UNIVERSITY OF PAVIA

Doctorate in Biomedical Sciences

Department of Molecular Medicine

Unit of Biochemistry

MECHANISMS OF COMPLEMENT EVASION IN GROUP B *STREPTOCOCCUS*

Supervisors:

Prof. Giampiero Pietrocola

Dr. Monica Fabbrini

Experimental PhD thesis by:

Stefania Giussani

XXXI Cycle, A. Y. 2017/2018

SG is a student at the University of Pavia and participates in a post graduate studentship program at GSK. This work was sponsored and funded by Novartis Vaccines and Diagnostics Srl; in March 2015 the Novartis non-influenza Vaccines business was acquired by the GSK group of companies. The sponsor was involved in all stages of the study conduct and analysis.

Table of contents

Thesis Summary

The objective of this PhD thesis was to investigate the interplay between Group B *Streptococcus* (GBS) and the Complement System, and in particular the strategies used by this important human pathogen to interfere with the different Complement pathways and effectors. The study had two main aims and the obtained results are presented in two separate chapters, both including a short background introduction and a discussion.

The first aim was to elucidate the involvement of the Classical, Lectin and Alternative Complement pathways in GBS clearance, and the role of the Capsular Polysaccharide and its Sialic acid component in protecting the microorganism from Complement attack. Moreover, this work was aimed at further investigating the mechanisms of Complement susceptibility in GBS, both in absence and presence of specific antibodies. The highly encapsulated COH1 strain belonging to serotype III, the most prevalent in GBS neonatal infections worldwide, and its acapsular isogenic mutant COH1 Δ*cpsE* were selected for this study. The relative role of each Complement pathway was investigated by C3b deposition, C1q binding and Opsonophagocytosis experiments. The relevance of the Classical pathway was investigated by using C1q-depleted Complement sources and Calcium/Magnesium depletion, and the contribution of the Alternative Pathway was assessed by using a Factor B-depleted Complement source. The role of Sialic acid in Complement deposition was studied by comparing serotype III GBS treated with Neuraminidase to the wild type and acapsular mutant strains. From this study we concluded that the GBS capsule strongly inhibits Complement activation by the Classical Pathway and also the following cascade amplification by the Alternative Pathway. This work could serve as a model to better elucidate the role of the capsule in other GBS serotypes, whose response to Complement attack could be different on the basis of the different capsule composition. Having a clear picture on how the Complement acts on GBS will help identifying new tools to counteract infections both in newborns and adults.

In the second part of this work we investigated a new role for a small secreted GBS polypeptide, named CIP (Complement Interfering Protein), previously studied for its ability to inhibit the Classical and Lectin Complement Pathways. Indeed Pietrocola and coworkers demonstrated that CIP was able to interact with C4 and C4b Complement components, and to prevent the formation of the C4b2a convertase, thus leading to a decreased C3b deposition on the bacterial surface. On the basis of the ability of this GBS protein to interact also with other Complement factors, the present study was aimed at functionally and biochemically characterizing CIP binding to C3 and its C3b and C3d degradation fragments. We first investigated the possible CIP binding region for C3d by Hydrogen-Deuterium Exchange-Mass Spectrometry (HDx-MS) analysis, and aligned CIP sequence to the ones of other bacterial C3d-binding proteins to detect common aminoacidic patterns. Moreover we aimed at characterizing CIP ability to interfere with the interaction between C3d and its CR2/CD21 receptor on B cells. Since CR2/CD21 clusters together with the B Cell Receptor (BCR) on the B cell surface, C3d-opsonized antigens can be simultaneously bound by these two surface receptors. This event leads to enhanced B cell activation and intracellular signaling. In this work we studied the effect of CIP on C3d-CR2/CD21 binding both *in vitro* and *ex vivo*, by using an immortalized B cell line. Moreover, we gained evidence CIP ability to interfere with the CD21-mediated stimulation of B cells by monitoring the intracellular Calcium mobilization.

In this respect, our study could represent a valuable contribution to both the characterization of molecular Complement evasion mechanisms and the development of CIP-based therapeutics.

Introduction

Streptococcus agalactiae

Description and History

Streptococcus agalactiae is a Gram positive bacterium, also referred to as Group B *Streptococcus* (GBS) on the basis of the classification done by Rebecca Lancefield in 1933 (1). GBS is a catalase negative facultative anaerobe whose colonies form typical narrow β-hemolytic halos when cultured on blood agar plates. (2). GBS was grouped into ten serotypes (Ia, Ib, II to IX) on the basis of the structure of its Capsular Polysaccharide (CPS). Nevertheless some isolates are still considered as Non-Typeable (NT) because they do not react with specific anti-capsular serum. Whole Genome Sequence analysis of NT strains revealed the presence of mutations in one or more genes which have been predicted to abolish capsule expression (3). The frequencies of detected NTs in GBS epidemiological studies have recently decreased with the development of serotyping methods of improved sensitivity (4, 5).

GBS was first isolated in 1889 and initially studied as the cause of bovine mastitis (5). Indeed, although commonly isolated from healthy postpartum women, it was not considered a human pathogen, but a commensal of the lower digestive and urogenital tracts of women (6). In the 1960s GBS was recognized as an important neonatal pathogen, and was identified as the leading cause of sepsis, pneumonia and meningitis in infants under 2 months of age, both in the US and Europe (7).

Transmission and Pathogenesis

GBS in normally found in the intestine, vagina and rectal area of 10-30% of healthy pregnant women and the infection can be transmitted vertically from mothers to newborns *in utero* or during delivery (8). GBS can spread through the rupture of membranes into the amniotic fluid, allowing bacteria to colonize the respiratory tract of the neonate causing pneumonia. From the lungs, GBS can then penetrate into the blood stream and overcome the Blood Brain Barrier causing meningitis (**Fig. 1**). Infections in infants can be divided into GBS Early-Onset Disease (EOD; between birth and 6 days of age) or Late-Onset Disease (LOD; between 7 and 89 days of age) (9). If maternal colonization is a prerequisite for EOD, which mainly manifests as respiratory failure or pneumonia, LOD acquisition is not completely understood, although vertical transmission, nosocomial acquisition and prematurity are recognized as risk factors (10).

The transmission of functional antibodies from mother to child is considered the fundamental step for the prevention of GBS infections (11). Children born from mothers without GBS-specific antibodies are more prone to acquire the GBS disease, both during delivery and hospitalization. Moreover GBS strains isolated from infected neonates have been found in the mother's breast milk suggesting this route as a possible mechanism of colonization (12, 13).

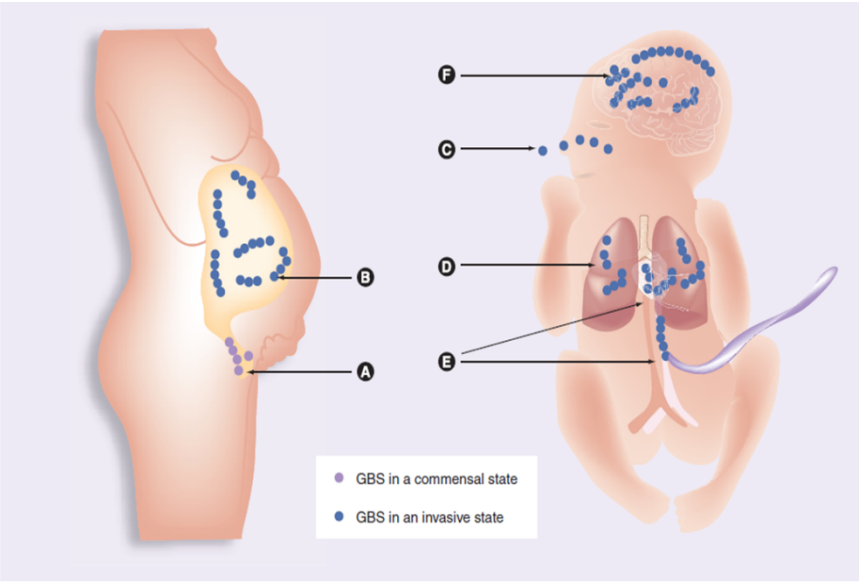

Figure 1_ Lifecycle of Group B Streptococci as a neonatal pathogen. (A) GBS resides as a commensal in genital and lower gastrointestinal tracts of women. (B) GBS can infiltrate the

intrauterine compartment in pregnant mothers who are asymptomatic carriers. (C) Newborn aspirate GBS in utero or during birth. (D) GBS invades the neonatal lung causing pneumonia. (E) From the lung, GBS gains access into the bloodstream of the neonate causing sepsis and invades multiple neonatal organs including the heart (F) GBS penetrates the blood–brain barrier causing meningitis. Adapted from Rajagopal L., 2009, *Future Microbiol.* 4(2)201–221.

Distribution and epidemiology

GBS colonization rates range from 6.5 to 36% and are similar worldwide, while the serotype distributions differ depending on the geographic area (14). Recent work confirmed that the most common serotypes in maternal GBS disease worldwide are Ia, Ib, II, III and V (**Fig. 2A**) (15, 16), among which the serotype III accounts for about 50% of neonatal disease globally (17). In particular, 60% of meningitis cases are caused by type III isolates belonging to the hypervirulent Sequence Type (ST)17 lineage, whose epidemiological relevance is not only linked to its capsular polysaccharide, but also to the expression of additional protein factors (17, 18).

Several studies from 1998 to 2017 showed that the distribution of Group B *Streptococcus* serotypes was strikingly similar across the WHO regions and no evidence was shown that distribution had changed over the past 30 years (16, 19) (**Fig. 2B**). The incidence of infant GBS disease was strikingly low in Asia, with the lowest incidence in Southeast Asia. The reasons for this difference could be a lower prevalence of hypervirulent type III GBS in these regions and to incomplete case ascertainment due to a high number of home births.

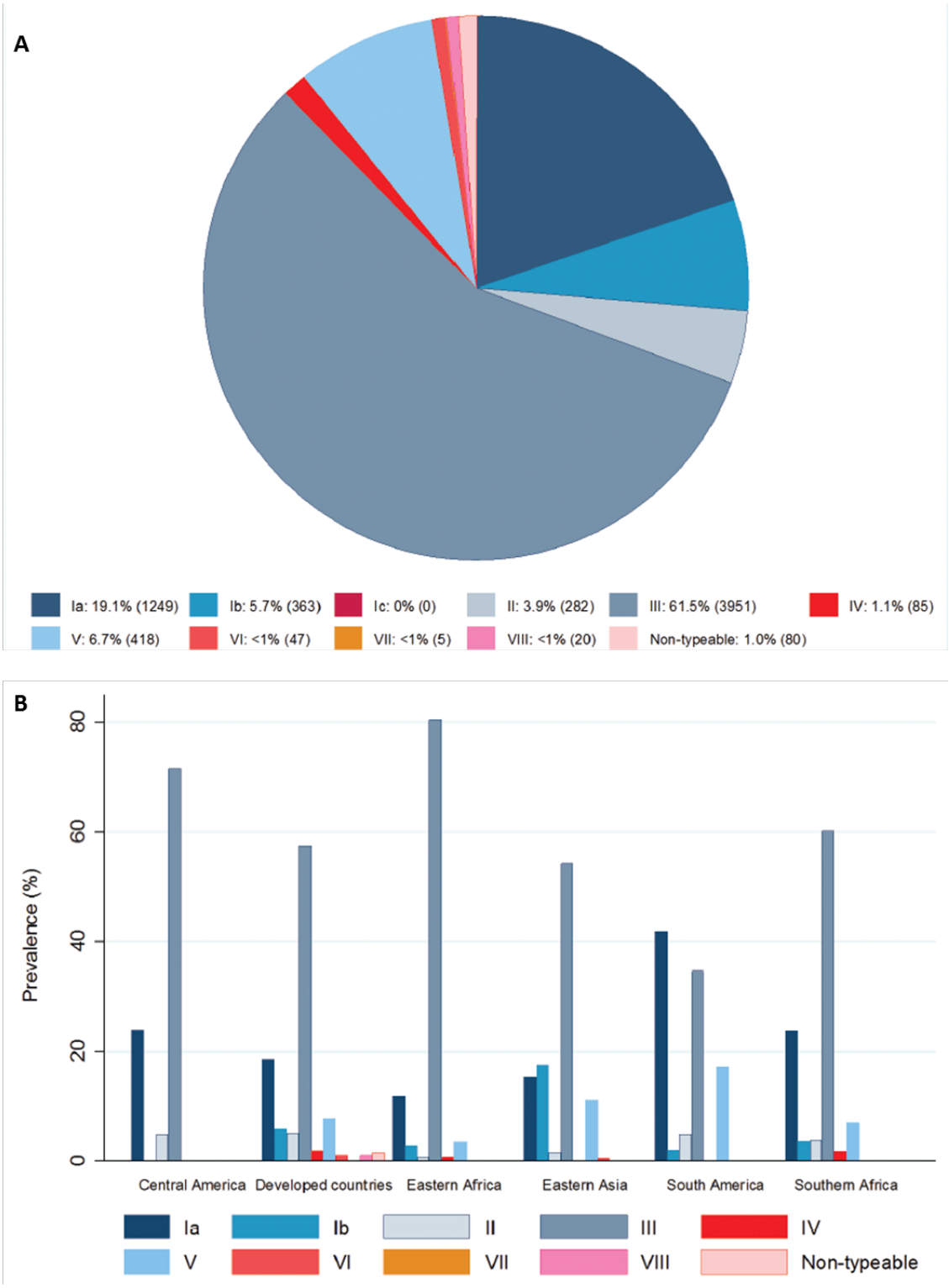

Figure 2. Global distribution of Group B Streptococcus serotypes in invasive disease in young infants (N = 6500 isolates). ***Panel A***: Prevalence of GBS serotypes presented as percentage (number of cases). ***Panel B***: Distribution of GBS serotypes by region. From Madrid *et al.*, 2017, *Clinical Infectious Diseases* 65(S2):S160–72**.**

Treatment of GBS infections

In the US, the detection and treatment of EODs has been regulated by the Centre for Disease Control (CDC) through the introduction in 1996 of guidelines for detection and prophylactic treatment of GBS infections (9). These measures, which were subsequently updated in 2002, recommend the universal screening of pregnant women for GBS carriage and a *peripartum* antibiotic prophylaxis, mainly by intravenous infusion of penicillin or erythromycin. This event coincided with the declining rates of early-onset neonatal disease especially from the 90s (**Fig. 3**). The efficacy of intrapartum antibiotic prophylaxis (IAP) for EODs prevention was demonstrated in clinical trials. Nevertheless prophylactic treatments have yet not reduced the incidence of GBS-induced LODs in children (20) and the prevention of these infections is still challenging.

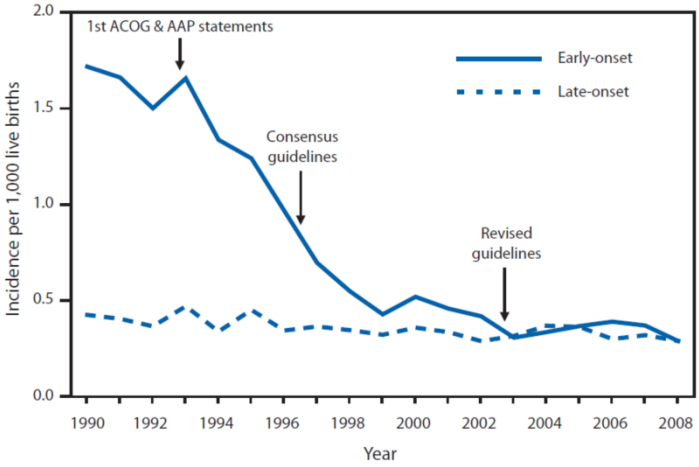

Figure 3. Incidence of early- and late-onset invasive group B streptococcal (GBS) disease (1990–2008) and activities for prevention of GBS disease. ACOG = American College of Obstetricians and Gynecologists; AAP = American Academy of Pediatrics. Adapted from Jordan H.T. *et al.*, 2008, *Pediatr Infect Dis J* 27:1057–64.

The use of prophylactic antimicrobial agents directed against GBS infections has raised concern for the development of antibiotic resistance. GBS is still susceptible to penicillin or ampicillin but the Minimum Inhibitory Concentration (MIC) of antibiotic effectiveness is increasing (21). Moreover recent studies reported several mutations in the GBS genome that enable it to counteract the action of antimicrobial agents (21). One such example is the ribosomal alteration mediated by ribosomal methylases, which alter the binding site for erythromycin thus rendering the microorganism resistant to this antibiotic. Moreover Da Cunha and colleagues reported the genetic basis for the spreading of few successful GBS clones that have acquired a conjugative element conferring resistance to tetracycline after the introduction of this antibiotic in 1948 (7).

Beside the major concern for GBS infections in newborns, a 7-years study conducted in the US from 1999 to 2005 (**Fig. 4**) demonstrated the incidence of GBS-induced diseases also in older adults (≥65 years of age) (22), especially if already immunocompromised (comorbidity) (23). GBS induced-diseases are also frequently nosocomial and are commonly related to the use of intravenous catheters.

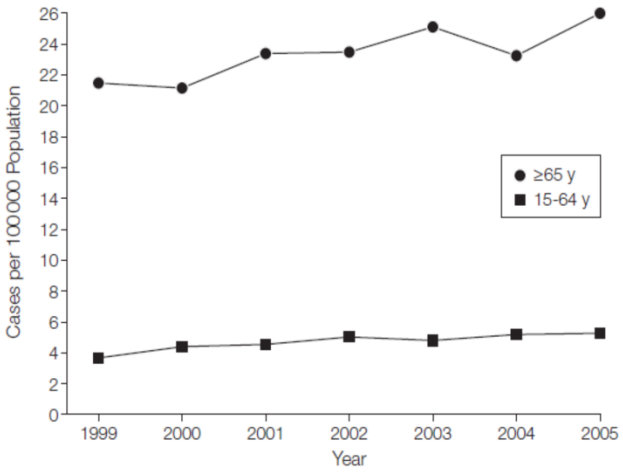

Figure 4. Incidence of Invasive Group B streptococcal disease among adults (≥ 15 y) in US areas, 1999-2005. Adapted from Phares et al., 2008, *JAMA*

For these reasons the design of a vaccine against GBS is considered an ideal solution, although no vaccine is currently available. The premise for vaccine creation consists in trying to elicit protective levels of antibodies in childbearing age women to allow placental passage of these GBS-specific antibodies. This would protect neonates throughout the period of disease risk (0-89 days of life) (24). The main difficulty in developing a globally effective GBS vaccine is the existence of different serotypes, whose geographical distribution is heterogeneous.

The first human clinical trials were conducted in the '80s by immunizing 40 pregnant women at a mean gestation of 31 weeks with a single 50 μg dose of purified native CPS-III, but only 60% of patients showed a significant IgG response (25). Further studies in which monovalent preparations were coupled to Tetanus Toxoid resulted in higher IgG titers, although there was no cross protection among serotypes in pre-clinical assays. In order to achieve a 95% coverage in Europe and North America a multivalent preparation that includes serotypes Ia, Ib, II, III and V would be needed (26). To further extend the vaccine coverage, a vaccine targeting both Capsular Polysaccharide and surface proteins would be highly recommendable (26).

The Human Complement System

The Complement system is a complex innate immune surveillance system that represents the first line of defence against invading pathogens and inflammation. The Complement system consists of a plethora of plasma or membrane proteins, whose activation initiates a cascade that leads to pathogen opsonitazion, inflammatory responses and homeostasis maintenance.

The Complement cascade depends on three distinct pathways, *i.e.* Classical (CP), Lectin (LP) and Alternative (AP), each leading to a common terminal outcome (27), which mainly depends on the nature of the microorganism. Gram negatives, which are characterized by a thin peptydoglycan layer, are subjected to both killing by neutrophils and lysis via the formation of the Membrane Attack Complex (MAC), that forms pores right in the membrane of bacteria.

In this thesis we'll focus on GBS and its interplays with the human Complement system. In particular, we will analyze Complement cascade initiation, C3b deposition and neutrophils-mediated killing of GBS. Moreover we will discuss the role of Complement in bridging innate and acquired immune responses.

Complement cascade initiation and C3b deposition

The main difference among the three pathways resides in their route of activation: the CP is normally initiated by the binding of the C1 complex to antibodies that are bound to antigens on the surface of bacteria (**Fig. 5**). The C1 complex consists of C1q and two molecules each of C1r and C1s. The binding of the recognition subcomponent C1q to the Fc portion of immunoglobulins results in autoactivation of the serine protease which cleaves C4 and C2 to form a C4b2a convertase.

C4b2a convertase also forms during the LP, which differs from the CP only in the initiation step. Indeed, the LP is triggered by the binding of either mannose-binding lectin (MBL) or ficolins, in complex with MBL-associated serine proteases (MASP1,-2, -3), to an array of carbohydrate groups on the surface of a bacterial cell. The serine protease activity of Lectin-MASP complex is responsible for the cleavage of C4 and C2, generating the C4b2a convertase. Apart from the activation mechanism, LP and CP share the same Complement factors during the cascade: their C4bC2a C3 convertase causes the cleavage of C3 to its active C3b form. C3b subsequently deposits on bacterial surface, opsonizing the microorganism and rendering it susceptible to phagocytosis or lysis (**Fig. 5**) (28).

Regarding the AP, in healthy individuals it is permanently active at low levels through a process termed “tickover” of C3. This mechanism is needed to continuously control and attack possible pathogens. The tickover, which occurs spontaneously at a rate of approximately 1% of total C3 per hour, generates a conformationally altered C3, designated C3(H2O), that is capable of binding Factor B. This interaction renders Factor B accessible to Factor D cleavage into Ba and Bb, and the new C3(H2O)Bb complex is able to cleave inactive C3 molecules to the active C3b form. Active C3b either associates to Factor Bb to form C3bBb, the AP C3 convertase, or covalently bind to the surface of pathogens via its thioredox domain (TED). Both interactions start C3b deposition on bacteria, which is the first key Complement step where all three pathways converge (**Fig. 5**) (29).

