this work protein microarray was confirmed to be a very reliable method, since the two out of two hits investigated more deeply were also confirmed by downstream analysis.

In summary, this work hopefully contributed in the understanding of a precise molecular mechanism that *S. aureus* uses for complement evasion, as well as shed light on human receptors targeted by *N. meningitidis* NadA, contributing in the understanding of molecular mechanisms used for pathogenesis. In addition this work possibly contributed to technical advances in the identification of novel host-pathogen, and widely, protein-protein interactions.

References

1. Carroll, M.V. and R.B. Sim, *Complement in health and disease.* Adv Drug Deliv Rev, 2011. **63**(12): p. 965-75.
2. Walport, M.J., *Complement. First of two parts.* N Engl J Med, 2001. **344**(14): p. 1058-66.
3. Walport, M.J., *Complement. Second of two parts.* N Engl J Med, 2001. **344**(15): p. 1140-4.
4. Lambris, J.D., D. Ricklin, and B.V. Geisbrecht, *Complement evasion by human pathogens.* Nat Rev Microbiol, 2008. **6**(2): p. 132-42.
5. Bardoel, B.W. and J.A. Strijp, *Molecular battle between host and bacterium: recognition in innate immunity.* J Mol Recognit, 2011. **24**(6): p. 1077-86.
6. Okumura, C.Y. and V. Nizet, *Subterfuge and sabotage: evasion of host innate defenses by invasive gram-positive bacterial pathogens.* Annu Rev Microbiol, 2014. **68**: p. 439-58.
7. Zipfel, P.F., T. Hallstrom, and K. Riesbeck, *Human complement control and complement evasion by pathogenic microbes--tipping the balance.* Mol Immunol, 2013. **56**(3): p. 152-60.
8. Potempa, M. and J. Potempa, *Protease-dependent mechanisms of complement evasion by bacterial pathogens.* Biol Chem, 2012. **393**(9): p. 873-88.
9. Serruto, D., et al., *Molecular mechanisms of complement evasion: learning from staphylococci and meningococci.* Nat Rev Microbiol, 2010. **8**(6): p. 393-9.
10. Zipfel, P.F. and C. Skerka, *Staphylococcus aureus: the multi headed hydra resists and controls human complement response in multiple ways.* Int J Med Microbiol, 2014. **304**(2): p. 188-94.
11. Lewis, L.A. and S. Ram, *Meningococcal disease and the complement system.* Virulence, 2014. **5**(1): p. 98-126.
12. Welsch, J.A. and S. Ram, *Factor H and neisserial pathogenesis.* Vaccine, 2008. **26 Suppl 8**: p. I40-5.
13. Lepage, P. and B. Dan, *Infantile and childhood bacterial meningitis.* Handb Clin Neurol, 2013. **112**: p. 1115-25.
14. Zipfel, P.F., R. Wurzner, and C. Skerka, *Complement evasion of pathogens: common strategies are shared by diverse organisms.* Mol Immunol, 2007. **44**(16): p. 3850-7.
15. McKenzie, R., et al., *Regulation of complement activity by vaccinia virus complement-control protein.* J Infect Dis, 1992. **166**(6): p. 1245-50.
16. Rosengard, A.M., et al., *Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement.* Proc Natl Acad Sci U S A, 2002. **99**(13): p. 8808-13.
17. Rooijakkers, S.H. and J.A. van Strijp, *Bacterial complement evasion.* Mol Immunol, 2007. **44**(1-3): p. 23-32.
18. Oda, T., et al., *Inactivation of chemotactic activity of C5a by the serratial 56-kilodalton protease.* Infect Immun, 1990. **58**(5): p. 1269-72.
19. Chmouryguina, I., et al., *Conservation of the C5a peptidase genes in group A and B streptococci.* Infect Immun, 1996. **64**(7): p. 2387-90.
20. Tsao, N., et al., *Streptococcal pyrogenic exotoxin B cleaves properdin and inhibits complement-mediated opsonophagocytosis.* Biochem Biophys Res Commun, 2006. **339**(3): p. 779-84.
21. Pace, D., A.J. Pollard, and N.E. Messonier, *Quadrivalent meningococcal conjugate vaccines.* Vaccine, 2009. **27 Suppl 2**: p. B30-41.
22. Masignani, V., et al., *Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870.* J Exp Med, 2003. **197**(6): p. 789-99.