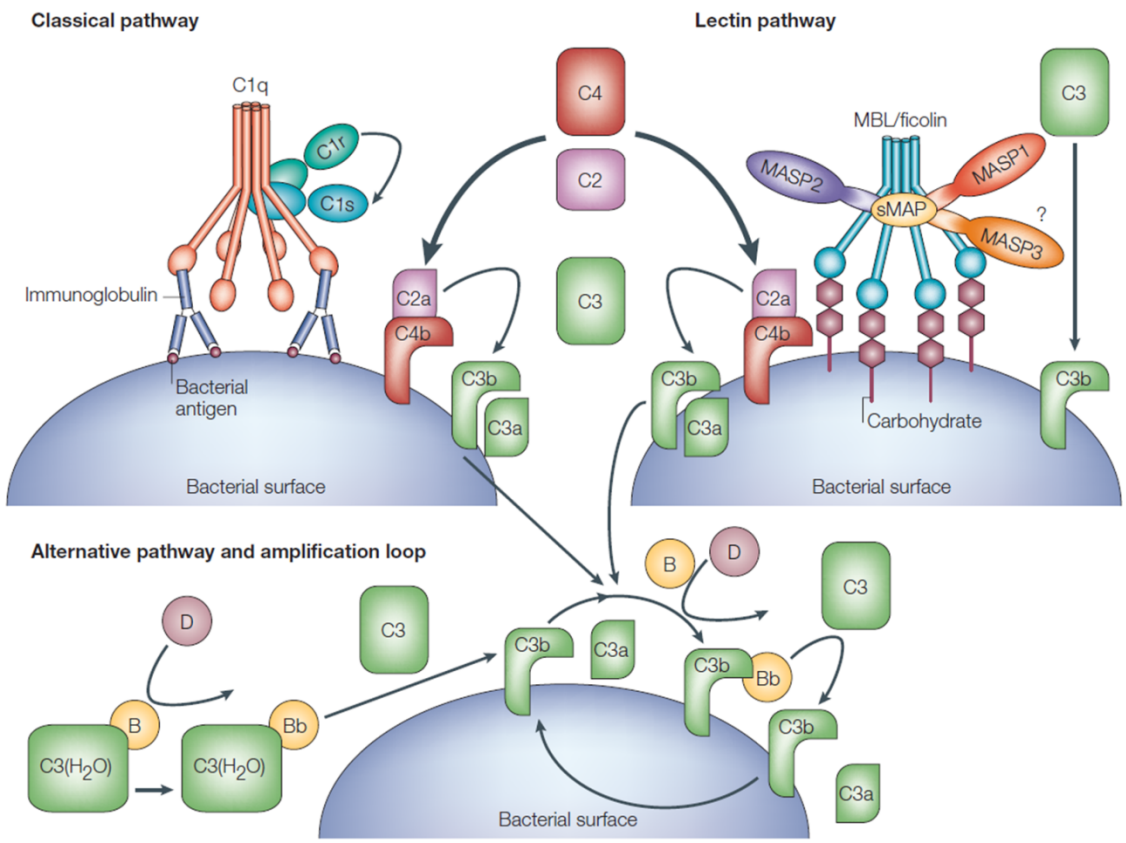

Figure 5_Pathways for Complement activation. The **Classical** and **Lectin Pathways** share the same Complement effectors during the cascade, with the only exception of their activation route. The **Alternative Pathway** is initiated by the low-grade activation of C3 by hydrolysed C3 (C3(H_2O)) and activated factor B (Bb). C3b attachment to the cell surface leads to a cascade amplification loop. Image adapted from Foster T. J., 2005, *Nat Rev Microbiol* 3(12):948-58

Termination of the cascade

The Classical and Lectin pathways C4b2a convertase cleaves C3 to C3b, which binds to, and causes the destruction of, invading bacteria (30). C3b could also associate to C4b2a, forming the C4b2a3b complex, which is a C5 convertase that cleaves this Complement component into C5a and C5b. C5a acts both as chemoattractant and anaphylotoxin, recalling PMNs to the infection site. C5b stimulates the aggregation of the downstream Complement components (C6-C9) to form the Membrane Attack Complex (MAC) on bacterial surface which causes the lysis of the microorganisms.

MAC formation is mainly observed for Gram negative bacteria. Gram positives are resistant to lysis and bacterial killing relies upon C3b deposition-mediated opsonophagocytosis (27).

Complement Regulation and C3b decay

C3b formation is tightly regulated to prevent an aberrant activation of the immune response. Complement inhibitors have overlapping targets, and are directed towards both C3 convertases and C3b. Complement regulators could be either soluble or membrane-associated (i.e. decay-accelerating factor; DAF). Here we will focus on the ones that are relevant to the thesis.

The Classical and Lectin Complement pathways are regulated by the soluble C4 Binding Protein (C4BP), a 500 kDa glycoprotein characterized by an octopus-like structure. C4BP acts as the cofactor of Factor I (FI), the serine protease that cleaves and inactivates C4b and C3b, forming iC4b and iC3b (27).

The Alternative pathway is strictly regulated by Factor H (FH), that engages most effectively with C3b or C3bBb when these are attached to self surfaces that carry specific polyanionic markers such as glycosaminoglycans (GAGs) and sialic acid. Factor H is a cofactor of Factor I, the serine protease that inactivates C3b forming iC3b, which remains anchored to bacterial surface through its TED domain. iC3b undergoes a series of further degradation steps that result in the surface-bound fragments C3dg (~39 kDa) and C3d (~34 kDa) (**Fig. 6**). C3d corresponds almost precisely to the original TED domain and is a ligand for Complement Receptor 2

(CR2) (27, 31), a 140-kDa type I glycoprotein expressed on the surface of all mature B cells, follicular dendritic cells (FDCs), epithelial cells, and a subset of thymocytes and immature peripheral blood T cells (32).

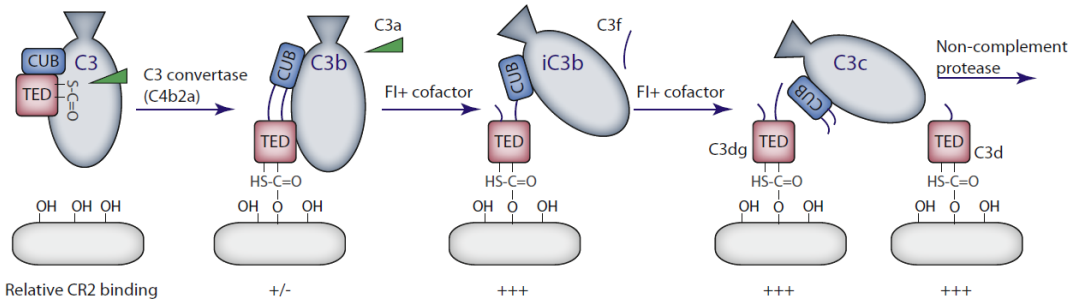

Figure 6. Structure-based rapresentation of the processes involved in the covalent tagging of antigen with C3b and its subsequent degradation to to the CR2 Ligands iC3b, C3dg, and C3d. C3 activation to C3b, induces the exoposition of its Thioredoxin Domain (TED), which covalently binds to the bacterial surface through a thioester bond. C3b degradation via Factor I and other plasma proteases leads to the formation of CR2 ligands (iC3b, C3dg and C3d). Adapted from Carroll *et al*., 2012, *Immunity Rev*. 37(2):199-207.

Opsonization and killing

The Complement-mediated elimination of GBS by the host, primarly results in opsonophagocytic killing by polymorphonuclear leukocytes (PMNs) (33). In this work we will investigate GBS resistance to opsonophagocytosis and the role of the three Complement pathways in this mechanism.

The deposition of C3b on the bacterial surface, tags microorganism for phagocytosis. Polymorphonuclear leukocytes, together with erythrocytes, B and T cells, display on their membrane the Complement Receptor 1 (CR1/CD35), which is able to bind both the C4b and C3b (34).

Indeed neutrophils express both the CR1 and the Fc-gamma receptor for IgG, which operate in synergism to promote uptake of microbial particles, leading to internalization and destruction in the lysosomes (**Fig. 7**) (34).

To experimentally determine the the role of Complement in GBS clearance, a killing-based opsonophagocytosis assay (OPKA), will be used. The assay will measure the number of bacterial colonies surviving phagocytic killing in the presence of specific Complement sources.

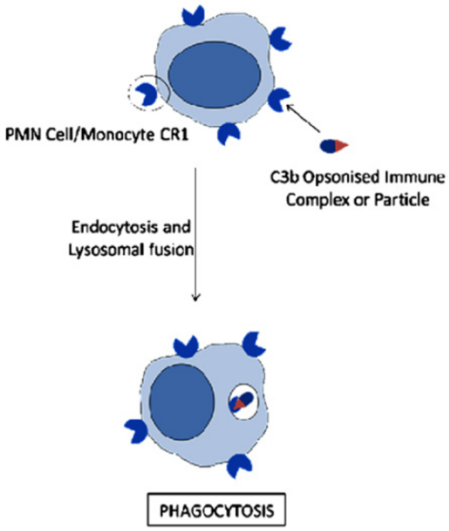

Figure 7. Polymorphonuclear (PMN) Cells are involved in antigen phagocytic uptake. The Complement Receptor 1 (CR1) on PMNs (i.e. Neutrophils) mediate the uptake of C3b-opsonized microorganisms through the interaction between C3b and CR1. Adapted from Khera R. *et al*., 2009, *Mol Immunol.* 46(5):761-772.

The relevance of Complement in adaptive immunity

In addition to its relevance in innate immunity, it was discovered that the Complement system could play a role in adaptive immunity. Servis and collaborators demonstrated that the interaction between C3d and CR2 (also known as CD21) on B cells, could promote B cell proliferation and activation. The inhibition of this interaction through a C3d-specific monoclonal antibody impaired the above mentioned effects (35).

Later studies described the mechanism through which B cells are stimulated by C3d-bound antigens. CR2/CD21 is part of the B cell co-receptor complex together with CD19 and CD81, the former being a signal-amplifying molecule (**Fig. 8**). By interacting simultaneously with CR2/CD21 and the antigen-specific B cell receptor (BCR), C3d crosslinks the two membrane complexes and promotes a strong intracellular signaling cascade (*i.e.* phosphorilation cascade and intracellular Calcium increase) that increases B cell activation a thousand-fold (36, 37).

In addition to B lymphocyte surface activation, CR2 is also involved in antigen trafficking in the lymph nodes, which is necessary for subcapsular antigen capture and transfer into the B cell follicle to promote humoral immune responses (37).

GBS bacteria have evolveded an immune evasion mechanism to inhibit or downregulate this signal amplification step. In the second part of this thesis GBS activity in counteracting the bridging between innate and adaptive immunity will be further elucidated.

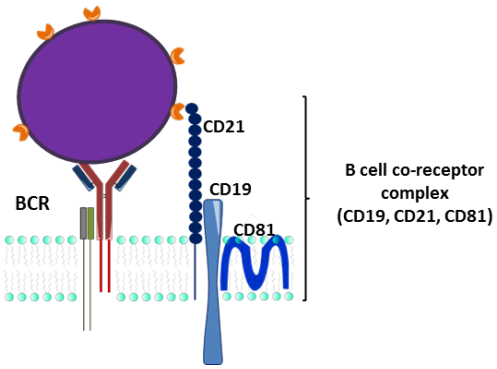

Figure 8. Schematic rapresentation of the B Cell Receptor (BCR) and its B cell co-receptor complex. The B Cell Receptor recognizes epitopes on the C3d-oponized antigens. C3d is bound by CR2/CD21 receptor on the B cell surface and serves as a cross-linker for the two membrane receptor complexes. This interaction triggers a strong intracellular signaling that promotes B cell proliferation and activation, as well as antigen uptake.

GBS Complement evasion systems

S. agalactiae shows a high tropism for the human lower gastrointestinal and genitourinary tracts, as an asymptomatic colonizer. To do so, this microorganism has evolved an array of virulence factors that confer the capability to escape the host immune system, both disguising it and preventing the activation of the immune response (38).

These molecular players are also involved in GBS pathogenesis and help it go through several key steps of the infectious process, including adherence to and invasion of different host epithelia and endothelial barriers, the escape from the immune system and the activation of inflammatory response (6).

This thesis focuses on the best characterized virulence factors that play a fundamental role in immune evasion.

Capsular Polysaccharide

GBS ability to survive in the host is driven by a huge array of virulence factors that allows its diffusion and protects it from the host immune system. The most prevalent and studied virulence factor is the thick Capsular Polysaccharide (CPS) that surrounds the bacterium and whose structure distinguishes ten different serotypes.

The CPS is covalently bound to the cell wall peptidoglycan, thus creating the mucoid capsule layer that covers the bacterial surface (39, 40). The CPS structure comprises repeating units constituted of 5–7 monosaccharides, depending on the serotype, and is encoded by 16 to 18 genes that are contained in the cps operon (41), which is transcribed as a single transcript (42).

Three main regions can be identified in the cps operon (**Fig. 9**): one dedicated to polysaccharide elongation and cell wall attachment (*cpsA* to *D*); a central region (*cpsE* to *L*) that determines capsule biosynthesis, serotype and that encodes glycosyltransferases and a polymerase; the last part of the operon that comprises four genes (*neuA* to *neuD*) for Sialic acid biosynthesis and linkage to the CPS (40, 42).

Some differences in csp operon composition can be found among different GBS serotypes, but *cpsA* to *–E*, *cpsL* as well as *NeuB*, *-C*, *-D*, and *-A*, are conserved in all ten serotypes (41). Although the physical structure and composition of each serotype-specific CPS is unique, all GBS strains share a terminal α2,3-linked Sialic acid, the N-

acetylneuraminic acid (Neu5Ac) (43), which varies in O-acetylation levels among serotypes. This chemical modification is performed by NeuD, and it was demonstrated that a delicate balance between O-acetylation and de-O-acetylation is necessary for bacterial survival in the host. Indeed the rate of GBS virulence is inversely proprotional to the quantity of O-acetyl groups on capsular Neu5Ac (44).

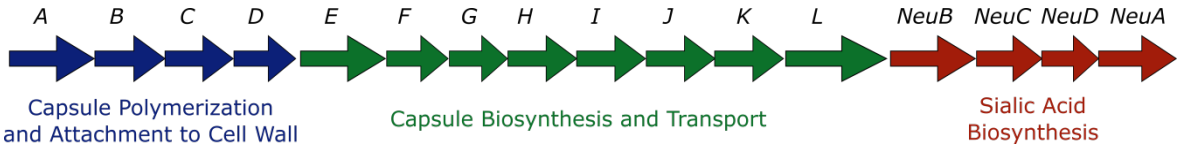

Figure 9. General organization of the Capsule Biosynthesis operon in GBS.

Despite the importance of the CPS, little is known about how GBS regulates the CPS expression during the different steps of the pathogenesis. Studies have shown that the CPS is important for biofilm formation in an *in vitro* model (45), and for interfering with adhesion and invasion of cultured epithelial and endothelial cells (46, 47). These observations suggest that GBS may regulate the expression of the CPS in response to the host environment.

Analysis of GBS transcriptome in different growth conditions has shown that the transcription of the cps operon is reduced when GBS is grown in human blood instead of laboratory medium (48). Moreover differences in doubling time during GBS growth have been observed to correlate with the amount of CPS produced. Fast-growing GBS appears to produce more CPS than slow-growing GBS (49).

It was also observed that when GBS is grown with a short doubling time, it invades epithelial cells more efficiently (50). Similarly to GBS, other bacteria such as *Streptococcus pneumoniae* and *Neisseria meningitidis* present this dual role of the CPS (51, 52). However, the detailed mechanism used to regulate the expression of this important virulence determinant is not established in any bacteria.

Relation between bacterial Sialic acid and human Siglecs

Sialic acids (Sias) are nine-carbon backbone monosaccharides primarily expressed by vertebrates as well as by some microbial pathogens, including GBS. It serves as decoy to resemble host "self" and avoid immune recognition (53). Sialic acid moieties linked to the CPS, together with the de-syalilated CPS, have been studied for their role in the prevention of Complement deposition (54), which a key step in the activation of both cellular innate and adaptive immune responses. This aspect will be further discussed in Part I of this thesis.

Mammalian immune cells express an array of receptors, whose function is to regulate the response to an extracellular stimulus by interacting with Sialic acids: the Sia-recognizing ImmunoGlobulin (Ig) superfamily LECtins (Siglecs) are a family of structurally related type I transmembrane proteins that display one V-set Ig domain, which is responsible for recognizing sialylated glycoconjugates, and a variable number of structural C2-set Ig domains (43). The intracellular portion of Siglecs is mainly constituted by Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) or ITIM-like domains. Siglecs can be divided into two groups based on sequence similarity and evolutionary conservation. The first group includes sialoadhesin, CD22, myelin-associated glycoprotein (MAG) and Siglec-15, and has orthologues in all mammals but low sequence similarity. The second group includes the CD33-related Siglecs, which differ in composition between species but share high sequence similarity in their extracellular regions (**Fig. 10**).

Figure 10. Siglec-family proteins in humans. The cell-expression patterns are indicated on the basis of antibody labelling. The brackets indicate low levels of expression.

Abbreviations: B, B cells; Ba, basophils; cDCs, conventional dendritic cells; Eo, eosinophils; GRB2, growth-factor-receptor-bound protein 2; Mac, macrophages; Mo, monocytes; MyP, myeloid progenitors; N, neutrophils; ND, not determined; NK, natural killer cells; OligoD, oligodendrocytes; pDCs, plasmacytoid dendritic cells; Schw, Schwann cells; Troph, trophoblasts.

Adapted from Crocker *et al.*, 2007, *Nat Rev Immunol*, 7(4):255-266

Carlin *et al.*, demonstrated that all GBS serotypes are able to interact with CD33-related Siglecs in a Sia-dependent manner (43). CD33-related Siglecs have been linked to inhibition of cellular activation and proliferation, as well as induction of apoptosis and pro-inflammatory cytokines. The interaction between Sias and their receptors triggers a signaling cascade, which is dependent on the ITIM and ITIM-like intracellular motifs that recruite the SHP1, SHP2 and SOCS3 tyrosine phosphatases, the last being a negative regulator of Siglecs inhibitiory functions (55).

Serotypes Ia and Ib strongly interact with hSiglec-5 and -7, while hSiglec-9 is the preferential target for serotype III. Confocal microscopy experiments on GBS confirmed this interaction on neutrophils (**Fig. 11**) (43). The Sias O-actetylation levels were reported to influence the engagement of Siglecs by GBS. An excess of Sia O-acetylation leads to the attenuation of GBS virulence in *in vivo* experiments (44). It has also been demonstrated that GBS interaction with hSiglec-5 and -9 reduces neutrophil oxidative burst, NET formation and bactericidal activity.

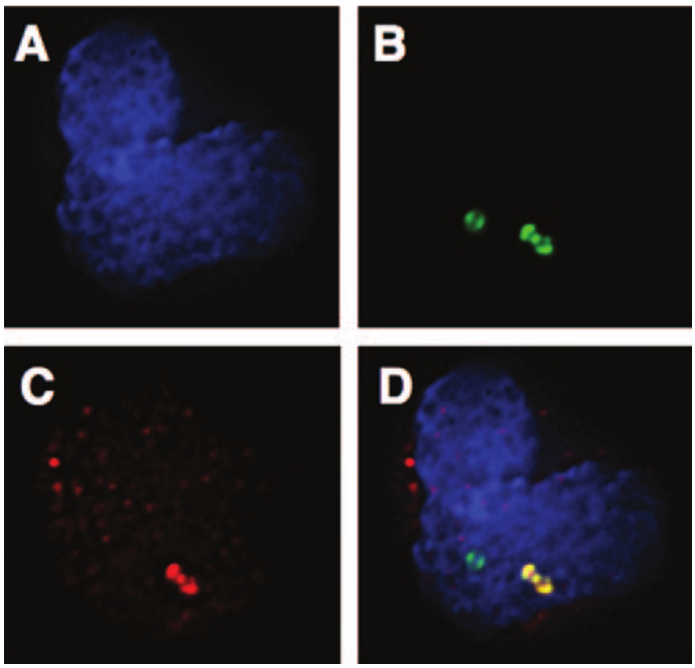

Figure 11. GBS attached to the surface of human neutrophils colocalizes with hSiglec-9. A) Nuclei of neutrophils; B) FITC-labeled GBS; C) hSiglec-9 on neutrophils; D) merge of previous pictures. Adapted from Carlin *et al.*, 2007, *J Bac*, 189(4):1231-1237.

Interestingly, in addition to the Sia-dependent Siglec engagement, it was discovered that some GBS strains can interact with hSiglec-5 through the surface-anchored β protein, which promotes bacterial attachment to the macrophage surface and impairment of the phagocytic activity. In addition, engagement of Siglec-5 by β protein increased SHP phosphatase recruitment to Siglec-5 and blunted neutrophils activation (56). Additional *in vivo* experiments in mice demonstrated that knock out mutants for Siglec E (a functional paralog of hSiglec-9) significantly enhanced macrophage inflammatory and bactericidal activity against GBS. Animals lacking Siglec-E expression appeared to benefit by the elevated inflammatory and bactericidal responses upon a low dose GBS infection. In contrast, after a lethel dose challenge, a detrimental overwhelming inflammation was observed in the Siglec-E-deficient mice.

A Sia-independent GBS-Siglecs interaction was also investigated. We report the example of the Sialoadhesin. Sialoadhesin has been identified as a receptor interacting with red blood cell receptors to modulate host immune responses through

the regulation of cell–cell interactions (57). However, since Sialoadhesin recognizes the same Sia epitopes utilized by the sialylated pathogens, it also plays a role in controlling invasive bacterial infections. Chang et al., demonstrated that GBS–sialoadhesin interaction facilitated the phagocytic and bactericidal activity of macrophages *in vitro*, and the efficient capture and control GBS dissemination *in vivo* upon systemic intravenous challenge (57, 58).

The delicate interplay between GBS Sias and Siglecs, represents an example of host-pathogen co-evolution to gain the best fitness advantage for survival within the host or defense against the pathogen.

Protein virulence factors involved in interference of immune responses

GBS success as a pathogen does not solely depend on the CPS, but also on a plethora of protein antigens, both Cell Wall Anchored (CWA) or secreted, that allow adherence and persistence in different host niches. This thesis focuses on GBS proteins that interfere with the key steps of the Complement cascade, a common immune mechanism among Gram positive and negative bacteria (59, 60).

One important strategy for Complement escape consists in covalently binding Complement regulatory proteins to the microbial surface, thus decreasing Complement activation and enhancing GBS survival in the host (61). These virulence factors find their targets at the key steps of the Complement cascade, namely C3 convertases formation, C3b deposition and C5a anaphylotoxin formation. Moreover there are evidences of bacterial proteins that interfere with Complement as a mediator of innate and adaptive immune responses.

Factor H binding proteins

One of the most common Complement evasion strategies consists in Factor H binding. Apart from Sialic acid, GBS also displays some proteins on its surface that are able to bind Factor H, the Complement modulator that induces C3b degradation by stabilizing Factor I. The binding of Factor H to the GBS surface not only enhances the inactivation of the Complement cascade, aiding the bacteria in evasion of phagocytic clearance, but might also influence other activities such as adhesion and invasion of host cells (*i.e.* brain endothelial cells to cause bacterial meningitis) (62, 63).

GBS expresses the β protein, a 125 kDa surface protein initially identified as one of the two components of the *c* antigen in type I GBS, together with the alpha protein (64). The β protein is mainly expressed by serotypes Ia, Ib, II and V, and absent in serotype III. It is known to interact via different binding regions with the Fc portion of Immunoglobulin A and with Factor H (65). Moreover human Siglec-5 was also studied for its interaction with the N-terminal domain of GBS β protein (66). This protein-dependent interaction with Siglec-5 promoted bacterial attachment to the

macrophage surface but impaired the cell's phagocytic activity (56). Genomic and structural data revealed that the β protein shares a high degree of similarity towards *S. pneumoniae* Hic protein, another bacterial virulence determinants that binds Factor H (59).

The same activity was investigated for the Streptococcal Histidine Triad (SHT), a 100 kDa protein that was recently studied by Maruvada *et al.* (63). Streptococcal Histidine Triad (SHT) gene is located in a pathogenicity island and in close proximity to the *scpB* and *lmb* virulence genes (63).

C5a peptidase

The Streptococcal C5a Peptidase (ScpB), is considered another important GBS virulence determinant and is highly conserved among human isolates. It cleaves C5a, which generates from C5, thus abolishing the chemotactic ability of this 74-residue polypeptide. C5a is an anaphylotoxin that attracts neutrophils (PMNs) to the infection sites, promoting histamine release and capillary permeability (67). C5a peptidase is highly conserved among streptococci and it was demonstrated that mice immunized against ScpB were able to promote the clearance of GBS introduced in their lungs (68).

BibA

Bacteria evolved several virulence mechanisms in order to exploit host Complement regulation mechanism: besides Factor H, which downregulates C3b formation, C4-Binding Protein (C4BP) is an inhibitor of both Classical and Lectin Pathways and promotes the cleavage of C4b and C3b. Several GBS strains and serotypes express BibA, a 630 aminoacids protein that was shown to bind C4BP and to increase the virulence of this microorganism (69).

Secreted virulence determinants

Besides the above mentioned CWA proteins, Pietrocola and colleagues recently studied a small secreted virulence determinant that interferes with the Complement cascade. It was named Complement Interfering Protein (CIP) for its ability to interact with C4 and C4b Complement components, thus preventing the formation of the Classical and Lectin C3 convertase (70).

CIP is encoded in a phage-derived region and is expressed by all the ST17 hypervirulent strains. It is considered a protein chimera as it shows partial homology to other small secreted bacterial proteins, in particular Extracellular Adherence Protein (Eap) and Extracellular Fibrinogen Binding protein (Efb), both from *Staphylococcus aureus*. CIP ability to bind to C4 and C4b resembles the Eap, that is known to prevent C4b2a convertase formation (71). Further structural studies conducted by Woehl and colleagues demonstrated that, despite the similarity of the of the CIP and Eap proteins, their binding sites on the C4b did not concide. Indeed the two proteins do not compete for C3b binding in competition experiments (72).

Chapter I:

Complement pathways involved in antibody-mediated opsonophagocytic killing of Group B *Streptococcus*

Background and Aim of the Study

GBS virulence depends on an array of factors (10), among which the capsular polysaccharide (CPS) that surrounds the bacteria is the best studied. CPS prevents phagocytic killing by interfering with Complement deposition on the bacterial surface and could represent a valuable GBS vaccine target (26). However, not all GBS strains express the capsular polysaccharide as, due to mutations in the capsule genes, up to 15% of colonizing isolates and of those causing adult invasive disease are naturally unencapsulated (73).

An important component of the capsule is the Sialic Acid (SA), present in all GBS serotypes. SA is considered one of the main components in the prevention of C3b deposition on GBS surface (54, 74). It has been demonstrated that the amount of Sialic acid present on the surface of mammalian erythrocytes correlates with their susceptibility to lysis. Rabbit erythrocytes, for example, display a low amount of Sialic acid on their membrane and are considered Complement activating particles (54, 75). Surface-associated Sialic acid modulates the Alternative Pathway activity by increasing the affinity of Factor H to C3b relative to Factor B, thus preventig the formation of the C3bBb convertase (76, 77).

The delicate balance among specific antibody concentration, capsule content and Sialic acid, influences the final outcome of Complement attack. The role of the different Complement pathways in the GBS clearance process is still under debate. Baker *et al.* (78) observed that some GBS strains belonging to serotypes Ia and III could undergo opsonophagocytosis in the absence of CPS- or protein-specific human sera. This antibody-independent killing was abolished in the presence of MgEGTA, which inihibits the Classical and Lectin pathways by chelating Calcium. Based on these observations, Baker and colleagues hypothesized a role for the Classical Pathway in antibody-independent GBS killing.

Further studies focusing on the role of Sialic acid on the inhibition of Complement activation, showed that removal of this sugar from the GBS surface through Neuraminidase treatment, leads to a strong activation of the Alternative Pathway and increased susceptibility to opsonophagocytic killing. Moreover it was demonstrated that high amounts of capsule-specific antibodies could hide SA

moieties on the GBS surface thus allowing Complement deposition via the Alternative Pathway, in an antibody-dependent mechanism (76).

Further, the Lectin Pathway (LP) was hypothesized as a potential mechanism for initiating opsonization of GBS with sera devoid of CPS-specific antibodies, as the C3 convertase of the LP (C4b2a) is generated by a mechanism that is both Calcium-dependent and antibody-independent (79). The LP shares its downstream effectors with the CP, but not its initiators. The CP starts by the binding of C1q to the antibodies present on bacterial surface, whereas the LP exploits Lectins that interact with glucidic residues on bacterial membranes, thus forming a complex with MBL/MASP (30). Three different lectins, which impact the LP, have been described: mannose-binding lectin (MBL), L-ficolin, and H-ficolin (80). Among them, MBL shows the lowest affinity towards the GBS capsule, while L-ficolin strongly interacts with both Lipotheicoic Acid (LTA) and N-acetylglucosamine (GlcNac) residues on GBS surface (79). By investigating the role of L-ficolin in initiating the Complement cascade on GBS, Aoyagi *et al.* (81) demonstrated that this lectin was able to bind both the wild type and acapsular mutant strains, and suggested Lipoteichoic Acid (LTA) as the possible Lectin target. They observed the formation of the C4b2a convertase by the Lectin Pathway, which led to the amplification of the cascade through the Alternative Pathway.

This work was aimed at further investigating the mechanisms of Complement activity on GBS, both in absence and presence of specific antibodies. The highly encapsulated serotype III COH1 strain, which is the most prevalent serotype in GBS neonatal infections worldwide, and the acapsular isogenic mutant COH1 Δ*cpsE* were selected for this study.

The contribution of each Complement pathway against GBS was investigated by C3b deposition, C1q binding and Opsonophagocytosis experiments. The relevance the Classical pathway was investigated through the use of C1q-depleted Complement sources and Calcium/Magnesium depletion. The contribution of the Alternative Pathway was assessed by using a Factor B-depleted Complement source. The role of Sialic acid in Complement deposition was studied by comparing serotype III GBS treated with Neuraminidase to the wild type and acapsular mutant strains.

Materials and Methods

Bacterial strains and culture media

Serotype III GBS clinical isolates COH1 (high capsule content) and its COH1 Δ*cpsE* (unencapsulated isogenic mutant)(63) were used in this study. Bacteria were grown in Todd-Hewitt Broth (THB, Becton Dickinson, Sparks, MD) at 37˚C in static conditions to an approximate OD of 0.6 (Abs600nm).

Proteins and human sera

For C3b deposition experiments, different human sera were tested as Complement sources or as GBS-specific IgG source. Human sera from GBS infected patients were used as IgG source after inactivation at 56°C for 30 minutes and had different IgG titers against CPS III, as measured by anti-PSIII ELISA. The Complement sources were complete Human Complement (HC), C1q-depleted human serum (Quidel, San Diego, CA) to inhibit CP, and Factor B-depleted serum to inhibit AP (CompTech Inc., Texas, USA). The depleted Complement sources were restored by adding C1q (Calbiochem, Germany) and Factor B (CompTech Inc., Texas, USA).

Growth and differentiation of HL-60 cells

HL-60 cells belong to a promyelocytic leukemia cell line. They were purchased from the American Type Culture Collection (CCL-240) and cultured in RPMI 1640 Glutamax (Invitrogen), supplemented with 10% heat inactivated Fetal Calf Serum (FCS, HyClone).

Cells were grown and differentiated to neutrophils four days before the experiments in growth medium supplemented with 0.78% Dimethyl Formamide (DMF, Sigma), according to Romero-Steiner *et al*. (82).

OpsonoPhagocytic Killing Assay (OPKA) on GBS

The OPKA reaction was performed in 96 well Nunc polypropylene microtiter plates (ThermoFisher), in a total volume of 125 µl HBSS. For each reaction mixture, 12.5 µl of human immune sera (0.25 - 0.08 - 0.025 µg/ml of PSIII-specific IgG) or reaction buffer, GBS bacteria (25 µl), differentiated HL-60 cells (75 µl) and human Complement (12.5 µl) were added. Control reactions were performed in the presence of heat inactivated Complement or in the absence of effector cells.

COH1 and COH1 Δ*cpsE* bacterial suspensions were prepared by directly diluting frozen aliquot stocks. One ml aliquot of frozen bacteria ($OD600_{nm}$=0.5) was thawed at RT, diluted in 9 ml of PBS and centrifuged at 3000 rpm for 10 min. The pellet was suspended in an appropriate volume of HBSS with Calcium and Magnesium in order to obtain a final concentration of ~600 CFU/µl (~$1.5x10^4$ CFU/well). COH1 was resuspended in 28 ml, while its acapsular isogenic mutant in 18 ml of buffer. Bacteria were then further diluted 1:2 in HBSS-1% Normal Rabbit Serum (Sigma) and dispensed in plates. The HL60 effector cells were used at a concentration of $2.6x10^7$/ml with a GBS to cells ratio varying from 25:1 to 40:1. The reaction plate was incubated for 1 h at 37 °C with shaking at 600 rpm by a Thermomixer (Eppendorf).

T0 reactions were diluted 1:100 in sterile water and plated in tilt onto Trypticase Soy Agar-5% blood sheep plates (TSA) (BD Biosciences). T60 reactions were diluted 1:20 and 1:200 in sterile water. Ten microliters of each dilution were then plated on TSA plates and incubated overnight at 37 °C + 5% CO_2 in order to determine Colony Forming Units at T0 and T60. The OPKA titer was expressed as the reciprocal serum dilution leading to 50% killing of bacteria, and percent of killing was calculated using this formula: killing (%)=[(mean CFU at T0−mean CFU at T60)/mean CFU at T0]x100. Data were then elaborated using GraphPad Software.

Paraformaldehyde treatment of bacteria

Bacteria were plated onto TSA plates and incubated overnight (~16 h) at 37°C. The following day 25 isolated colonies for each strain were selected and stirred into THB. Bacteria were grown as previously described and the growth was followed by measuring the absorbance at 600 nm, using THB as blank control. Bacterial growth was monitored until an OD of 0.6 was reached. At that point, 50 ml of bacteria were collected, centrifuged 10 minutes at 3000 rpm, treated with 25 ml of PBS, 0.1% paraformaldehyde (PFA) for 1 h at 37°C and then stored overnight at 4°C. Bacteria were then tested for inactivation by plating on TSA Blood Agar plates, and counted at FACS (SOS I).

Neuraminidase treatment of COH1

COH1 strain was inoculated and grown o/n at 37°C in THB, without shaking. The culture was diluted to OD=0.05 in 50 ml of THB and then grown until OD=0.6. Bacteria were centrifuged (3000 rpm, 10 minutes at 4°C) and resuspended in 0.5 ml of PBS pH 6.0 and 10 μl of Neuraminidase (α(2-->3,6,8,9) Neuraminidase from *Arthrobacter urefaciens*, Sigma). This enzyme had a specific activity of ≥135 units/mg protein, where one unit of Neuraminidase hydrolyzed 1 μmole of 4-methylumbelliferyl α-D-N-acetylneuraminide per minute at pH 5.0. The incubation was left 1 hour at 37°C. Bacteria were washed twice and then fixed in PFA 1% for 1 hour 37°C.

Inactivation of bacteria was assessed by plating on TSA-Blood Agar plates and then counted on a FACS (SOSI).

C3b Deposition on GBS and FACS analysis

C3b deposition experiments on GBS were performed on PFA-treated COH1, COH1 Δ*cpsE* and Neuraminidase treated COH1 by Flow Cytometry. Bacteria (25 µl of 4x10^{7}cells/ml) were incubated with 12.5 µl of heat-inactivated human serum diluted in HBSS with or without Calcium and Magnesium (Invitrogen). In the C3b deposition set up experiments a rabbit PSIII-specific serum was used at 1:30, 1:90, 1:270 and 1:810, together with 20% Baby Rabbit Serum (Cederlane) as Complement source. In subsequent experiments, 25 µl of the different human Complement sources (C1q-, fB-depleted) were used. HBSS was added instead of IgG sources, as negative control. In other experiments, bacteria were incubated with different amounts of anti-PSIII human IgG that ranged from high (20 µg/ml) to low (1 µg/ml) or no titer.

The reactions were then left at room temperature for 15'. Bacteria were centrifuged at 3000 rpm for 7', washed with PBS, and the deposition revealed with a FITC-labeled Mouse anti-human C3b polyclonal antibody (Cederlane) diluted 1:100 in PBS. Incubation was kept in ice for 30' and washed twice with PBS. Fluorescence signals were measured by Flow cytometry (CANTO II) using the BD FACSDIVA Software, and analyzed through the FlowJo Software. The reported Mean Fluorescence Intensity (MFI) corresponds to the Geometric Mean of the fluorescence peak area which derives from the area under the peak (**Fig. 1**). In the Complement-restored samples, C1q or Factor B were added to the depleted Complement sources, taking into account the concentration of those factors in plasma (generally 50 µg/ml for C1q and 200 µg/ml for Factor B) and the final Complement concentration in the reaction (20%) (83).

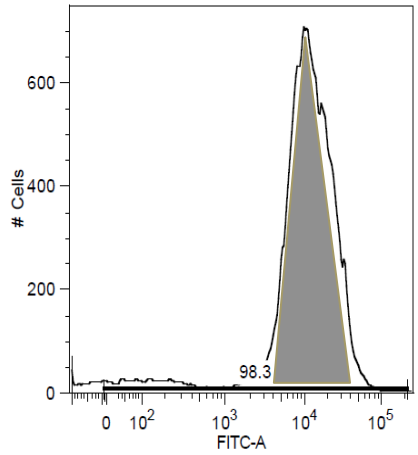

Figure 12. Example of Flow Cytometry fluorescence peak. Fluorescence is reported on the *x* axis, while the number of cell on the *y* axis. The C3b deposition values derive from the Geometric Mean of the grey area under the peak.

C1q binding

To evaluate whether C1q could bind to GBS surface in an antibody-dependent and –independent manner, a Flow Cytometry experiment was performed. Previously fixed and counted COH1, Δ*cpsE* and Neuraminidase Treated COH1 strains were used at $4x10^7$/ml in PBS. Bacteria were incubated with either 1 μg or 0.33 μg of a low anti-PSIII IgG titer human serum for 10 minutes at Room Temperature, and subsequently washed with PBS.

100 μl of C1q at 200 μg/ml were added to each well and incubated with bacteria for 15'. The plate was washed and incubated with a Phycoerithrin-labeled Mouse Anti-Human C1q (Cederlane) for 30' at 4°C (1:100). After two washes with PBS, samples were acquired on CANTO-II using the BDiva Software (BD Biosciences). Data were analyzed using the FlowJo software and are represented as the Geometric Mean of the Fluorescence Intensity.

Results

Contribution of the capsular polysaccharide (CPS) to Complement-mediated GBS phagocytic killing

To confirm the protective effect of the capsular polysaccharide against GBS phagocytosis, we first performed opsonophagocytic killing experiments using the highly encapsulated serotype III COH1 strain and its acapsular isogenic mutant COH1 Δ*cpsE*.

As shown in **Figure 2A** the acapsular mutant strain was completely killed by neutrophils both in the absence and in presence of specific antibodies. Conversely the wild type COH1 strain survived to phagocytic treatment in the absence of immune serum, and was killed only in the presence of PSIII-specific IgG.

We further investigated the susceptibility of the acapsular GBS to phagocytic killing in the absence of specific IgG using different Complement concentrations.

As shown in **Figure 2B**, the susceptibility of COH1 Δ*cpsE* to Complement alone was high. This strain was killed at concentrations as low as 0.007%, confirming a strong effect of the capsule in protecting GBS bacteria from Complement-mediated phagocytic killing.

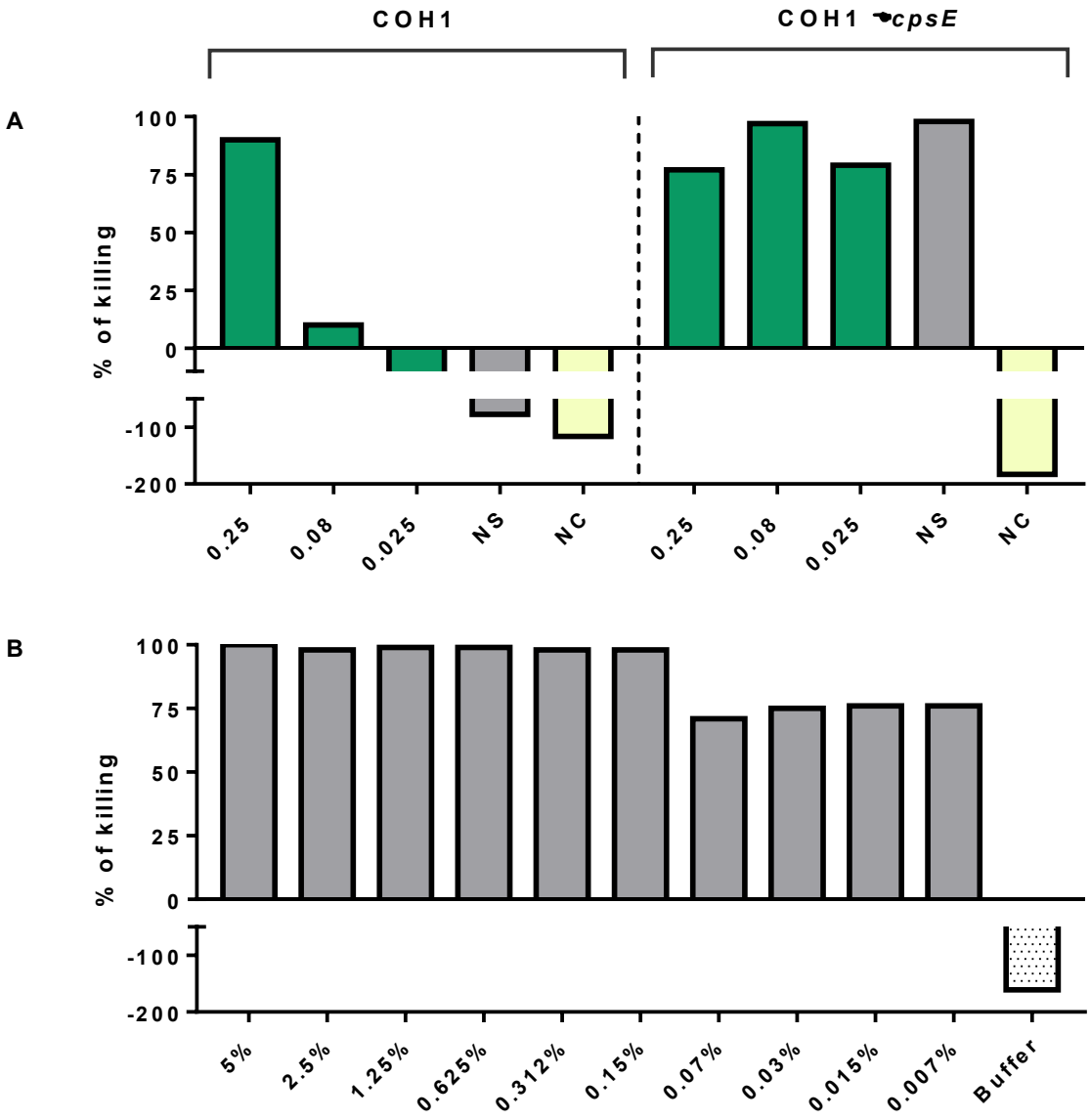

Figure 2. Opsonophagocytic killing of type III GBS in the absence and presence of serum. ***Panel A*****:** Opsonophagocytosis of COH1 and its acapsular mutant in the absence or presence of decreasing serum dilutions (0.25 - 0.08 - 0.025 μg/ml of PSIII-specific IgG). Green bars represent GBS killing in the presence of serum and Complement; grey bars represent killing in the absence of serum (NS) and in the presence of Complement, while yellow bars show GBS killing in the absence of Complement (NC) and in presence of PSIII-specific IgG. ***Panel B***: Opsonophagocytosis experiment conducted on COH1 acapsular mutant in the absence of immune sera and decreasing concentrations of human Complement (5%-0.007%).

Set-up of C3b deposition experiments on a highly encapsulated GBS type III strain

To better dissect the role of the capsule in protecting GBS from Complement attack, we assessed C3b deposition on wild type and unencapsulated COH1. C3b deposition on bacterial surface represents the fundamental step for Complement-mediated phagocytic killing.

We first analyzed the growth curves of GBS COH1 highly encapsulated type III strain and of its acapsular mutant (**Fig 3A**). The two curves were comparable as they showed similar slopes in the exponential part, although the wild type strain recovered from the stationary phase more quickly than the acapsular mutant. Indeed, COH1 reached the stationary phase at OD_{600} 2, and its acapsular mutant at OD_{600} 1.7.

We subsequently performed C3b deposition experiments on COH1 grown until early, mid- and late exponential phases (OD_{600} 0.3, 0.6 and 1 respectively). Bacteria were first incubated with serial dilutions of a rabbit PSIII-specific serum and with 20% Baby Rabbit Serum (Cederlane) as Complement source. C3b deposition was revealed as described in the Materials and Methods section. As shown in the upper panels of **Figure 3B**, the deposition signal in the presence of serum was detectable in all the tested growth points with no significant differences. No Complement deposition was observed on serotype III COH1 strain in the absence of specific serum (bottom panels **Figure 3B**). OD_{600} 0.6 was selected for further experiments.