23. Pizza, M., et al., *Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing.* Science, 2000. **287**(5459): p. 1816-20.
24. Serruto, D., et al., *The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens.* Vaccine, 2012. **30 Suppl 2**: p. B87-97.
25. Madico, G., et al., *The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance.* J Immunol, 2006. **177**(1): p. 501-10.
26. Seib, K.L., et al., *Factor H-binding protein is important for meningococcal survival in human whole blood and serum and in the presence of the antimicrobial peptide LL-37.* Infect Immun, 2009. **77**(1): p. 292-9.
27. Beernink, P.T. and D.M. Granoff, *Bactericidal antibody responses induced by meningococcal recombinant chimeric factor H-binding protein vaccines.* Infect Immun, 2008. **76**(6): p. 2568-75.
28. Gonzalez, R., et al., *Screening the mammalian extracellular proteome for regulators of embryonic human stem cell pluripotency.* Proc Natl Acad Sci U S A, 2010. **107**(8): p. 3552-7.
29. Chu, X., et al., *Role of Rot in bacterial autolysis regulation of Staphylococcus aureus NCTC8325.* Res Microbiol, 2013. **164**(7): p. 695-700.
30. Schluepen, C., et al., *Mining the bacterial unknown proteome: identification and characterization of a novel family of highly conserved protective antigens in Staphylococcus aureus.* Biochem J, 2013. **455**(3): p. 273-84.
31. Stemerding, A.M., et al., *Staphylococcus aureus formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcgammaR antagonists that inhibit IgG-mediated effector functions.* J Immunol, 2013. **191**(1): p. 353-62.
32. Prat, C., et al., *A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1.* J Immunol, 2006. **177**(11): p. 8017-26.
33. Margarit, I., et al., *Capturing host-pathogen interactions by protein microarrays: identification of novel streptococcal proteins binding to human fibronectin, fibrinogen, and C4BP.* FASEB J, 2009. **23**(9): p. 3100-12.
34. Galeotti, C.L., et al., *Surface interactome in Streptococcus pyogenes.* Mol Cell Proteomics, 2012. **11**(4): p. M111 015206.
35. Kang, M., et al., *Collagen-binding microbial surface components recognizing adhesive matrix molecule (MSCRAMM) of Gram-positive bacteria inhibit complement activation via the classical pathway.* J Biol Chem, 2013. **288**(28): p. 20520-31.
36. Agarwal, V., et al., *Binding of Streptococcus pneumoniae endopeptidase O (PepO) to complement component C1q modulates the complement attack and promotes host cell adherence.* J Biol Chem, 2014. **289**(22): p. 15833-44.
37. Schorey, J.S., M.C. Carroll, and E.J. Brown, *A macrophage invasion mechanism of pathogenic mycobacteria.* Science, 1997. **277**(5329): p. 1091-3.
38. Thwaites, G.E. and V. Gant, *Are bloodstream leukocytes Trojan Horses for the metastasis of Staphylococcus aureus?* Nat Rev Microbiol, 2011. **9**(3): p. 215-22.
39. Yiangou, Y., et al., *Isolation, characterization, and pharmacological actions of peptide histidine valine 42, a novel prepro-vasoactive intestinal peptide-derived peptide.* J Biol Chem, 1987. **262**(29): p. 14010-3.
40. Morampudi, V., et al., *Vasoactive Intestinal Peptide Prevents PKCepsilon-Induced Intestinal Epithelial Barrier Disruption During EPEC Infection.* Am J Physiol Gastrointest Liver Physiol, 2014: p. ajpgi 00195 2014.
41. Murchie, R., et al., *Protein tyrosine phosphatase sigma targets apical junction complex proteins in the intestine and regulates epithelial permeability.* Proc Natl Acad Sci U S A, 2014. **111**(2): p. 693-8.
42. Kita, T., et al., *Role of oxidized LDL in atherosclerosis.* Ann N Y Acad Sci, 2001. **947**: p. 199-205; discussion 205-6.