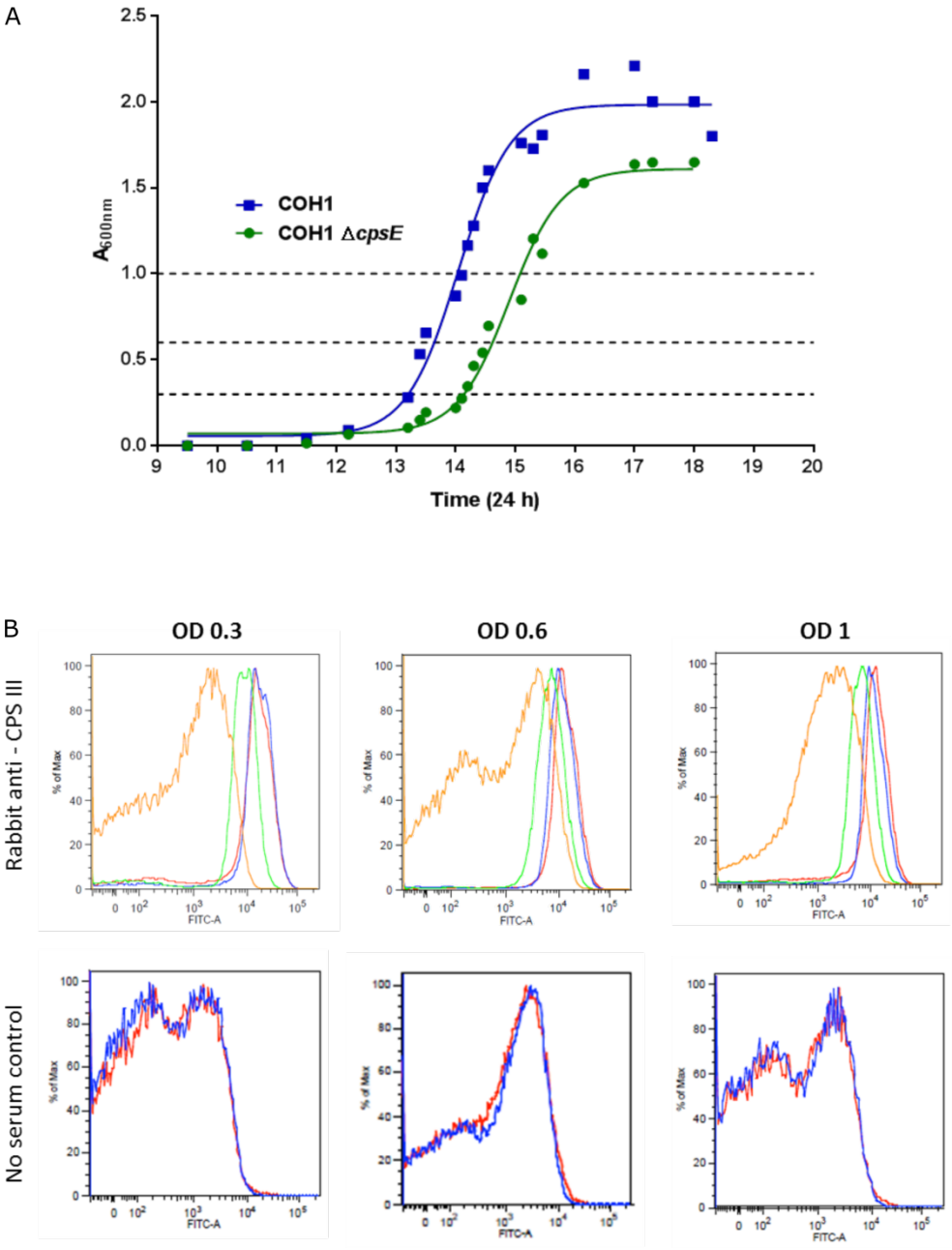

Figure 3. Growth curves and C3b deposition on COH1 at different time points. ***Panel A*****:** Growth curves of COH1 and COH1 Δ*cpsE*. Bacteria were grown in THB, at 37°C, static conditions. The dotted lines correspond to the growth points considered for C3b deposition experiments. ***Panel B:*** C3b deposition on COH1 at different growth points. Upper panels show C3b deposition on bacteria incubated with serial dilutions of PSIII-specific rabbit serum and stained with an anti-C3b FITC-labeled secondary antibody. Lower panels show the negative controls in which no IgG source was added. Sera dilutions: red 1:30; blue 1:90; green 1:270; orange 1:810.

C3b deposition on GBS COH1 and its isogenic acapsular mutant in the absence or presence of CPS III-specific antibodies

The highly encapsulated type III COH1 strain and its non-encapsulated isogenic mutant *ΔcpsE* grown at mid-exponential phase were then tested for C3b deposition using heat-inactivated human sera. The sera selected for these experiments belonged to individuals previously exposed to type III GBS and contained high (20 μg/ml) or low (1 μg/ml) IgG titer against CPS-III (78).

Bacteria were incubated in presence or absence of heat incativated immune sera and 20% non-immune serum as complete Human Complement source (HC). As shown in **Figure 4**, in the absence of specific IgG, C3b deposition on COH1 was very low. Conversely, the acapsular mutant was highly susceptible to Complement deposition. In the presence of PSIII-specific antibodies, the levels of C3b deposition on the acapsular mutant were higher than the wild type strain and similar to those observed in the absence of antibodies. Moreover the deposition signals were higher in the 1 μg/ml samples compared to the 20 μg/ml ones, suggesting a possible steric hindrance in presence of high amount of anti PSIII antibodies.

These results led us to conclude that the highly encapsulated strain was resistant to C3b deposition in the absence of specific IgG, while the unencapsulated strain was highly susceptible, confirming the importance of the capsule for GBS Complement evasion.

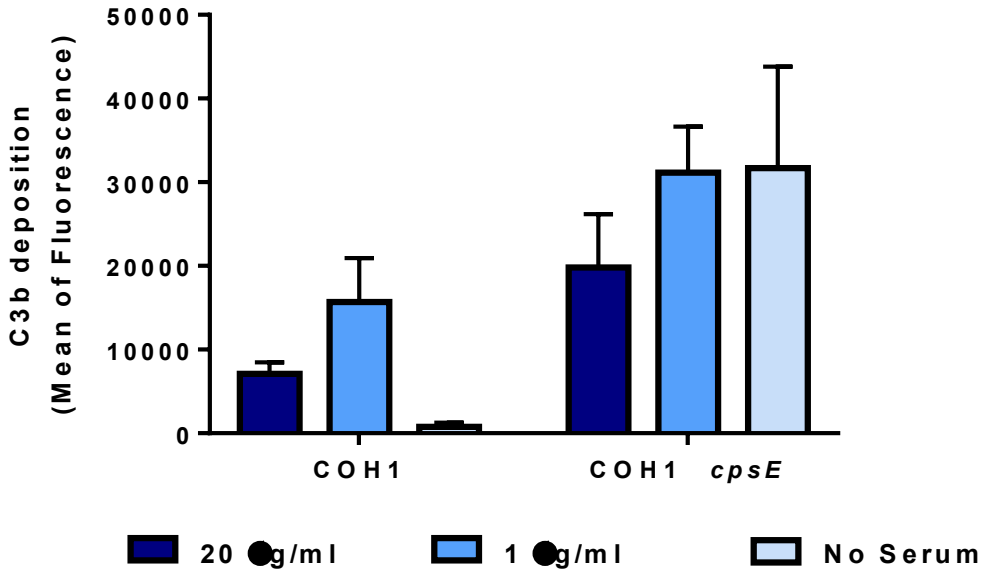

Figure 4. C3b deposition on COH1 and its acapsular isogenic mutant strain. C3b deposition on bacteria incubated with or without different PSIII-specific amounts of IgG (20 μg/ml; 1 μg/ml) and with 20% Normal Human Complement (NHC). C3b on bacterial surface was revealed with a FITC-labeled anti-C3b antibody and the deposition signal detected by Flow Cytometry. The graph shows the Geometric Mean of the FITC fluorescence.

Depletion of Complement Factors affects C3b deposition

To gain information on the contribution of the Classical and Alternative Pathways on GBS Complement susceptibility, we tested COH1 and its acapsular mutant for C3b deposition using C1q- and Factor B-depleted Complement sources. The two strains were incubated with or without human serum containing low (1 μg/ml) PSIII specific IgG concentrations.

Regarding the encapsulated strain (**Fig. 5A**), the absence of C1q totally abolished deposition. C3b binding was restored upon addition of C1q. Also in this case, the presence of antibodies resulted in higher C3b deposition compared to the No Serum control. Conversely, the absence of Factor B only partially lowered the deposition signal on COH1. Deposition occurred both in presence or absence of serum, suggesting that this Complement source contained GBS-specific antibodies.

Concerning the acapsular mutant, the absence of C1q completely abolished deposition in the absence and presence of the IgG sources (**Fig. 5B**). Deposition was restored by adding C1q, while the absence of Factor B did not have any effect.

Overall, these data led to the conclusion that the Capsular polysaccharide inhibits the activation of the Classical Pathway on serotype III GBS COH1.

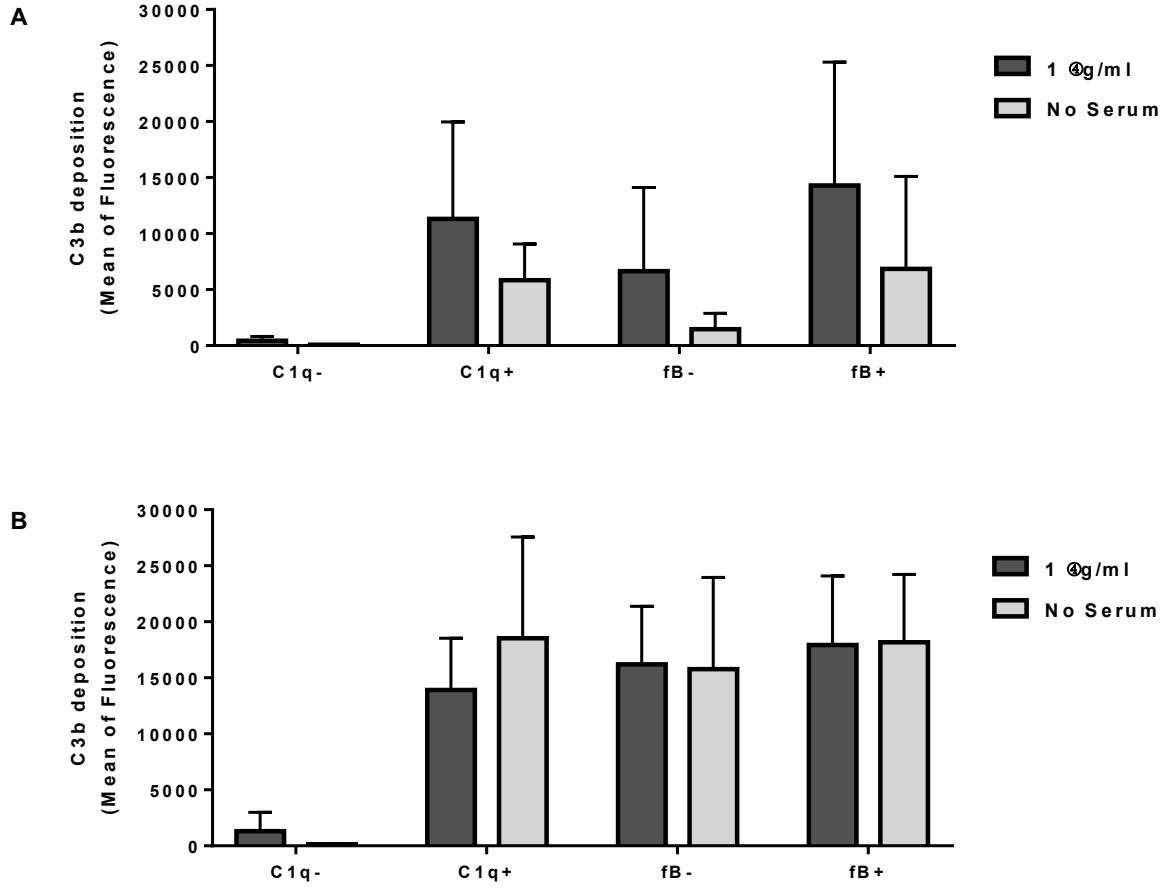

Figure 5. C3b deposition on COH1 and COH1 Δ*cpsE* strains with factors-depleted Complement sources. COH1 (***Panel A***) and COH1 Δ*cpsE* (***Panel B***) bacteria were incubated with or without sera displaying low IgG titer against PSIII and with 20% human Complement depleted and restored of C1q and Factor B (fB). C3b on bacterial surface was revealed with a FITC-labeled anti-C3b antibody and the deposition signal detected by Flow Cytometry. The graph shows the Geometric Mean of the FITC fluorescence.

C1q binding to GBS surface in the presence of a low anti-PSIII specific IgG titer serum

To further investigate the possible activity of C1q, C1q binding experiments were performed both on wild type COH1 and its acapsular isogenic mutant. Bacteria were first incubated with heat-inactivated human serum (contanining 1 μg/ml of PSIII-specific antibodies) or without serum and subsequently added 200 μg/ml of C1q (Calbiochem). The fluorescence signal indicative of C1q binding was detected through the use of a Phycoeritrin-labeled monoclonal antibody against C1q.

As shown in **Figure 6**, C1q binding to COH1 did not occur in the absence of specific serum, and was low in the presence of IgG. Regarding the acapsular mutant, C1q binding occurred only in presence of human serum.

The observed C1q binding to COH1 Δ*cpsE* in the presence of human serum suggests that this serum contained IgG directed to GBS proteins in addition to the anti capsular antibodies. The assay sensitivity did not allow to detect any C1q binding in absence of antibodies.

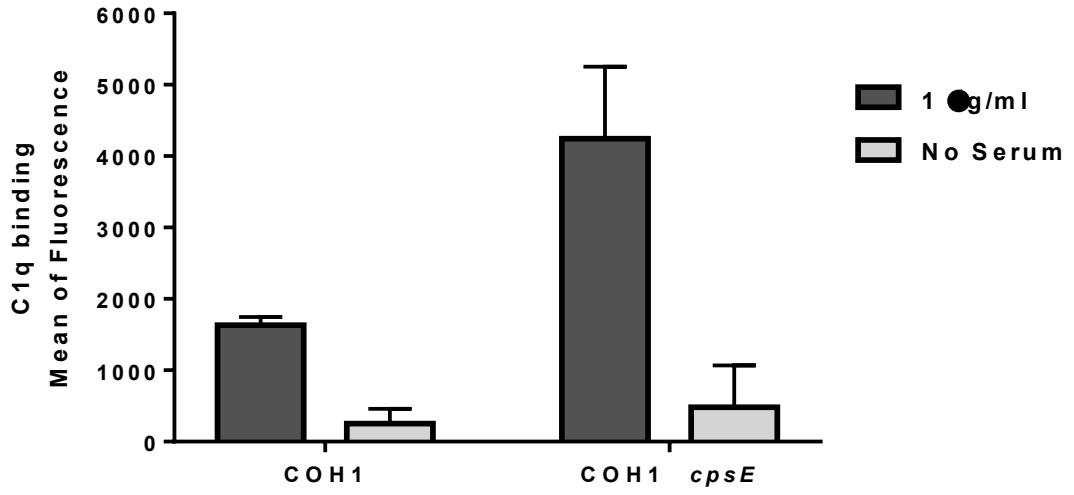

Figure 6. C1q binding on COH1 and its acapsular isogenic mutant strain. GBS bacteria were incubated with or without heat-inactivated human serum (1 μg/ml of PSIII-specific IgG) and with 200 μg/ml of C1q. Binding was revealed with an anti-C1q monoclonal antibody labeled with Phycoerithrin and fluorescence was analyzed by Flow Cytometry.

Evaluation of the role of the Sialic acid in GBS Complement evasion strategies

Our data provided evidence about the role of the capsular polysaccharide in the prevention of C3b deposition on GBS. Nevertheless, in order to better elucidate the the contribution of Sialic acid in the modulation of the Complement cascade, we performed C3b deposition experiments in the absence of a GBS-specific IgG source on GBS COH1 previously treated with Neuraminidase, a glycoside hydrolase that cleaves the glycosidic linkages of neuraminic acids (*i.e.* Sialic acid), and compared it to the untreated control.

The results obtained for C3b deposition on COH1 (**Fig. 5A**) were consistent with the ones deriving from previous experiments, in which the deposition signal was abolished in the absence of C1q and Factor B. Moreover in the presence of complete Complement source (C1q- and Factor B-restored conditions), deposition was detectable (**Fig. 7**). Regarding the Neuraminidase Treated strain, the absence of C1q and Factor B abolished C3b binding, while after addition of Complement effectors deposition levels were higher compared to wild type COH1, and similar to those observed for the acapsular mutant (**Fig. 5B**).

Previous work from Fearon *et al.* (54) demonstrated that Sialic acid is able to interfere with the Complement cascade. They hypothesized that this inhibitory activity was due to the interaction with Factor H (fH), and to the ability to control the Complement cascade activation by inhibiting the Alternative Pathway. However these results provided evidence on the role of Sialic acid in interfering with not only with Alternative, but also with the Classical Pathway by the prevention of C3b binding.

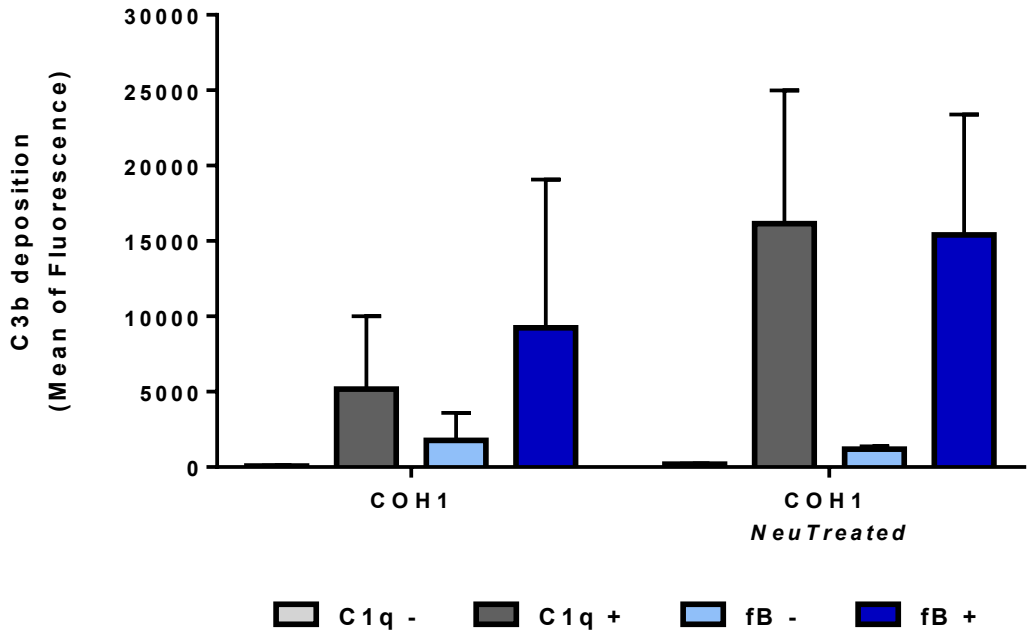

Figure 7. C3b deposition on COH1 and its Neuraminidase Treated control. Type III COH1 strain and its Neuraminidase Treated control were tested for C3b deposition in the absence of GBS-specific IgG. Bacteria were incubated with factors-depleted Complement sources and with their restored controls. C3b on bacterial surface was revealed with a FITC-labeled anti-C3b antibody and the deposition signal detected by Flow Cytometry. The graph shows the Geometric Mean of the FITC fluorescence.

Contribution of the Alternative Pathway to C3b deposition

To further investigate the role of the capsular polysaccharide and its Sialic acid component in protecting COH1 from Complement deposition via the Classical and Alternative pathways, C3b deposition experiments in the absence of specific antibodies were performed on COH1 Δ*cpsE* and Neuraminidase treated strains, with or without Calcium and Magnesium. In fact, the Classical and Lectin pathways require Calcium and Magnesium to enable the formation and functionality of their C3 convertases (30, 84).

As shown in **Figure 8**, the absence of Calcium and Magnesium totally abolished the C3b deposition signals both for COH1 Δ*cpsE* and Neuraminidase treated strains in all tested conditions.

In the control tests performed in presence of Calcium and Magnesium, the data matched those presented in **Figures 5B** and **7**. In particular C3b deposition occurred both for COH1 Δ*cpsE* and Neuraminidase treated strains when using Complement sources containing both C1q and Factor B. When C1q was lacking, no deposition occurred on either strain, while in the absence of Factor B deposition was observed only for the acapsular mutant.

The above experiments confirmed that the Sialic acid containing type III capsular polysaccharide protects GBS COH1 from Complement deposition both via the Classical and Alternative Pathways by the prevention of C3b binding.

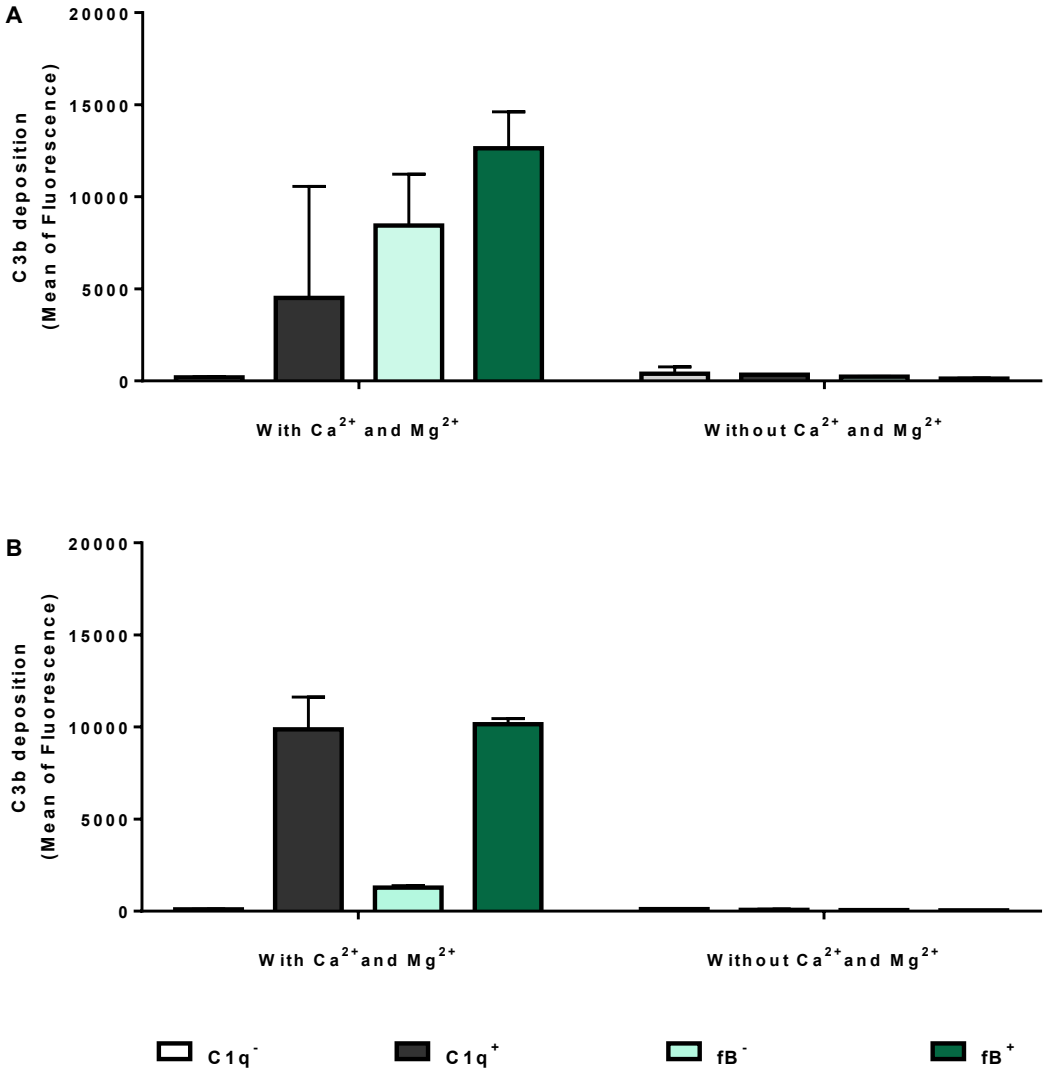

Figure 8. C3b deposition on COH1 Δ*cpsE* and on the Neuraminidase Treated control. Unencapsulated (***Panel A***) and Neuraminidase Treated COH1 (***Panel B***) bacteria were incubated with C1q- or Factor B-depleted Complement sources in the absence or presence of Calcium and Magnesium. C1q- and Factor B-restored controls were also set. The experiments were performed in the absence of PSIII specific IgG. C3b on bacterial surface was revealed with a FITC-labeled anti-C3b antibody and the deposition signal detected by Flow Cytometry. The graph shows the Geometric Mean of the FITC fluorescence.

Discussion

There is compelling evidence that the GBS capsule inhibits C3b deposition through its glycosidic components, among which the Sialic acid is the most studied (74, 76). This work was focused on the role of capsular polysaccharide in protecting GBS from Complement attack. The followed approach was based on opsonophagocytic killing and C3b deposition experiments with the aim of elucidating the relative role of the Classical and Alternative Pathways in initiating GBS Complement-mediated clearance. The experiments were conducted using complete and C1q- or Factor B-depleted human Complement sources and buffers containing or devoid of Calcium and Magnesium. For our experiments we used the COH1strain belonging to the highly relevant GBS serotype III, its unencapsulated mutant COH1 Δ*cpsE* and Neuraminidase Treated wild type COH1.

Our results highlight a striking susceptibility of the GBS acapsular mutant to Complement-mediated phagocytic killing, even in the absence of specific antibodies, compared to the wild type strain. Conversely wild type bacteria were only killed in presence of GBS-specific IgG.

Complement susceptibility of the COH1 Δ*cpsE* strain was confirmed by C3b deposition experiments, with much higher signals compared to the wild type. Again, C3b deposition on the acapsular strain took place regardless of the presence of an IgG source. An important role of Sialic acid in protecting GBS bacteria from C3b deposition was underlined in C3b deposition experiments using the Neuraminidase Treated COH1.

It has been hypothesized that the protective effect of the capsular polysaccharide and its Sialic acid moiety is mainly associated with an interference with the Alternative Pathway by Factor H hijacking (76). Moreover, recent studies underlined the involvement of the Lectin Pathway in the innate immune response against this microorganism (81). It has also been reported that GBS defences against Complement can be counteracted by a high concentration of anti-capsule antibodies via the Alternative Pathway (76).

Conversely to what has been previously stated, our results showed that only the absence of C1q abolished C3b deposition on unencapsulated COH1, suggesting the

relevance of the Classical Pathway as initiator of the Complement cascade. C1q binding experiments did not provide direct evidence of C1q deposition on COH1 Δ*cpsE* and we hypothesize that this could be due to a lower sensitivity of this assay compared to the highly amplified C3b deposition. Conversely, the absence of Factor B never prevented C3b deposition on COH1 Δ*cpsE*, either with or without IgG. Therefore the Alternative Pathway does not seem to play a central role in initiating Complement attack on this acapsular strain.

To further characterize the role of Sialic acid in GBS Complement evasion, COH1 strain was treated with Neuraminidase, in order to remove the N-acetylneuraminic acid (Neu5Ac) moieties on its capsular polysaccharide. Our results confirmed high C3b deposition on this strain in the presence of complete Human Complement. Complement attack was totally abolished if C1q or Factor B were absent, suggesting a role of Sialic acid in protecting GBS against both the Classical and Alternative Pathways.

The absence of Calcium and Magnesium affected antibody-independent deposition on the unencapsulated and Neuraminidase Treated strains, confirming a key role of the capsule and it Sialic acid compoment in protecting GBS from C3b deposition via the Classical pathway.

Overall, these results highlight a relevant Complement inhibitory activity for capsular Sialic acid. It was recently demonstrated that the capacity of C1q to bind antibodies is influenced by the glycosilation levels of the Fc portion (85). The higher susceptibility of Neuraminidase Treated COH1 to C3b deposition in the absence of antibodies, could be explained by the fact that C1q is able to directly bind to the surface of bacteria, thus activating the Complement cascade.

In summary, we conclude that the capsule strongly inihibits Complement activation by the Classical Pathway and the following cascade amplification by the Alternative Pathway. Further experiments aimed at investigating the role of the Lectin Pathway are still needed. This will provide a complete overview on the susceptibility of GBS to Complement attack.

This work could serve as a model to better elucidate the role of the capsule in other GBS serotypes, whose response to Complement attack could be different on the basis of the different capsule composition. Having a clear picture of how the

Complement acts on GBS will help identifying new tools to counteract infections both in newborns and adults.

Chapter II:

Complement Inhibitory Protein (CIP) from *Streptococcus agalactiae* at the interface between innate and acquired immune response

A manuscript including the results presented in this section has been published in the FASEB J:

Giussani S., Pietrocola G., Donnarumma D., Norais N., Speziale P., Fabbrini M., Margarit I. (2018). The *Streptococcus agalactiae* complement interfering protein combines multiple complement-inhibitory mechanisms by interacting with both C4 and C3 ligands.
doi: 10.1096/fj.201801991R

Background and Aim of the Study

Group B *Streptococcus* is endowed with a number of virulence factors that facilitate the interactions with multiple targets, to penetrate host barriers and to counteract innate immune responses. Complement effectors serve as first line of defense against GBS infections, promoting phagocytic killing by neutrophils and macrophages (86). Therefore, the pathogen has evolved several mechanisms to counteract Complement activation in order to colonize and invade its host. Since the C3b deposition on the bacterial surface is the first fundamental step of the Complement cascade which engages all the three pathways, GBS has evolved several mechanisms to counteract this event. GBS primarly acts by preventing either the initiation of the Complement Cascade, or the formation of the C3 convertases (C4b2a for the Classical and Lectin Pathways and C3bBb for the Alternative Pathway) (87). One such example is the β protein, which promotes the decay of the Alternative Pathway by hijacking Factor H (59).

In addition to the well-known GBS Cell Wall Anchored (CWA) proteins, some GBS isolates express a recently identified small secreted virulence determinant that is able to modulate C3 convertase formation. This protein, named Complement Interfering Protein (CIP), is a 153-residues, 15 kDa polypeptide encoded in a phage-derived region, which is located in close proximity to recombination hot spots and to other hypothetical CWA proteins of unknown function (70). The corresponding gene was first annotated as *san_2130* (now COH1_1804) in the genome sequence of the GBS serotype III ST-17 COH1 strain and has been detected both in human and bovine isolates. Interestingly, a subset of strains from another human streptococcal pathogen, the group A *Streptococcus* (GAS), expresses a protein displaying 46% identity with CIP. The GBS *cip* gene and its GAS homolog are present in a similar phage-derived genomic region named RD2 that was acquired by horizontal transfer and is integrated into a tRNA gene flanked by direct repeats (70), suggesting inter-species lateral transfer.

Pietrocola and coworkers demonstrated that CIP was able to interact with both C4 and C4b Complement factors, inhibiting the Classical and Lectin Pathways (70). The formation of the C4b2a convertase was indeed prevented by CIP, while the

Alternative Pathway convertase (C3bBb) formation was not affected. Sequence analysis revealed that CIP shares a low identity (~15%) with the Extracellular Adherence Protein (Eap) from *Staphylococcus aureus*, a protein that has ability to interact with C4 and C4b Complement effectors (71). Despite the sequence and functional similarities between the two proteins, recent work demonstrated that CIP and Eap do not compete for C4b binding, which indicates that they do not bind to the same site on the C4b ligand (72).

CIP also presents partial homology with the C-terminal region of staphylococcal Extracellular fibrinogen binding protein (Efb), with the Efb-homologous protein (Ehp) and with the domain IV of Staphylococcal binding IgG protein (Sbi) (~12% identity and ~18% similarity with all of them). These three proteins are incapable of binding C4 ligands, but can interact with the C3 central component of Complement and its fragments C3b, iC3b, C3dg and C3d (87, 89, 90). Moreover Efb and Ehp exert an inhibitory function on the Alternative Pathway, as the interaction with C3b prevents the formation of the C3bBb convertase (88).

The Complement system is not only a means through which bacterial infections are controlled, but also a critical step of the immune response that can connect innate and adaptive immunity. C3b tags Gram positive microorganisms for phagocytosis, and its degradation by Factor I and other proteases leads to the formation of smaller fragments that act as mediators of the immune response. In particular C3d, the last C3b degradation fragment, is a small 35 kDa protein that is able to interact with Complement Receptor 2 (CR2, also known as CD21) on the surface of B cells. CR2/CD21, together with CD19 and CD81, constitutes the B cell co-receptor complex. C3d-opsonized bacteria are simultaneously bound by the CR2/CD21 receptor and by the antigen-specific B Cell Receptor (BCR). The crosslink between the two membrane receptors triggers a signaling cascade via the intracellular domains of BCR and CD19 component. This leads to enhanced B cell activation, proliferation and actin rearrangements (36). Therefore C3d acts as a molecular adjuvant for the B cell response.

The present study was aimed at functionally and biochemically characterizing CIP interaction with C3 and its subfragments C3b and C3d. The effect of CIP on C3d-CR2/CD21 binding both *in vitro* and *ex vivo* was studied by determining whether CIP could interfere with the CD21-mediated stimulation of B cells (monitoring the intracellular signaling pathways). Finally the possible CIP binding region for C3d was studied by Hydrogen-Deuterium Exchange-Mass Spectrometry analysis. In this respect, our study could represent a valuable contribution to both the characterization of molecular Complement evasion mechanisms and the development of CIP-based therapeutics.

Materials and Methods

Antibodies and reagents

C3, C3b and C3d were purchased from Merck (Darmstadt, Germany). Rabbit polyclonal antibody against C3 was provided by Abcam (Cambridge, MA, USA) and C3-depleted human serum was purchased from Sigma (Saint Louis, Missouri, USA). Soluble CR2/CD21 for ELISA assays were purchased from Sino Biologicals (Wayne, PA, Stati Uniti). The monoclonal antibody against C3d was purchased from ThermoFisher (Waltham, Massachusetts, USA). Biotinylated C3d was created with the Biotin Type A Conjugation Kit (Abcam, Cambridge, MA, USA), Streptavidin was purchased from Sigma (Saint Louis, Missouri, MO, USA) and Biotinylated anti-Human-IgM was provided by BioLegend (San Diego, California, CA, USA).

Cell cultures and media

Raji Cells (ATCC, Manassas, Virginia, USA) were cultured in RPMI (ThermoFisher, Waltham, Massachusetts, USA) supplemented with 10% Fetal Calf Serum (GE Healthcare, Buckinghamshire, UK) and Pen/Strep (Sigma, Saint Louis, Missouri, USA). B cells were isolated from human PBMCs (MAT Biotech B.V., The Netherlands) using the Human B Cell Purification Kit II, following manufacturer's instructions (Miltelnyi Biotec, Germany).

Western blot analysis of C3 binding to CIP

Affinity-purified His-tagged CIP was subjected to 12.5% SDS-PAGE (5 μg/lane), electro-blotted onto a nitrocellulose membrane, and incubated with 1% (v/v) normal or C3-depleted human serum (Sigma, Saint Louis, Missouri, USA). The membrane was probed with goat anti-C3 serum (Complement Technology, Texas, USA) and HRP-conjugated mouse anti-goat Abs (Dako, Glostrup, Denmark).

C3 and its C3b and C3d fragments (Merck, Darmstadt, Germany) were loaded onto a 12.5% SDS-PAGE gel and electro-blotted. The membrane was overlaid with CIP diluted to 1 μg/ml in PBS-Milk 3% -Tween 20 0.05% followed by primary rabbit anti-CIP antiserum and then a goat HRP-conjugated anti-rabbit Abs (1:1000).

Nunc MaxiSorp Microtiter plates (ThermoFisher, USA) were coated with 100 ng of C3, C3b or C3d and incubated overnight at 4°C in 50 mM carbonate buffer (pH 9.5). The wells were washed three times with PBS supplemented with 0.1% (v/v) Tween 20 (PBST), blocked with 2% (w/v) Bovine Serum Albumin (BSA) in PBST for 1 h at 22°C, and probed with increasing concentrations of CIP, followed by incubation with rabbit anti-CIP Ab (1:1000), and then with a HRP-conjugated goat anti-rabbit IgG (1:1000). The signal was revealed by HRP enzymatic activity. To calculate the relative affinity association constant (K_A) of CIP for C3, C3b and C3d human ligands, the data were fitted using the following equation: A = Amax [L]K_A/(1 + K_A[L]), where [L] is the molar concentration of ligand. The reported dissociation constants (K_D) were calculated as reciprocals of the K_A values. The assays were performed at least 2 times for each protein, and the K_D values obtained were reproducible in all cases.

For ELISA detection of C3d/CIP and C3d/CD21 complexes, 250 ng of C3d were surface coated onto microtiter plates and CIP or CD21 were diluted in a buffer containing increasing NaCl concentrations, and added to the wells. The complex formation was revealed through rabbit anti-CIP or anti-CD21 serum (1:1000) and goat anti-rabbit-HRP.

ELISA for CIP interaction with C3, C3b and C3d Complement factors

Nunc MaxiSorp Microtiter plates (ThermoFisher, Waltham, Massachusetts, USA) were coated with 100 ng of C3, C3b and C3d and incubated overnight at 4°C in 50 mM carbonate buffer (pH 9.5). The wells were washed three times with PBS supplemented with 0.1% (v/v) Tween 20 (PBST), blocked with 2% BSA in PBST for 1 h at 22°C, and probed with increasing concentrations of CIP, followed by incubation with rabbit anti-CIP Ab (1:1000), HRP-conjugated goat anti-rabbit IgG (1:1000) and detection of HRP enzymatic activity.

To assess the inhibitory effect of NaCl on the formation of C3d/CIP and C3d/CD21 complexes, 250 ng of C3d were surface coated onto a microtiter plate and CIP diluted in a buffer containing increasing NaCl concentrations, was added to the wells. The complex formation was revealed through a rabbit anti-CIP serum (1:1000) and detection of goat anti-rabbit-HRP enzymatic activity.

SPR analysis of CIP binding to C3d

The affinity of the interaction between CIP and C3d was evaluated using a BIAcore X100 instrument (GE LifeSciences). Human C3d was covalently immobilized on a dextran matrix CM5 sensor chip surface by using a C3d solution (30 μg/ml in 50 mM sodium acetate buffer [pH 5]) diluted 1:1 with N-hydroxysuccinimide and N-ethyl-N9-(3-dimethylaminopropyl)carbodiimide hydrochloride. The excess of active groups on the dextran matrix was blocked using 1 M ethanolamine (pH 8.5). On another flow cell, the dextran matrix was treated as described above but without any ligand to provide an uncoated reference flow cell. The running buffer used was PBS containing 0.005% (v/v) Tween 20. A two-fold linear dilution series (5 μM up to 0.0390 μM) of CIP in running buffer were passed over the ligand at the flow rate of 45 ml/min and all the sensorgrams were recorded at 22°C. Surface regeneration was achieved by injecting a solution of 25 mM NaOH. Association and dissociation kinetics parameters (K_a and K_d) and the equilibrium dissociation constant K_D were estimated with a 1:1 interaction model (Langmuir model) by nonlinear fitting, using BIAevaluation 1.0 software.

ELISA: Dose-dependent inhibition of CIP binding to CR2/CD21

Microtiter wells were coated with 100 ng of CR2/CD21 and incubated overnight at 4°C in 50 mM carbonate buffer (pH 9.5). The wells were washed three times with PBS supplemented with 0.1% (v/v) Tween 20 (PBST), blocked with 2% BSA in PBST for 1 h at 22°C, and then probed with C3d (100 ng/well) preincubated with serial dilutions of CIP in PBS, followed by incubation with anti-C3 goat polyclonal Ab (1:2000 - Complement Technology, Texas , USA) and HRP-conjugated rabbit anti-goat IgG (1:1000 – Dako, Glostrup, Denmark) and detection of HRP enzymatic activity.

Competitive ELISA

Microtiter wells were coated with 100 ng of soluble CR2/CD21 (Sino Biologicals, Wayne, PA, USA) and incubated overnight at 4°C in 50 mM carbonate buffer (pH 9.5). The wells were washed three times with PBST, blocked with 2% (w/v) BSA in PBST for 1 h at 22°C, and then probed with C3d (100 ng/well) pre-

incubated with serial dilutions of CIP in PBS, followed by incubation with anti-C3 goat polyclonal Ab (1:2000, Complement Technology Texas, USA) and then HRP-conjugated rabbit anti-goat IgG (1:1000, Dako, Glostrup, Denmark). Binding of the secondary antibody was revealed by HRP enzymatic activity.

Flow Cytometry analysis of C3d interaction with CR2/CD21 on Raji and on purified human B cells

C3d biotinylation was performed through the Biotin Type A Conjugation Kit and subsequently dialized to get rid of excess biotin. Biotinylated C3d (bC3d) was incubated with streptavidin for 30' at 37°C; the bC3d-SA complex was subsequently incubated with His-Tag purified CIP diluted to 1, 3, 6, or 9 μM and left for 30' at 37°C. Fib3 was used at 9 μM as negative control. The complex was then added to Raji Cells ($5x10^5$/sample) and the mixture was left 15 minutes at 37°C. Bound C3d was then stained with a monoclonal mouse anti-C3d (1:100) and revealed with a PE-labeled anti-mouse secondary antibody (Jackson Immunologicals, West Grove, Pennsylvania, USA). Both incubations were left for 10 minutes at 37°C and two washes in PBS-FCS 1% were performed after each incubation. Cells were fixed with Cytofix (BD Biosciences, Franklin Lakes, New Jersey, USA) for 20 minutes at 4°C and then analysed at CANTOII Flow Cytometer through BD FACSDIVA Software (BD Biosciences).

The Geometric Mean of the peaks was calculated through the FlowJo Software (BD Biosciences). The signal obtained from the samples with no CIP added, were considered as the 100% of C3d bound to CR2/CD21 on Raji. The other signals were normalized over the 100%. The graph shows the residual C3d binding, which derives from the Mean Fluorescence Intensity values of the peaks analyzed at the FlowJo Software. This experiment represents the mean of three independent experiments.

The same protocol was used to assess C3d-CR2 inhibition on B cells purified from human PBMCs.

Calcium Mobilization on Raji cells

Raji B cells ($1x10^6$/ml) were incubated with Fura Red (Molecular Probes, ThermoFisher) in PBS-EDTA 2mM at 37°C for 40 min and then washed twice in PBS-

EDTA 2 mM. The stimulus was formed by incubating for 30' at 37°C the biotinylated C3d 0.1 μM, Streptavidin 1 μM and the Biotinylated anti-human-IgM 0.2 μM. To assess the inhibition of intracellular signalling, increasing CIP concentrations (0.5, 5, 20 μM) were preincubated with biotinylated C3d prior to stimulus formation. Fib3 at 20 μM was used as negative control. Each signaling measurement was performed with 500 μl of cells ($5x10^5$/sample) at LSRII Flow Cytometer (BD Biosciences). After establishing a baseline for 60 seconds, Raji cells were stimulated by the addition of the preformed stimuli (100 μl). Changes in Fura Red fluorescence emissions were recorded using an LSRII (BD Bioscience). Data were analysed through the FlowJo software and plotted as function of time and Fura Red emission ratio.

Hydrogen Deuterium Exchange-Mass Spectrometry (HdX-MS)

Sample preparation, digestion and separation for HDX-MS analysis was performed as previously described (89). The CIP-C3d complex was formed by adding 45 pmol of CIP and C3d (molar ratio of 1:1), incubated for 30 min at room temperature and then for 10 min on ice. The deuteration was initiated by diluting the sample with deuterated PBS and performed on ice, as previously described (89). At different times of deuteration, samples were removed for quenching and dissociation of the protein/protein complex and immediately frozen in liquid nitrogen. A control experiment without C3d was performed using the same conditions.

Labeled samples were thawed rapidly to 0°C and injected into a Waters nanoACQUITY ultra-performance liquid chromatographic system with HDX technology. Samples were digested online using a Poroszyme Immobilized Pepsin Cartridge (ThermoFisher) and the generated peptides were trapped, concentrated, desalted and separated on a reverse-phase ACQUITY UPLC BEH C18, 1.7 μm, 1.0x100mm (Waters, Milford, Massachusetts, USA).

Mass spectra were acquired in resolution mode (m/z 100–2,000) on a Waters Synapt-G2 mass spectrometer equipped with a standard ESI source. The identity of each peptide was confirmed by mass spectrometry elevated energy MS^E, as previously reported (89). Data were processed using Protein Lynx Global Server 2.5 (Waters, Milford, Massachusetts, USA), and each fragmentation spectrum was inspected manually to confirm the assignment. The DynamX software (Waters,

Milford, Massachusetts, USA) was used to select the peptides considered for the analysis and to extract the centroid mass of each of them and for each charge state as a function of the labeling time. Only the peptic peptides present in at least four repeated digestions of the unlabeled proteins were considered for the analysis.

Sequence alignment and statistical analysis

Sequences of Efb-C and Sbi were downloaded from UniProtKB and sequence alignment was performed using Geneious software (Biomatters Ltd, Auckland, New Zealand) setting BLOSUM62 Matrix to calculate sequence identities and similarities. Statistical analysis was performed using GraphPad Prism Software (La Jolla, California, USA).

Results

The GBS CIP protein interacts with C3 and its C3b and C3d fragments

We first investigated whether CIP showed a binding capacity towards C3 present in human serum by Far Western blot assays. A Histidine-tagged recombinant form of CIP was highly purified from *E. coli*, analysed by SDS-PAGE (**Figure 1A**) and electro-blotted onto nitrocellulose membranes. The membranes were incubated with normal or C3-depleted human serum before immunostaining with a polyclonal anti-C3 antibody (**Figure 1B**, *lanes 1* and *2* respectively). A single protein band with an apparent molecular weight of 15 kDa in agreement with the monomeric form of CIP was revealed on the membrane incubated with normal serum, whereas no signal was detected in the absence of C3, evidencing a specific interaction between CIP and C3.

Dose-dependent saturable interactions between CIP and C3, C3b or C3d ligands were confirmed by ELISA. In these experiments, surface-coated C3, C3b and C3d were overlaid with increasing concentrations of purified CIP, followed by incubation with anti-CIP mouse serum and HRP-conjugated secondary antibody (**Figure 2**). Apparent K_D values of 94.65 ± 0.0074 nM, 98.05 ± 0.0010 nM and 92.06 ± 0.0076 nM were estimated for C3, C3b and C3d respectively.

We previously demonstrated that CIP can bind C4b with a similar affinity to the one here obtained for C3 and its subfragments (70). Therefore, we sought to investigate by competitive ELISA whether CIP binding to its C4b and C3b ligands could occur at different sites of the GBS protein.