43. Kita, T., et al., *Oxidized-LDL and atherosclerosis. Role of LOX-1.* Ann N Y Acad Sci, 2000. **902**: p. 95-100; discussion 100-2.
44. Mehta, J.L., et al., *Inhibition of LOX-1 by statins may relate to upregulation of eNOS.* Biochem Biophys Res Commun, 2001. **289**(4): p. 857-61.
45. Sawamura, T., et al., *An endothelial receptor for oxidized low-density lipoprotein.* Nature, 1997. **386**(6620): p. 73-7.
46. Shimaoka, T., et al., *LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria.* J Immunol, 2001. **166**(8): p. 5108-14.
47. Campbell, L.A., et al., *Chlamydia pneumoniae binds to the lectin-like oxidized LDL receptor for infection of endothelial cells.* Microbes Infect, 2012. **14**(1): p. 43-9.
48. Wu, Z., et al., *LOX-1 deletion improves neutrophil responses, enhances bacterial clearance, and reduces lung injury in a murine polymicrobial sepsis model.* Infect Immun, 2011. **79**(7): p. 2865-70.
49. Honnappa, S., et al., *An EB1-binding motif acts as a microtubule tip localization signal.* Cell, 2009. **138**(2): p. 366-76.
50. Shaw, P.J., et al., *CD4(+) and CD8(+) T cell-dependent antiviral immunity requires STIM1 and STIM2.* J Clin Invest, 2014. **124**(10): p. 4549-63.
51. Zhang, H., et al., *STIM1 calcium sensor is required for activation of the phagocyte oxidase during inflammation and host defense.* Blood, 2014. **123**(14): p. 2238-49.
52. Schwan, C., et al., *Clostridium difficile toxin CDT hijacks microtubule organization and reroutes vesicle traffic to increase pathogen adherence.* Proc Natl Acad Sci U S A, 2014. **111**(6): p. 2313-8.
53. Hyser, J.M., et al., *Activation of the endoplasmic reticulum calcium sensor STIM1 and store-operated calcium entry by rotavirus requires NSP4 viroporin activity.* J Virol, 2013. **87**(24): p. 13579-88.
54. Usmani, S.M., et al., *Molecular basis of early epithelial response to streptococcal exotoxin: role of STIM1 and Orai1 proteins.* Cell Microbiol, 2012. **14**(3): p. 299-315.
55. Marin-Grez, M., J.T. Fleming, and M. Steinhausen, *Atrial natriuretic peptide causes pre-glomerular vasodilatation and post-glomerular vasoconstriction in rat kidney.* Nature, 1986. **324**(6096): p. 473-6.
56. Komatsu, Y., et al., *C-type natriuretic peptide (CNP) in rats and humans.* Endocrinology, 1991. **129**(2): p. 1104-6.
57. Hodes, A. and D. Lichtstein, *Natriuretic hormones in brain function.* Front Endocrinol (Lausanne), 2014. **5**: p. 201.
58. Blier, A.S., et al., *C-type natriuretic peptide modulates quorum sensing molecule and toxin production in Pseudomonas aeruginosa.* Microbiology, 2011. **157**(Pt 7): p. 1929-44.
59. van der Poll, T., et al., *Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia.* J Infect Dis, 1997. **176**(2): p. 439-44.
60. Capecchi, B., et al., *Neisseria meningitidis NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells.* Mol Microbiol, 2005. **55**(3): p. 687-98.
61. Nagele, V., et al., *Neisseria meningitidis adhesin NadA targets beta1 integrins: functional similarity to Yersinia invasin.* J Biol Chem, 2011. **286**(23): p. 20536-46.

Abbreviation list

4CMenB: 4 component meningococcus type B vaccine formulation

AP: complement alternative pathway

AR2G: Amine Reactive 2nd Generation biosensor

BLI: Bio-Layer Interferometry

C1q: complement component C1q

C1qA: complement component C1q subcomponent A

C1qB: complement component C1q subcomponent B

C1qC: complement component C1q subcomponent C

C1r: complement component 1r

C1s: complement component 1s

C2: complement component C2

C3: complement component C3

C4: complement component C4

C4BP: C4 binding protein

C5: complement component C5

C6: complement component C6

C7: complement component C7

C8: complement component C8

C9: complement component C9

CCP: complement control protein

CFH: complement factor H

CFI: complement factor I

CHIPS: chemotaxis inhibitory protein of *S. aureus*

ClfA: clumping factor A

ClfB: clumping factor B

CP: complement classical pathway

CPP: complement control protein

CR1: Complement receptor type I (CD35)

CRD: C-type carbohydrate recognition domain

CRP: C Reactive Protein

CTLD or **CLECT**: C-type lectin-like domain

CUB: complement C1r/C1s, Uegf, Bmp1 domain

CWA protein: Cell Wall Anchored protein

Cy5: cyanine 5

DAF: decay-accelerating factor (CD55)