Surface-coated C3b or C4b were overlaid with purified CIP in the absence or presence of equimolar concentrations of soluble C4b or C3b, followed by incubation with anti-CIP mouse serum and HRP-conjugated secondary antibody. As shown in **Figure 3A**, soluble C4b inhibited binding of CIP to immobilized C4b but not to C3b. Further, soluble C3b inhibited binding of CIP to immobilized C3b but not to C4b (**Fig. 3B**). The above data suggest that the double binding capacity of CIP towards C4 and C3 ligands relies on two independent interaction sites.

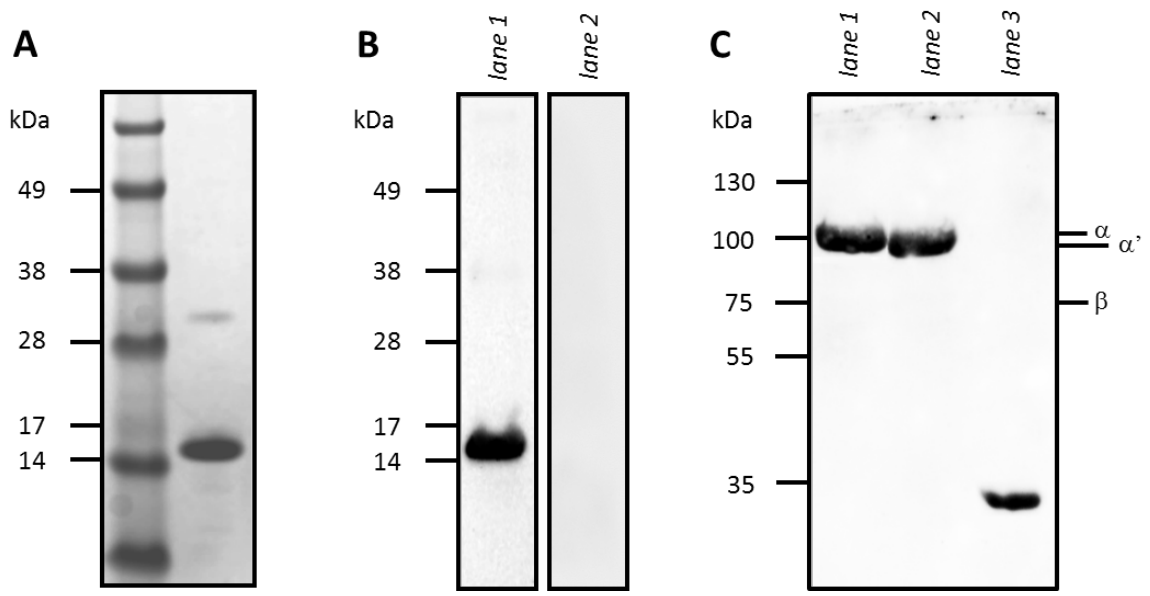

Figure 1: Far Western blot analysis revealing the interaction between CIP and C3, C3b, C3d. ***Panel A*****:** SDS-PAGE analysis of affinity-purified His-tagged CIP used in this study. ***Panel B*****:** CIP was subjected to SDS-PAGE, electro-blotted onto a nitrocellulose membrane and incubated with normal (*lane 1*) or C3-depleted human serum (*lane 2*); the membrane was probed with anti-C3 serum and HRP-conjugated

secondary antibody. ***Panel C*****:** C3, C3b and C3d (*lanes 1-3* respectively) were loaded onto a SDS-PAGE and the electro-blotted membrane was overlaid with CIP followed by a primary anti-CIP serum and then a HRP-conjugated secondary antibody to reveal the CIP binding. The expected molecular weight of C3 α, C3 β, C3b α', and C3d are indicated on the right**.**

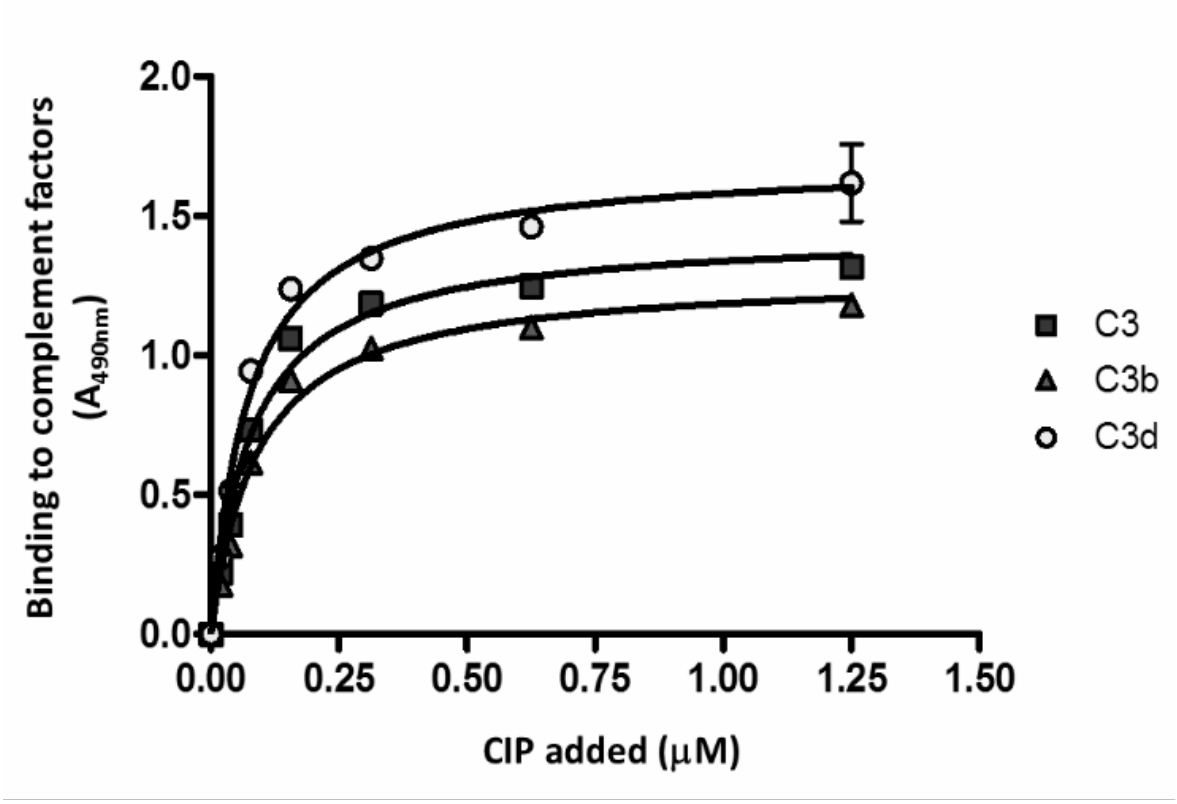

Figure 2: Dose-dependent binding of CIP to surface-coated C3, C3b and C3d. Microtiter wells were coated with 100 ng of Complement factors and incubated with increasing concentrations of CIP; bound protein was detected by anti-CIP polyclonal serum, followed by HRP-conjugated secondary antibody.

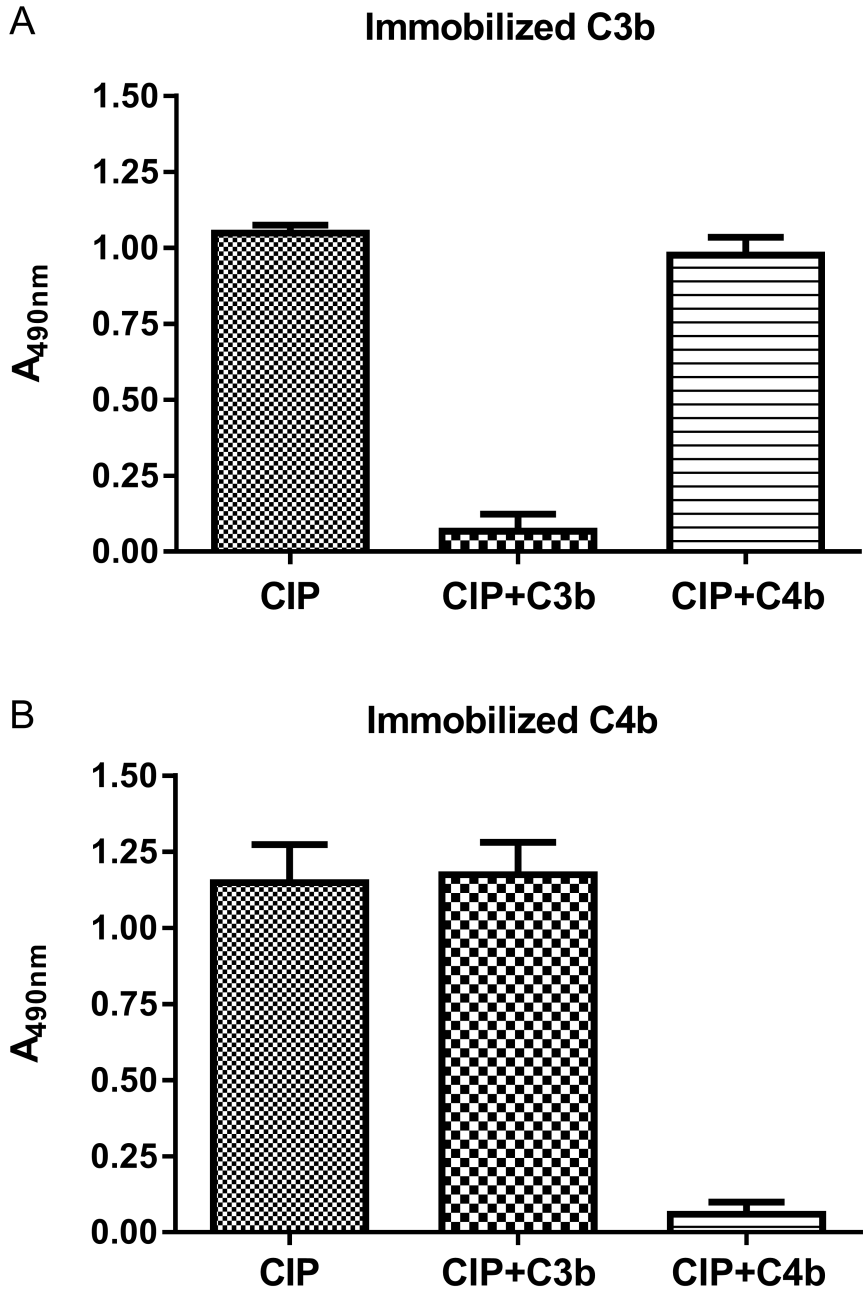

Figure 3. Competitive inhibition of CIP interaction with C3b and C4b. ***Panel A***:100 ng of C3b were coated on a 96-well plate and overlaid with 0.6 μM of CIP preincubated with equimolar amounts of C3b or C4b, followed by incubation with anti-CIP polyclonal IgG and then anti-mouse HRP-conjugated antibody. ***Panel B*** shows a similar experiment in which 100 ng of C4b were immobilized and overlaid with 0.6 μM of CIP preincubated with equimolar amounts of C4b or C3b.

Biochemical characterization of the interaction between CIP and C3d

The stoichiometry of the interaction between CIP and C3d, the smallest fragment of C3, was investigated by Size-Exclusion Chromatography. When C3d and CIP were loaded separately onto the column two peaks corresponding to 34 and 15 kDa monomers were detected. After co-incubation of equimolar concentrations of CIP with C3d, one peak of approximately 50 kDa, the estimated molecular weight of the C3d/CIP complex, was eluted from the solution suggesting a binding ratio of 1:1 between the two proteins (**Figure 4A**).

Surface Plasmon Resonance (SPR) analysis of the interaction between CIP and C3d confirmed a concentration dependent binding closely fitting to a Langmuir 1:1 kinetic model (**Figure 4B**). Purified C3d was immobilized onto the surface of a dextran chip, and CIP was added in concentrations ranging from 0.039 to 5 μM. The GBS protein bound to C3d in a dose-dependent manner, with a measured apparent K_D of 7.9 ± 0.62 x 10^{-8} M (K_{on} 1.08 +/-0.25 x 10^4 $M^{-1}s^{-1}$, K_{off} 8.5 +/-0.46 x 10^{-4} s^{-1}).

The interaction between C3d and CR2/CD21 is known to be ionic strength–dependent (90). Co-crystal structural analysis highlighted essential salt bridges and hydrogen bond interactions between positively charged residues in the surface patches of CR2/CD21 SCR 1 and 2 domains and a C3d concave acidic pocket (91).

CIP is a highly positively charged protein with 20 Lysin and 8 Arginine residues out of 153 and an isolectric point of 9.62. We investigated the effect of ionic strength on the interaction between CIP and C3d. As shown in **Figure 5,** binding of surface-coated C3d to both soluble CR2/CD21 and CIP was inhibited at increasing NaCl concentrations. These results suggested that CIP positively charged residues could interact with the acidic pocket of C3d, as previously observed for CR2/CD21, Efb and its C-terminal Efb-C fragment, as well as Sbi.

Recent structural studies revealed that the staphylococcal proteins Efb-C and the domain IV of Sbi are folded in a three-helix-bundle conformation and identified the residues directly involved in the interaction with C3d (92). The Circular Dichroism spectrum of CIP in the far-UV indicated a prevalence of alpha helix secondary structure, with a maximum positive molar ellipticity at around 7800 deg*x*cm^2*x*$dmol^{-1}$ and a maximum negative molar ellipticity at around -4500 deg*x*cm^2*x*$dmol^{-1}$ (**Figure 6**).

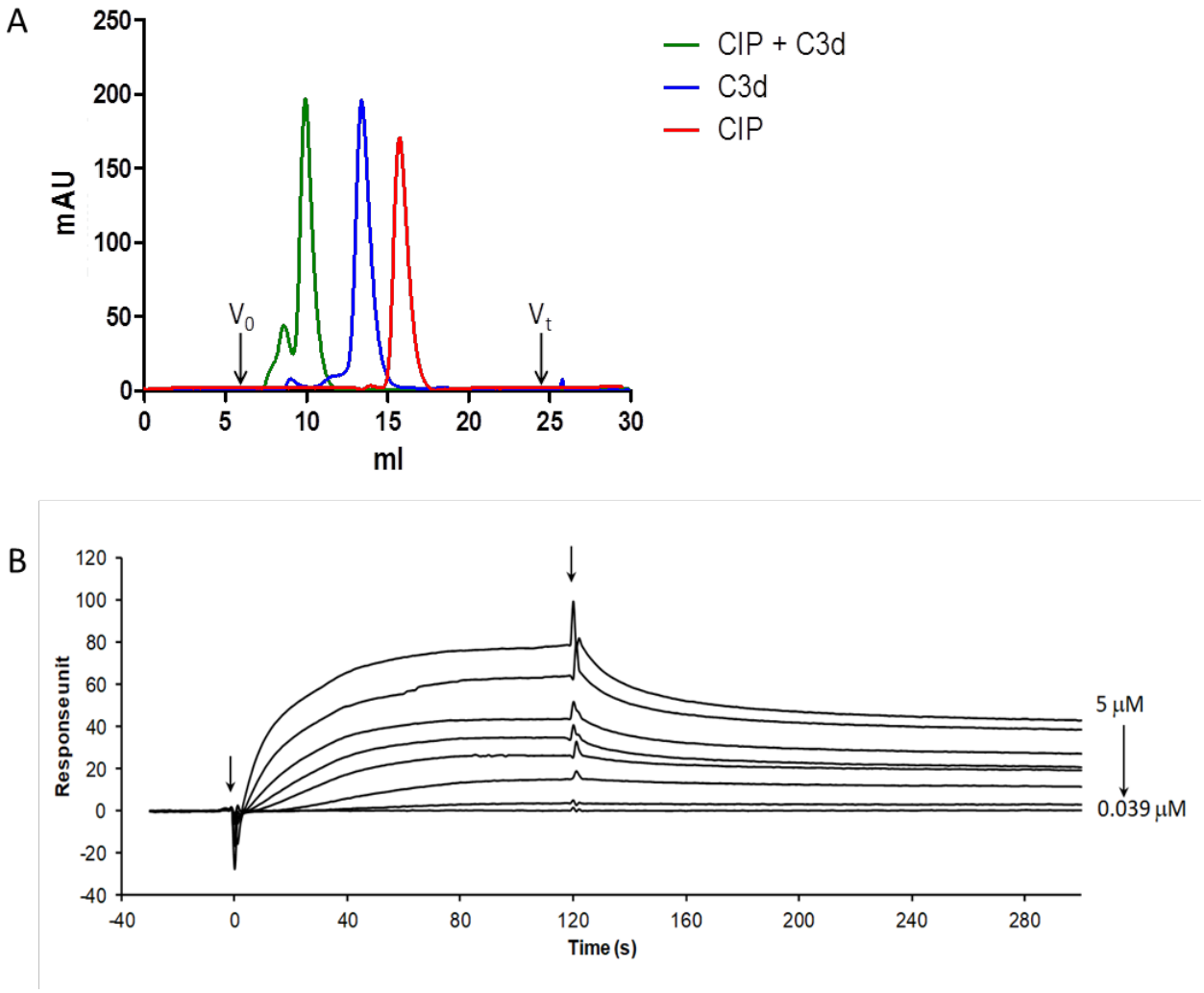

Figure 4. Analysis of the interaction between CIP and C3d by Size Exclusion Chromatography and by Surface Plasmon Resonance. ***Panel A***: CIP and C3d were loaded separately or mixed in equimolar concentration onto a A Superdex column; the molecular weight of eluted peaks was determined from a calibration curve. ***Panel B***: Two-fold linear dilution series (0.039–5 µM) of CIP were injected over the C3d surface (250 response units) of a CM5 sensor chip. The obtained sensorgrams were normalised versus the response obtained when the recombinant GBS protein was flowed over uncoated chips. Each sensorgram was evaluated using the BIA 3.0 software provided with the system. The panel shows one representative of three experiments where the start and the end of injection are indicated by arrows.

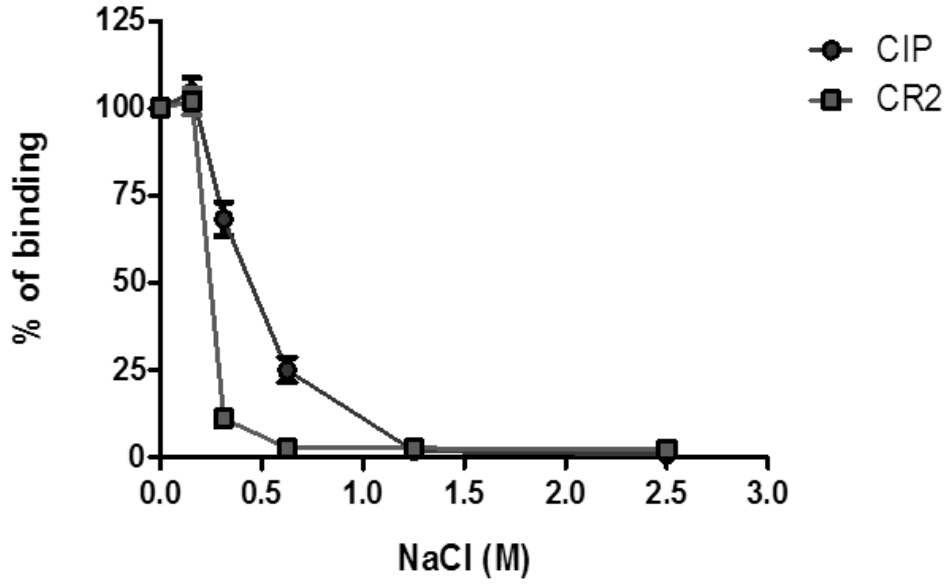

Figure 5. Ionic strenght dependent interaction of CIP with C3d assessed by ELISA. Microtiter wells were coated with 250 ng of C3d. The wells were probed with CIP or CD21 diluted in a buffer containing increasing concentrations of NaCl (0-2.5 M). Complex formation was revealed through anti-CIP or anti-CD21 polyclonal IgG, followed by HRP-conjugated secondary antibody. The 100% binding corresponds to the interaction between CIP and C3d in the absence of NaCl.

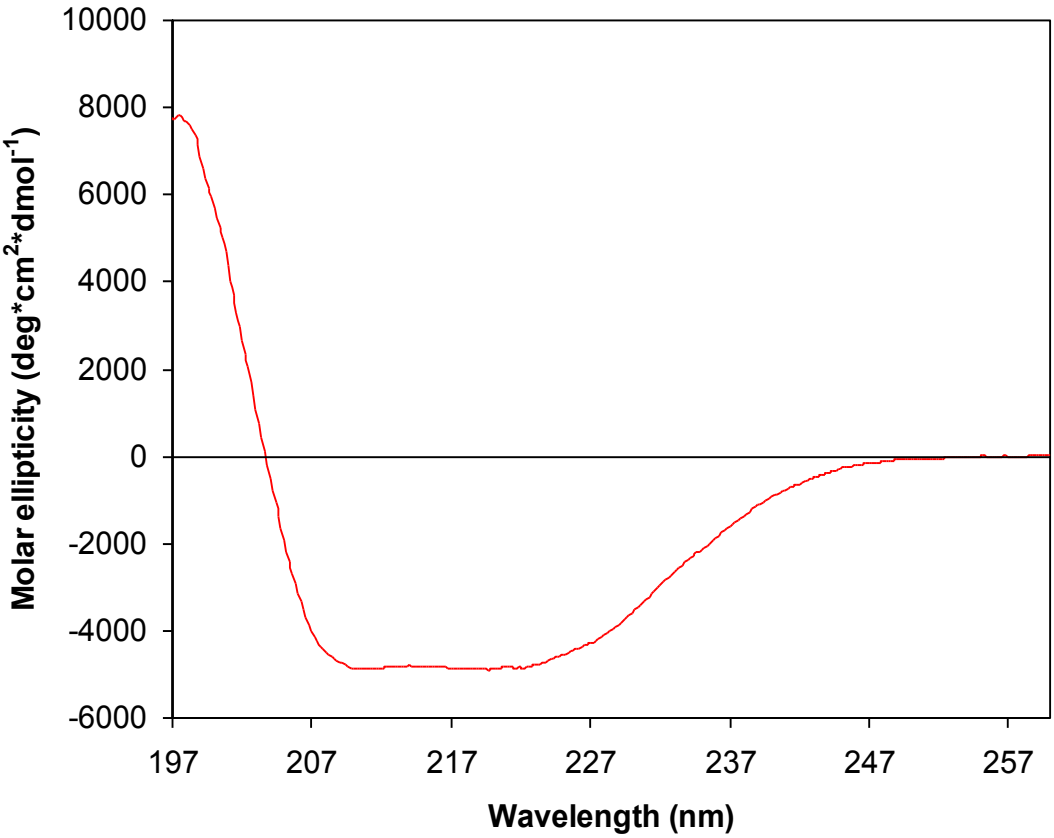

Figure 6. CIP secondary structure prediction through Circular Dichroism analysis. Secondary structure prediction revealed a high proportion of α-helices distributed throughout the protein; far-UV CD spectra of the protein showed double minima at 222 and 208 nm and a single maximum at 193 nm. The deconvolution of the CD spectra by using three independent procedures showed that the protein is mainly composed of α-helices (46 %) and the remaining part of β-sheets (36%) and random coil (18%) structures.