DLS: Dynamic Light Scattering

Eap: extracellular adherence protein

Ebps: elastin-binding protein

ECM: extracellular matrix

EDC: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide

Efb: extracellular fibrinogen-binding protein

EGF: epidermal growth factor

Ehp: Efb-homologous protein

Emp: extracellular matrix protein-binding protein

EsxA: ESAT-6 Secretion Systems protein A

EsxB: ESAT-6 Secretion Systems protein B

FACS: Fluorescence -Activated Cell Sorting

FBG: fibrinogen binding domain

Fc: fragment crystallizable of IgG

FcγR: fragment crystallizable gamma receptor

fHbp: factor H binding protein

FLIPr: formyl-peptide receptor like-1 inhibitory protein

FLIPr- like: formyl-peptide receptor like-1 inhibitory protein - like

FnBA: fibronectin-binding protein

FPR: formyl-peptide receptor

FPRL1: formyl-peptide receptor like-1

GAS: group A *Streptococcus*

GBS: group B *Streptococcus*

GPCRs: G-protein coupled receptor

GST tag: Glutathione S-transferase tag

HDL: High Density Lipoprotein

HEK293: human embryonic kidney cell line

His-tag: histidine tag

HLA: Hemo-Lysin Alpha

hsp70: heat shock protein 70

hsp90: heat shock protein 90

IgG: immunoglobulin G

IMD: Invasive meningococcal disease

IsdA: ron-regulated surface determinant protein A

IsdB: ron-regulated surface determinant protein B

KD: dissociation constant

LOS: lipo-oligosaccharides

LOX-1: Lectin-like Oxidized LDL receptor-1

LP: complement lectin pathway

LPS: lipo-polysaccharides

LytM: Glycyl-glycine endopeptidase

mAb: Monoclonal antibody

MAC: membrane attack complex

MASP: Mannan-binding lectin serine protease or mannose-associated serine protease

MCP: membrane cofactor protein (CD46)

MDRS: Multi Drug Resistant Strains

MenB: Meningococcus type B

MFI: mean fluorescence intensity

MSCRAMMs: Microbial Surface Component Recognizing Adhesive Matrix Molecules

Msf: meningooccal surface fibril

MW: molecular weight

NadA: Neisserial adhesin A

NadA3 FL: NadA variant 3 full length protein

NadA3: NadA variant 3

NEAT: Near iron Transporter

NHS: *N*-hydroxysuccinimide

NspA: neisserial surface protein A,

NspA: neisserial surface protein A

OMV: outer membrane vesicles

Opc: opacity protein

oxLDL: oxidized Low Density Lipoprotein

PAMPs: pathogen-associated molecular patterns

PorA: porin A

PTPRS: Protein Tyrosine Phosphatase Receptor type sigma isoform 4

PVDF: Polyvinylidene fluoride

RCA: regulators of complement activity

RGD: domain: arginin- glycine-aspartic acid domain

SA: Streptavidin biosensor

Sbi: staphylococcal binder of immunoglobulin

SCIN: staphylococcal complement inhibitor

SCR : short consensus repeat

SdrB: Serine-Aspartate Repeat containing protein B

SdrC: Serine-Aspartate Repeat containing protein C

SdrD: Serine-Aspartate Repeat containing protein D

SE-HPLC/MALLS: size exclusion- high performance liquid chromatography/multi angle laser light scattering

SP domain: serine protease domain

SpA: Staphylococcal protein A

SSL-10: superantigen-like 10

SSL-7: superantigen-like 7

TAA: trimeric autotransporter adhesion

TLR4: toll-like receptor 4

WHB: whole human blood

ACKNOWLEDGEMENTS

CURRICULUM VITAE

LUIGI ANGELO DOMENICO SCIETTI
via F. Cavallotti 92, 26813, graffignana (LO), Italy
born on 29 December 1986

EDUCATION

2015	Visiting scientist (January 2015 - March 2015) at Novartis Vaccines and Diagnostic (Siena - Italy)
2012–2014	Ph.D. student in the protein-protein interaction group of Dr. Sabrina Liberatori at Novartis Vaccines and Diagnostic (Siena - Italy).
2009–2011	MSc student in the structural biology group of Prof. Andrea Mattevi, Università degli studi di Pavia (Pavia – Italy). Thesis title: "Biochemical and structural characterization of the LSD1-CoREST-Nucleosome complex"
2005-2009	student in the cell biology group of Prof. Carlo Pellicciari, Università degli studi di Pavia (Pavia – Italy). Thesis title: "Immunocytochemical study of the cisplatinum-induced organellar damage in rat neuroblastoma cells"
2000-2005	Highschool, Lodi - Italy

ATTENDED COURSES AND WORKSHOPS

2014 November	invited speaker at “Octet user’s meeting”
2014 September	selected speaker at “Siena Proteomic meeting”
2014 August	Wellcome Trust Advanced course on “protein-protein interactions and networks”
2014 July	selected speaker at Novartis “Research Days”
2014 (3days)	short course on “how to write a grant” (teacher: Dr. Aaron Zeichner)
2014 (3 days)	EIMID short course on “successful found raising in R&D”
July 2013 (2 weeks)	Short term visiting student in Novartis Basel at NIBR/NBC/PPA, Scharenberg lab, to carry on a collaboration study on protein microarray.
2013 (1day)	congress on “biomolecules, biopolymers and biohydrogel”
2012 (2days)	short course on “how to write a scientific paper” (teacher: Dr. Keith Veitch)