Mapping of the CIP peptide involved C3d binding

The C3d binding region on the CIP protein was investigated by Hydrogen Deuterium Exchange-Mass Spectrometry (HDx-MS). In this type of experiments, the interface between the binding partners can occlude solvent accessibility, therefore reducing deuterium exchange rate of the backbone amide hydrogens. After pepsin digestion of the protein of interest, the resulting peptides are compared for their masses and a consistent mass shift of 1 Da is considered the threshold for a significant exchange of one deuterium atom (94).

The CIP protein was incubated alone or in presence of C3d in a deuterated solution for different periods of time. Eleven CIP peptides, corresponding to 76% coverage of the mature protein, were generated from subsequent pepsin digestion (**Figure 7A**). Deuterium incorporation in the recovered peptides was monitored by Mass Spectrometry. As shown in **Figure 7B** a consistent difference of 1 Da in deuterium uptake was detected for fragment 96-127. No differences in exchange ratio were detected for any of the remaining peptides, including those partially overlapping with the region 96-127, and starting from amino acid 96 up to 121. The data suggested that the CIP aminoacid/s involved in the C3d interaction were located in the stretch between residues 122 to 127 that contains an Arginine residue (R123).

Of note, as shown in **Figure 8**, the R123 included in this CIP region was separated by 6 residues from an Asparagine (N130). The two residues align to the R131 and N138 present on the alpha helix 2 of the staphylococcal Efb/Efb-C and to R231 and N238 of Sbi-IV, for which a direct involvement in C3d binding was previously demonstrated by co-crystalization and mutagenesis experiments (92, 94). **Figure 8** also highlights additional aminoacid identities/similarities between the three C-terminal region of the three C3d binding proteins. This analysis highlighted 10% and 12% identities and 15% and 20% similarity between CIP and Efb-C or Sbi respectively.

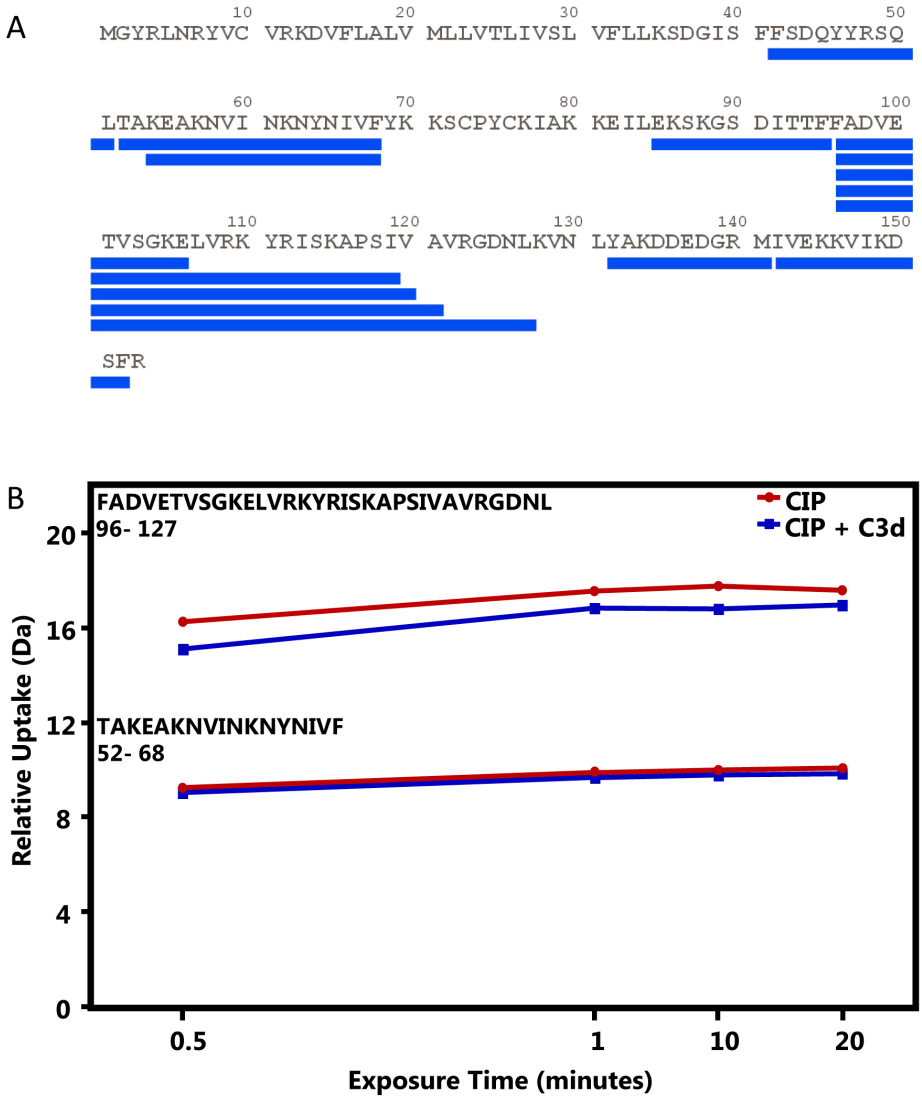

Figure 7. **HDx-MS analysis of CIP interaction with C3d.** ***Panel A***: Peptide coverage of the CIP protein: each blue line represents a peptide analyzed in the HDx experiment. ***Panel B***: Deuterium uptake over 20 minutes of the peptide 96-127 in the absence (red curve) or presence (blue curve) of C3d.

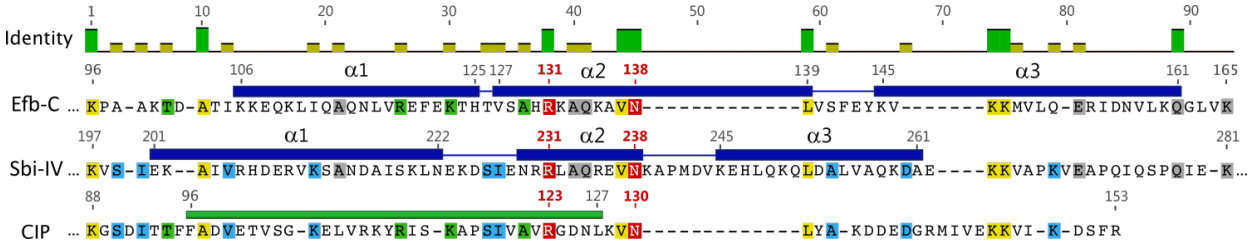

Figure 8. **Sequence alignment among the C-terminal regions of three bacterial C3d-binding proteins.** Partial amino acid sequence comparison between Efb-C, Sbi-IV (from *S. aureus* USA300 and NCTC 8325 strains respectively) and CIP (from GBS COH1) Identical aminoacids among the three proteins are indicated in yellow; identical aminoacids shared by Efb-C and CIP are in green, by Sbi and CIP in light blue and by Efb-C and Sbi in gray; red aminoacids outline the key residues for C3d binding on Efb-C and Sbi, while the red numbers indicate their position The alpha helices of Efb-C and Sbi are indicated by the blue lines; the green line on CIP covers the fragments detected in the HdX experiment. The alignment was performed by Geneious software.

CIP interaction with C3d inhibits the formation of C3d-CR2/CD21 complexes

As previously reported, CIP inhibits the formation of the C4b2a Classical and Lectin C3 convertase, but not the C3bBb Alternative pathway convertase (70). We hypothesized that the interaction between CIP and C3d could interfere with the formation of the C3d-CR2/CD21 complex and reduce the kinetics of antibody-mediated B cell intracellular signaling, as already observed for the staphylococcal Efb (95).

To investigate the capacity of CIP to interfere with the interaction between C3d and CR2/CD21, we performed a competitive ELISA experiment where C3d was pre-incubated with increasing concentrations of CIP and overlaid on microtiter plates coated with CR2/CD21, followed by polyclonal anti-C3 and secondary antibody incubation. The GBS fibrinogen binding protein Fib3 (96) was used as negative control. As shown in **Figure 9**, CIP inhibited the C3d-CD21 interaction in a dose-dependent manner, while no inhibition was observed for Fib3 tested at the highest CIP concentration.

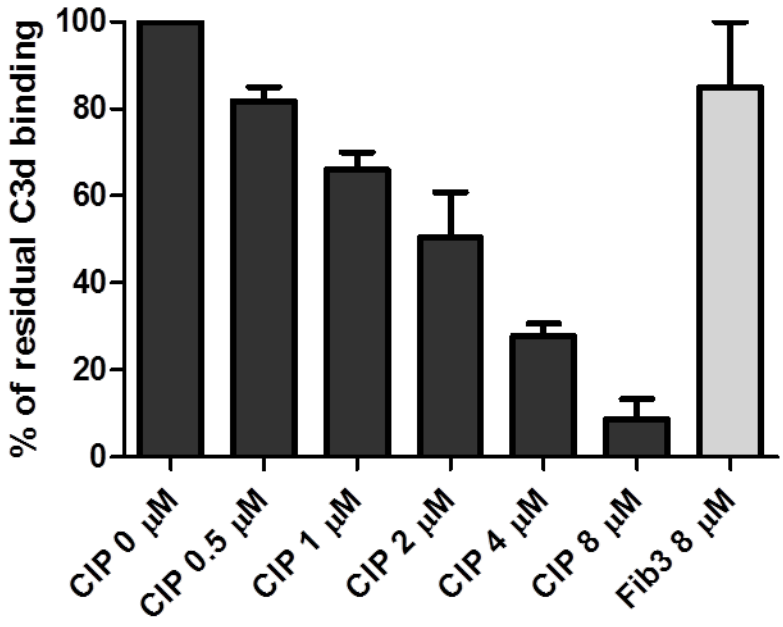

Figure 9. **ELISA competitive experiments showing that CIP inhibits the formation of the C3d-CR2/CD21 complex.** Microtiter wells were coated with 100 ng of CR2/CD21. The wells were probed with C3d preincubated with or without increasing concentrations of CIP or the highest concentration of Fib3 protein, followed by anti-C3 polyclonal Ab and HRP-conjugated secondary antibody to detect enzymatic activity. Data are expressed as percent of the absorbance values detected in the absence of competitor (CIP 0 μM).

CIP inhibits the formation of the C3d-CR2/CD21 complex on B cells

We subsequently investigated whether CIP could inhibit the interaction between soluble C3d and CR2/CD21 present on the B cell surface. It has been previously reported that C3d multimers can bind to CR2/CD21 on the Raji immortalized B cell line (95, 97).

Flow cytometry experiments confirmed binding of biotinylated C3d pre-incubated with Streptavidin (C3d-Biotin-SA) to Raji B cells (data not shown). Then, increasing concentrations of CIP were pre-incubated with the C3d-Biotin-SA complex before adding the mixture to Raji cells followed by Flow cytometry. As shown in **Figure 10A**, the presence of CIP interfered with C3d binding to the B cells in a dose-dependent manner. Conversely, no inhibition was observed using the highest concentration of the negative control Fib3.

CIP inhibition of C3d binding was also confirmed in a similar experiment using B cells enriched from human PBMCs, as evidenced by the dose dependent shift in fluorescence in the corresponding flow cytometry histograms (**Figure 10B**).

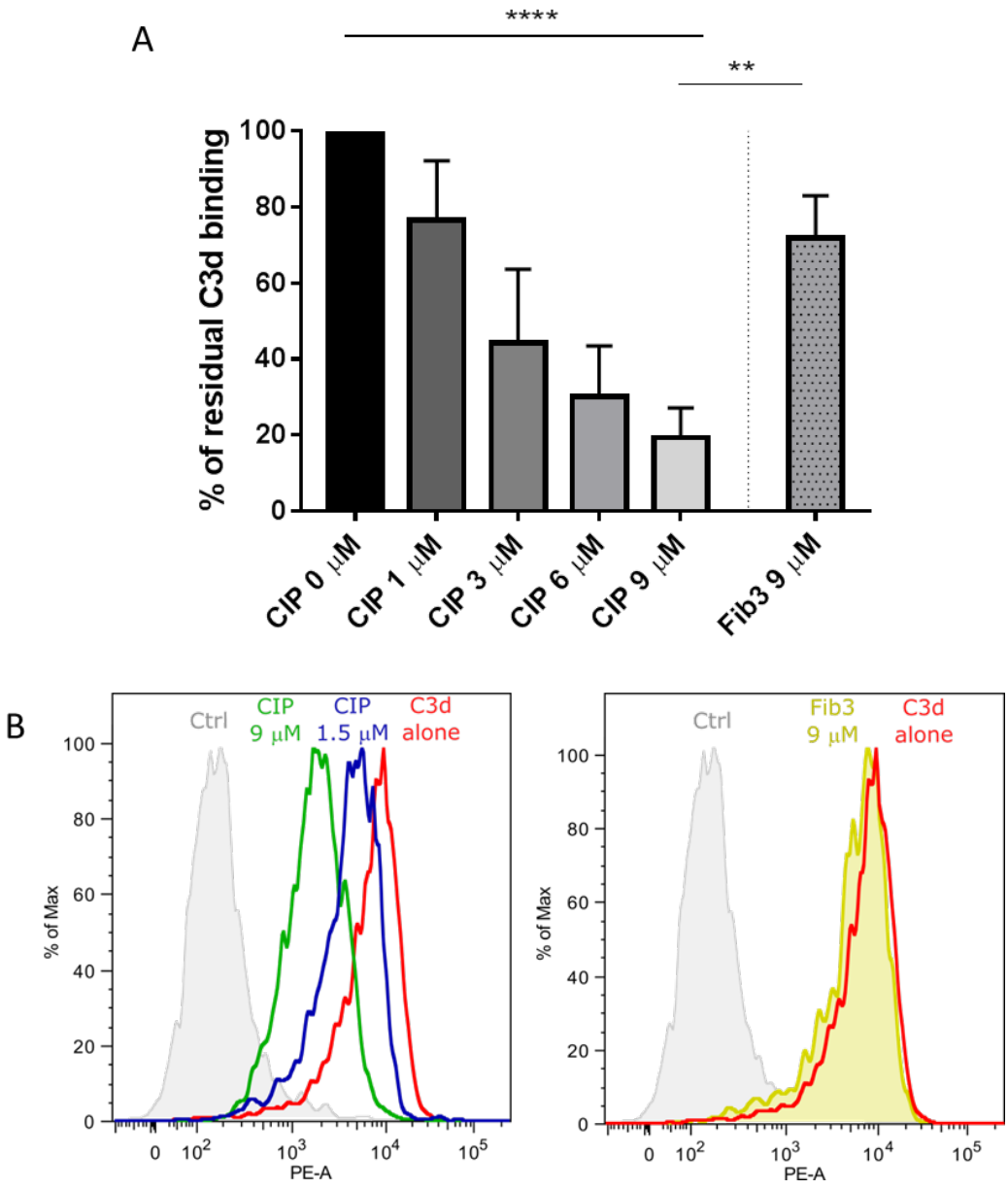

Figure 10. **Flow Cytometry analysis of C3d interaction with B cells in presence or absence of CIP**. ***Panel A:*** Biotinylated C3d was pre incubated with Streptavidin and then with 1-9 μM of CIP, 9 μM of Fib3 or buffer alone. Each mixture was added to Raji B cells and treated as described. Binding of the biotinylated C3d-SA complex to cells was revealed by Flow Cytometry using a C3d-specific monoclonal antibody and PE-labeled secondary antibody. Mean Fluorescence Intensity (MFI) values of the peaks were analyzed by the FlowJo Software. The graph shows the percent residual MFI values in presence of competitor compared to buffer alone derived from 4 independent experiments. ***Panel B:*** Flow Cytometry analysis of C3d binding to enriched B cells from human PBMCs in presence or absence of 1.5 μM or 9 μM CIP or 9 μM of Fib3; experimental conditions as in panel A.

CIP inhibits B cell intracellular signaling

The implications of CIP-C3d interaction on B cell intracellular signaling were investigated in experiments where C3d-Biotin-SA and Biotinylated anti-human IgM were combined to trigger intracellular Calcium increase, as depicted in the diagram shown in **Figure 11A**.

Before the addition of stimuli, Raji B cells were treated with Fura Red, a fluorophore that changes its emission wavelength when bound to Calcium. Changes in cellular fluorescence at different time points were recorded by Flow cytometry. The acquisition of fluorescent cells incubated with Biotin-anti-human-IgM only was used as sub-stimulatory baseline.

To assess inhibition, intracellular signaling was triggered in the absence or in presence of increasing concentrations of CIP and in the presence of the highest concentration of Fib3. As shown in **Figure 11B**, the CIP protein decreased intracellular Calcium release in a dose-dependent manner and the signal was completely abolished at 20 μM CIP concentrations (**Fig 11A**). Conversely, fluorescence was not affected by the presence of Fib3 (**Fig. 11C**).

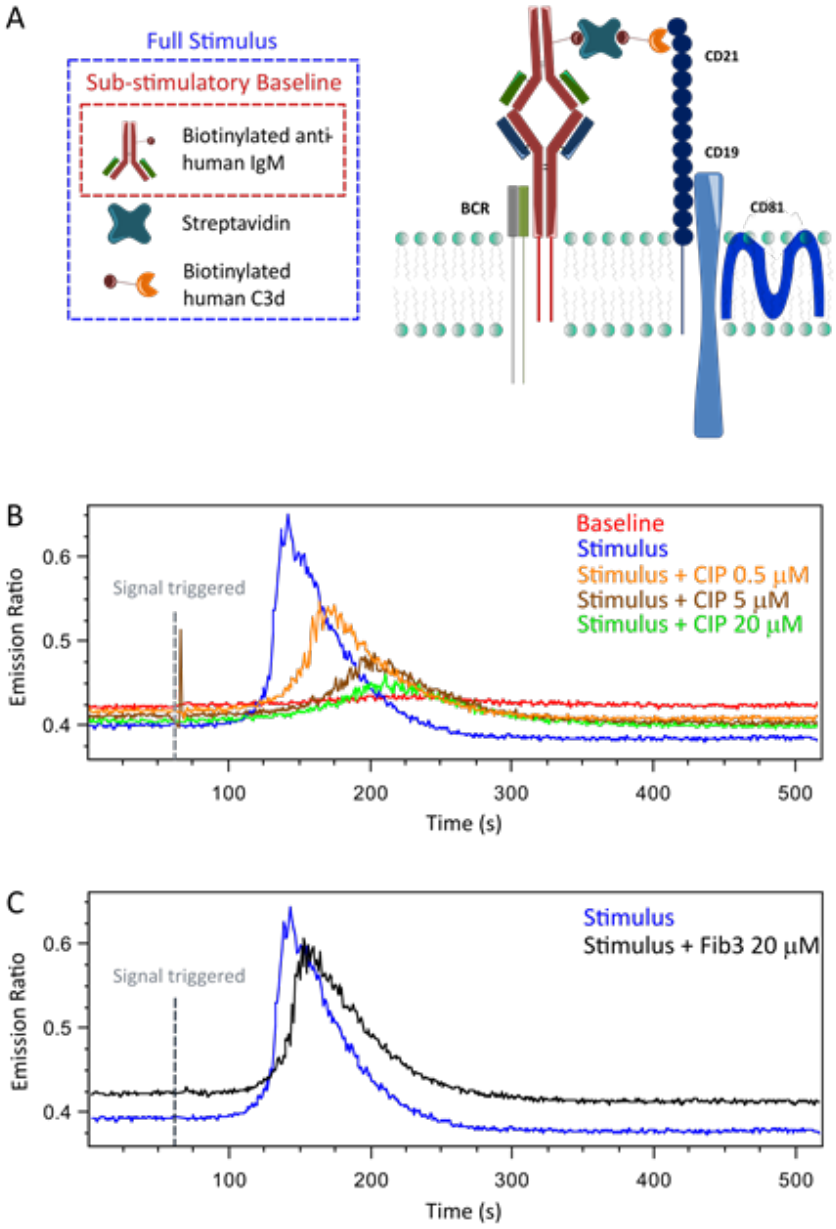

Figure 11. **Flow Cytometry analysis of B cell intracellular calcium mobilization in presence or absence of CIP**. ***Panel A***: Diagram representation of the stimulus that triggers intracellular Calcium increase (*blue box*); biotinylated anti-human-IgM antibody (*red box*) is used to measure the substimulatory baseline prior to each experiment. The stimulus is designed to cross-link the BCR to CR2/CD21. ***Panel B-C***: Raji cells were preincubated with Fura Red followed by the stimulus (see *Panel A*). 0.5-20 µM of CIP (*Panel B*) or 20 µM of Fib3 (*Panel C*) were preincubated with C3d-Biotin-SA to assess their inhibition effect; changes in cellular fluorescence were monitored during 540 seconds and analyzed by Flow Cytometry. One representative of three performed experiments is shown.

Discussion

The GBS CIP protein was first identified as a virulence factor secreted by Group B *Streptococcus,* showing a capacity to bind the Complement proteins C4 and C4b and to block the deposition of C3b on the bacterial surface via the Lectin and Classical Pathways (70). The present study identified as new ligands for the CIP protein the C3 central Complement component and its thioester containing effector fragments C3b and C3d, and revealed new Complement evasion mechanisms for this GBS protein.

By sequence homology comparisons with Staphylococcal secreted virulence factors, CIP appeared as a protein chimera showing partial similarity to the C4 binding protein Eap from *S. aureus* in its N-terminal region, and to the C3b/C3d binding proteins Efb and Sbi in its C-terminal region (70) (**Fig. 8**). The CIP C4 and C3 binding sites appeared to be independent based on competition experiments.

The interaction between Efb or Sbi with C3 induces conformational changes in C3b that abolish the C3bBb convertase function blocking downstream activation events (94, 98). Moreover, Sbi was shown to form a tripartite complex with C3b and Factor H that contributes to its inhibitory effect towards the Complement Alternative Pathway (98). A similar role of the GBS CIP protein in preventing the formation/activation of the C3bBb convertase could have been expected based on its C3b binding capacity, but was excluded in our previous investigations (70). The molecular basis for the different modulatory capacity towards the Alternative Pathway between CIP and the Efb and Sbi staphylococcal proteins will deserve further investigations. Here we focused our attention on the interaction with C3d, the last degradation fragment of C3, possibly containing the complete CIP binding site. A saturable univalent interaction between CIP and C3d was demonstrated by coupling ligand and receptor in solid phase supports and by size exclusion chromatography. SPR experiments confirmed an intrinsic affinity between CIP and C3d in the order of 0.1 μM, slightly superior to the K_D measured for the staphylococcal Sbi (92) and lower than the one reported for Efb (99).

Besides playing a key role in innate immune surveillance, the Complement system is implicated in the engagement of adaptive responses (31), and several *in vivo* studies support a role for Complement Receptors (CRs) in the acquisition of target antigens

(100). In particular, the C3d/C3dg physiological degradation products of C3 enhance B cell signaling by simultaneously binding the antigen-B cell receptor complex and the co-receptor complex formed by CR2/CD21, CD19 and CD81. This receptor co-ligation has a profound molecular adjuvant effect that lowers the threshold of antigen required for B cell activation by more than one thousand fold (101). The C3d-dependent crosslink also directs B cells towards their T cell boundary, where they further differentiate through clonal expansion, somatic hypermutation, affinity maturation and immunoglobulin class switch recombination (102). Furthermore, the retention of C3d-opsonized antigens through CR2/CD21 on Follicular Dendritic Cells (FDCs) in the lymph node is essential for the generation of high affinity antibodies and memory B cells (103).

Here we show that binding of CIP to C3d prevents the interaction of this protein with the CR2/CD21 B cell receptor both *in vitro* and *ex vivo* and that this interference effect results in decreased intracellular signaling, as measured by lower Calcium release. Therefore, the data point towards a new role for the CIP protein in counteracting adaptive immunity by modulating the activity of the C3d Complement effector, and may represent a new example of the co-evolution between the GBS microorganism and its human host.

The CIP concentrations used in our experiments were in the range of the 0.1 to 10 μM, and equimolar amounts of CIP and C3d were used for analyzing the interaction between the two proteins by Size Exclusion Chromatography. An approximate quantification of CIP by dot blot in the supernatant of a GBS neonatal strain grown in vitro to stationary phase in parallel to recombinant CIP, indicated concentrations of about 0.3-0.5 μM (data not shown). This value could represent an underestimation, as CIP is able to bind to the bacterial surface (70) not allowing a reliable quantification of the secreted form. We previously observed that CIP was differentially expressed in different growth media and its expression might be regulated also during infection, making difficult to draw definite conclusions on the physiological relevance of concentrations measured from an in vitro culture.

Deep genomics studies have indicated that lateral transfer and recombination are strongly implicated in the evolution of GBS. In particular, phage insertions generate inter-strain diversity and provide the pathogen with a number of virulence factors that facilitate its survival in the host (104). Of note, the gene coding for CIP is located

in a hot spot phage insertion region of about 20 kbp (70) also containing the *bspC* locus implicated in biofilm formation (105, 106). Interestingly, Group A *Streptococcus* isolates belonging to serotypes associated with maternal–fetal urogenital infections contain a similar phage insertion that also encodes a secreted protein 46% identical to CIP (70), suggesting inter-species lateral transfer.

Co-crystal structural analysis highlighted essential salt bridges and hydrogen bond interactions between a concave acidic pocket from C3d and positively charged residues in the surface patches of CR2/CD21 SCR 1 and 2 domains (91, 107). A similar type of interaction between C3d and cation residues present on the alpha helix 2 of the staphylococcal Efb and Sbi was demonstrated by co-crystalization and mutagenesis experiments (92, 94). Secondary structure predictions confirmed by CD analysis indicate that the C-terminal region of CIP also presents an alpha-helical structure. Interestingly, HDx-MS led to the identification of a CIP peptide interacting with C3d that contains an Arginine (R123) separated by six residues from an Asparagine (N130). The two residues perfectly matched the R131 and N138 present on the alpha helix 2 of the staphylococcal Efb/Efb-C and the R231 and N238 of Sbi. Unfortunately, the CIP peptide containing the N130 was not recovered by MS possibly due to the proximity of pepsin-sensitive residues. Two recombinant variants of CIP, the first containing a substitution of R123 with an Alanine (A) residue and the second a double mutant R123 to A plus N130 to A, were expressed in *E. coli* to experimentally confirm their role in C3d binding. Despite high expression levels, the two mutant proteins were poorly soluble in acqueous buffer and quickly precipitated. Future co-crystalization and mutagenesis experiments will be used to confirm the amino acid residues directly involeved in the interactions between CIP and C3d.

The gastrointestinal and urogenital GBS colonization sites are rich in Mucosal Associated Lymphoid Tissues (MALT) containing high numbers of B cells, where CIP could exert its immunomodulation effect (108).

Besides having an important role as ligand of CR2/CD21, C3d also binds the Complement Receptor 3 (CD11b/CD18) on Dendritic Cells and Macrophages via its Integrin I domain (109). A possible additional effect of CIP not investigated here could be the prevention of the interaction between C3d and CR3 on these antigen presenting cells, limiting the transport of GBS antigens to the Lymph nodes and their presentation to resting B cells.

To investigate the effect of CIP on GBS virulence in vivo, we tried to obtain an isogenic cip mutant in a GBS neonatal strain but were unsuccessful. Previous experiments had shown that GBS survival in human blood was enhanced after preincubation of the bacteria with recombinant CIP (70). It is tempting to speculate that the observed anti-phagocytic effect of CIP could rely on its capacity to bind both C4b and C3. Indeed, besides modulating the formation of C3b via the classical/lectin pathways as previously reported, the CIP protein could also prevent binding of complement labeled bacteria to CR3 and CR1/CD35 receptors in neutrophils.

Finally, it has been postulated that differentiation of autoreactive B cells could also involve co-receptor engagement of C3d along with B cell receptor binding to self-antigens (31). If this hypothesis will be confirmed, proteins capable of interfering with the C3d-CR2/CD21 interaction like CIP or their derived peptides could represent valuable therapeutic tools for the modulation of aberrant B cell responses to combat antibody-mediated autoimmune disorders.

References

1. Lancefield, R. (1933) Serological Differentiation of Human and Other Groups of Hemolytic *Streptococci*. *J Exp Med* **57**, 571-595
2. Scanziani, R., Dozio, B., Baragetti, I., Grillo, P., Colombo, L., De Liso, S., and Surian, M. (1999) Vaginal colonization with group B Streptococcus (*Streptococcus agalactiae*) and peritonitis in a woman on CAPD. *Nephrol Dial Transplant* **14**, 2222-2224
3. Teatero, S., Ferrieri, P., Martin, I., Demczuk, W., McGeer, A., Fittipaldi, N., and Burnham, C.-A. D. (2017) Serotype Distribution, Population Structure, and Antimicrobial Resistance of Group B Streptococcus Strains Recovered from Colonized Pregnant Women. *Journal of Clinical Microbiology* **55**, 412-422
4. Gibbs, R. S., Schrag, S., and Schuchat, A. (2004) Perinatal Infection Due to Group B Streptococcus. *Obstetrics and Gynecology* **104**, 1062-1076
5. G.P., K. (1997) *Streptococcus agalctiae* mastitis: a review. *Can Vet J* **38**, 429-437
6. Lindahl, G., Stalhammar-Carlemalm, M., and Areschoug, T. (2005) Surface proteins of Streptococcus agalactiae and related proteins in other bacterial pathogens. *Clin Microbiol Rev* **18**, 102-127
7. Da Cunha, V., Davies, M. R., Douarre, P. E., Rosinski-Chupin, I., Margarit, I., Spinali, S., Perkins, T., Lechat, P., Dmytruk, N., Sauvage, E., Ma, L., Romi, B., Tichit, M., Lopez-Sanchez, M. J., Descorps-Declere, S., Souche, E., Buchrieser, C., Trieu-Cuot, P., Moszer, I., Clermont, D., Maione, D., Bouchier, C., McMillan, D. J., Parkhill, J., Telford, J. L., Dougan, G., Walker, M. J., Consortium, D., Holden, M. T. G., Poyart, C., and Glaser, P. (2014) Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. *Nat Commun* **5**, 4544
8. Chiarot, E., Spagnuolo, A., Maccari, S., Naimo, E., Acquaviva, A., Cecchi, R., Galletti, B., Fabbrini, M., Mori, E., Ruggiero, P., Grandi, G., Fontana, M. R., Bensi, G., and Margarit, I. (2018) Protective effect of Group B Streptococcus type-III polysaccharide conjugates against maternal colonization, ascending infection and neonatal transmission in rodent models. *Sci Rep* **8**, 2593
9. Schrag S.J., Z. S., Farley M.M., Reingold A.L., Harrison L.H., Lefkowitz L.B., Hadler J.L., Danila R., Cieslak P.R., Schuchat A. (2000) Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. *The New England Journal of Medicine* **342**, 15-20
10. Rajagopal, L. (2009) Understanding the regulation of Group B *Streptococcus* virulence factors. *Future Microbiology* **4**, 201–221
11. Le Doare, K., Faal, A., Jaiteh, M., Sarfo, F., Taylor, S., Warburton, F., Humphries, H., Birt, J., Jarju, S., Darboe, S., Clarke, E., Antonio, M., Foster-Nyarko, E., Heath, P. T., Gorringe, A., and Kampmann, B. (2017) Association between functional antibody against Group B Streptococcus and maternal and infant colonization in a Gambian cohort. *Vaccine* **35**, 2970-2978
12. Le Doare, K., and Kampmann, B. (2014) Breast milk and Group B streptococcal infection: vector of transmission or vehicle for protection? *Vaccine* **32**, 3128-3132
13. Olver, W. J., Bond, D. W., Boswell, T. C., and Watkin, S. L. (2000) Neonatal group B streptococcal disease associated with infected breast milk. *Archives of disease in childhood. Fetal and neonatal edition* **83**, F48-49
14. Barcaite, E., Bartusevicius, A., Tameliene, R., Kliucinskas, M., Maleckiene, L., and Nadisauskiene, R. (2008) Prevalence of maternal group B streptococcal colonisation in European countries. *Acta Obstet Gynecol Scand* **87**, 260-271

15. Hall, J., Adams, N. H., Bartlett, L., Seale, A. C., Lamagni, T., Bianchi-Jassir, F., Lawn, J. E., Baker, C. J., Cutland, C., Heath, P. T., Ip, M., Le Doare, K., Madhi, S. A., Rubens, C. E., Saha, S. K., Schrag, S., Sobanjo-Ter Meulen, A., Vekemans, J., and Gravett, M. G. (2017) Maternal Disease With Group B Streptococcus and Serotype Distribution Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **65**, S112-S124
16. Madrid, L., Seale, A. C., Kohli-Lynch, M., Edmond, K. M., Lawn, J. E., Heath, P. T., Madhi, S. A., Baker, C. J., Bartlett, L., Cutland, C., Gravett, M. G., Ip, M., Le Doare, K., Rubens, C. E., Saha, S. K., Sobanjo-Ter Meulen, A., Vekemans, J., Schrag, S., and Infant, G. B. S. D. I. G. (2017) Infant Group B Streptococcal Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. *Clin Infect Dis* **65**, S160-S172
17. Campisi, E., Rosini, R., Ji, W., Guidotti, S., Rojas-Lopez, M., Geng, G., Deng, Q., Zhong, H., Wang, W., Liu, H., Nan, C., Margarit, I., and Rinaudo, C. D. (2016) Genomic Analysis Reveals Multi-Drug Resistance Clusters in Group B Streptococcus CC17 Hypervirulent Isolates Causing Neonatal Invasive Disease in Southern Mainland China. *Front Microbiol* **7**, 1265
18. Brochet, M., Couve, E., Zouine, M., Vallaeys, T., Rusniok, C., Lamy, M. C., Buchrieser, C., Tieu-Cuot, P., Kunst, F., Poyart, C., and Glaser, P. (2006) Genomic diversity and evolution within the species *Streptococcus agalactiae*. *Microbes Infect* **8**, 1227-1243
19. Edmond, K. M., Kortsalioudaki, C., Scott, S., Schrag, S. J., Zaidi, A. K. M., Cousens, S., and Heath, P. T. (2012) Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. *The Lancet* **379**, 547-556
20. Verani, J. R., McGee, L., and Schrag, S. J. (2010) Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control* **59**, 1-36
21. Verani J.R., M. L., Schrag S.J. (2010) Prevention of Perinatal Group B streptococcal disease. *MMWR* **59 (RR-10)**, 1-32
22. Christina R. Phares, Ruth Lynfield, Monica M. Farley, Janet Mohle-Boetani, Lee H. Harrison, Susan Petit, Allen S. Craig, William Schaffner, Shelley M. Zansky, Ken Gershman, Karen R. Stefonek, Bernadette A. Albanese, Elizabeth R. Zell, Anne Schuchat, and Schrag, S. J. (2008) Epidemiology of Invasive Group B *Streptococcus* in the United States, 1999-2005. *JAMA* **299**, 2056-2065
23. Ballard, M. S., Schonheyder, H. C., Knudsen, J. D., Lyytikainen, O., Dryden, M., Kennedy, K. J., Valiquette, L., Pinholt, M., Jacobsson, G., Laupland, K. B., and International Bacteremia Surveillance, C. (2016) The changing epidemiology of Group B *Streptococcus* bloodstream infection: a multi-national population-based assessment. *Infect Dis (Lond)* **48**, 386-391
24. Baker, C. J., and Edwards, M. S. (2003) Group B *Streptococcus* conjugate vaccines. *Arch Dis Child* **88**, 375–378
25. Baker, C. J., Rench, M., Edwards, M. S., Carpenter, R. J., Hays, B. M., and Kasper, D. (1988) Immunization of Pregnant Women with a Polysaccharide Vaccine of Group B *Streptococcus*. *N Engl J Med* **319**, 1180-1185
26. Johri, A. K., Lawrence, C. P., Glaser, P., Dua, M., Sharma, P. K., Grandi, G., and Rappuoli, R. (2006) Group B *Streptococcus*: global incidence and vaccine development. *Nat Rev Microbiol* **4**, 932-942
27. Merle, N. S., Church, S. E., Fremeaux-Bacchi, V., and Roumenina, L. T. (2015) Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol* **6**, 262
28. Reddy, Y. N., Siedlecki, A. M., and Francis, J. M. (2017) Breaking down the complement system: a review and update on novel therapies. *Curr Opin Nephrol Hypertens* **26**, 123-128
29. Thurman, J. M., and Holers, V. M. (2006) The Central Role of the Alternative Complement Pathway in Human Disease. *The Journal of Immunology* **176**, 1305-1310
30. Foster, T. J. (2005) Immune evasion by staphylococci. *Nat Rev Microbiol* **3**, 948-958

31. Carroll, M. C., and Isenman, D. E. (2012) Regulation of humoral immunity by complement. *Immunity* **37**, 199-207
32. Henson S.E., S. D., Boackle S.A., Holers V.M., Karp D.R. (2001) Generation of recombinant human C3dg tetramers for the analysis of CD21 binding and function. *J Immunol Methods* **258**, 97-109
33. Fabbrini, M., Sammicheli, C., Margarit, I., Maione, D., Grandi, G., Giuliani, M. M., Mori, E., and Nuti, S. (2012) A new flow-cytometry-based opsonophagocytosis assay for the rapid measurement of functional antibody levels against Group B Streptococcus. *J Immunol Methods* **378**, 11-19
34. Khera, R., and Das, N. (2009) Complement Receptor 1: disease associations and therapeutic implications. *Mol Immunol* **46**, 761-772
35. Servis, C., and Lambris, J. D. (1989) C3 synthetic peptide support growth of human CR2-positive lymphoblastoid B cells. *J Immunol* **142**, 2207-2212
36. Cherukuri, A., Cheng, P. C., and Pierce, S. K. (2001) The Role of the CD19/CD21 Complex in B Cell Processing and Presentation of Complement-Tagged Antigens. *The Journal of Immunology* **167**, 163-172
37. Holers, V. M. (2014) Complement and Its Receptors: New Insights into Human Disease. *Annual Review of Immunology* **32**, 433-459
38. Merle, N. S., Noe, R., Halbwachs-Mecarelli, L., Fremeaux-Bacchi, V., and Roumenina, L. T. (2015) Complement System Part II: Role in Immunity. *Front Immunol* **6**, 257
39. Deng L., K. D. L., Krick T.P., Wessels M.R. (2000) Characterization of the linkage between type III Capsular Polysaccharide and bacterial cell wall of Group B *Streptococcus*. *The Journal of Biological Chemistry* **275**, 7497–7504
40. Toniolo, C., Balducci, E., Romano, M. R., Proietti, D., Ferlenghi, I., Grandi, G., Berti, F., Ros, I. M., and Janulczyk, R. (2015) Streptococcus agalactiae capsule polymer length and attachment is determined by the proteins CpsABCD. *J Biol Chem* **290**, 9521-9532
41. Cieslewicz, M. J., Chaffin, D., Glusman, G., Kasper, D., Madan, A., Rodrigues, S., Fahey, J., Wessels, M. R., and Rubens, C. E. (2005) Structural and Genetic Diversity of Group B Streptococcus Capsular Polysaccharides. *Infection and Immunity* **73**, 3096-3103
42. Yamamoto, S., Miyake, K., Koike, Y., Watanabe, M., Machida, Y., Ohta, M., and Iijima, S. (1999) Molecular characterization of type-specific capsular polysaccharide biosynthesis genes of Streptococcus agalactiae type Ia. *J Bacteriol* **181**, 5176-5184
43. Carlin, A. F., Lewis, A. L., Varki, A., and Nizet, V. (2007) Group B streptococcal capsular sialic acids interact with siglecs (immunoglobulin-like lectins) on human leukocytes. *J Bacteriol* **189**, 1231-1237
44. Weiman, S., Uchiyama, S., Lin, F. Y., Chaffin, D., Varki, A., Nizet, V., and Lewis, A. L. (2010) O-Acetylation of sialic acid on Group B Streptococcus inhibits neutrophil suppression and virulence. *Biochem J* **428**, 163-168
45. Xia, F. D., Mallet, A., Caliot, E., Gao, C., Trieu-Cuot, P., and Dramsi, S. (2014) Capsular polysaccharide of Group B Streptococcus mediates biofilm formation in the presence of human plasma. *Microbes and infection / Institut Pasteur*
46. Hulse, M. L., Smith, S., Chi, E. Y., Pham, A., and Rubens, C. E. (1993) Effect of type III group B streptococcal capsular polysaccharide on invasion of respiratory epithelial cells. *Infect. Immun.* **61**, 4835-4841
47. Tamura, G. S., Kuypers, J. M., Smith, S., Raff, H., and Rubens, C. E. (1994) Adherence of group B streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components. *Infect. Immun.* **62**, 2450-2458
48. Mereghetti, L., Sitkiewicz, I., Green, N. M., and Musser, J. M. (2008) Extensive adaptive changes occur in the transcriptome of Streptococcus agalactiae (group B streptococcus) in response to incubation with human blood. *PloS one* **3**, e3143
49. Ross, R. A., Madoff, L. C., and Paoletti, L. C. (1999) Regulation of cell component production by growth rate in the group B Streptococcus. *J. Bacteriol.* **181**, 5389-5394

50. Malin, G., and Paoletti, L. C. (2001) Use of a dynamic in vitro attachment and invasion system (DIVAS) to determine influence of growth rate on invasion of respiratory epithelial cells by group B Streptococcus. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 13335-13340
51. Yother, J. (2011) Capsules of Streptococcus pneumoniae and other bacteria: paradigms for polysaccharide biosynthesis and regulation. *Annu. Rev. Microbiol.* **65**, 563-581
52. Kugelberg, E., Gollan, B., and Tang, C. M. (2008) Mechanisms in Neisseria meningitidis for resistance against complement-mediated killing. *Vaccine* **26 Suppl 8**, I34-39
53. Doran, K. S., and Nizet, V. (2004) Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. *Mol Microbiol* **54**, 23-31
54. Fearon, D. T. (1978) Regulation by Membrane Sialic Acid of β1H-dependent Decay-Dissociation of Amplification C3 Convertase of the Alternative Complement Pathway. *Proc. Natl. Acad. Sci. USA* **75**, 1971-1975
55. Crocker, P. R., Paulson, J. C., and Varki, A. (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* **7**, 255-266
56. Carlin, A. F., Chang, Y. C., Areschoug, T., Lindahl, G., Hurtado-Ziola, N., King, C. C., Varki, A., and Nizet, V. (2009) Group B Streptococcus suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. *J Exp Med* **206**, 1691-1699
57. Chang, Y. C., Olson, J., Louie, A., Crocker, P. R., Varki, A., and Nizet, V. (2014) Role of macrophage sialoadhesin in host defense against the sialylated pathogen group B Streptococcus. *J Mol Med (Berl)* **92**, 951-959
58. Chang, Y. C., and Nizet, V. (2014) The interplay between Siglecs and sialylated pathogens. *Glycobiology* **24**, 818-825
59. Jarva, H., Hellwage, J., Jokiranta, T. S., Lehtinen, M. J., Zipfel, P. F., and Meri, S. (2004) The Group B Streptococcal and Pneumococcal Hic Proteins Are Structurally Related Immune Evasion Molecules That Bind the Complement Inhibitor Factor H in an Analogous Fashion. *The Journal of Immunology* **172**, 3111-3118
60. Serruto, D., Rappuoli, R., Scarselli, M., Gros, P., and van Strijp, J. A. (2010) Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. *Nat Rev Microbiol* **8**, 393-399
61. Hovingh, E. S., van den Broek, B., and Jongerius, I. (2016) Hijacking Complement Regulatory Proteins for Bacterial Immune Evasion. *Front Microbiol* **7**, 1-20
62. Campbell, J. R., Baker, C. J., and Edwards, M. S. (1992) Influence of Serotype of Group B *Streptococci* on C3 Degradation. *Infection and Immunity* **60**, 4558-4562
63. Maruvada, R., Prasadarao, N. V., and Rubens, C. E. (2009) Acquisition of factor H by a novel surface protein on group B *Streptococcus* promotes complement degradation. *FASEB J* **23**, 3967-3977
64. Wilkinson, H. W., and Eagon, R. G. (1971) Type-specific antigens of group B type Ic streptococci. *Infect Immun* **4**, 596-604
65. Areschoug, T., Stalhammar-Carlemalm, M., Karlsson, I., and Lindahl, G. (2002) Streptococcal β protein has separate binding sites for human factor H and IgA-Fc. *J. Biol. Chem.* **277**, 12642-12648
66. Nordstrom, T., Movert, E., Olin, A. I., Ali, S. R., Nizet, V., Varki, A., and Areschoug, T. (2011) Human Siglec-5 inhibitory receptor and immunoglobulin A (IgA) have separate binding sites in streptococcal beta protein. *J Biol Chem* **286**, 33981-33991
67. Brown, C. K., Gu, Z. Y., Matsuka, Y. V., Purushothaman, S. S., Winter, L. A., Cleary, P. P., Olmsted, S. B., Ohlendorf, D. H., and Earhart, C. A. (2005) Structure of the streptococcal cell wall C5a peptidase. *Proc Natl Acad Sci U S A* **102**, 18391-18396
68. Shet, A., Kaplan, E., Johnson, D., and Cleary, P. P. (2004) Human immunogenicity studies on group A streptococcal C5a peptidase (SCPA) as a potential vaccine against group A streptococcal infections. *Indian J Med Res* **119 Suppl**, 95-98

69. Santi, I., Scarselli, M., Mariani, M., Pezzicoli, A., Masignani, V., Taddei, A., Grandi, G., Telford, J. L., and Soriani, M. (2007) BibA: a novel immunogenic bacterial adhesin contributing to group B Streptococcus survival in human blood. *Mol Microbiol* **63**, 754-767
70. Pietrocola, G., Rindi, S., Rosini, R., Buccato, S., Speziale, P., and Margarit, I. (2016) The Group B Streptococcus-Secreted Protein CIP Interacts with C4, Preventing C3b Deposition via the Lectin and Classical Complement Pathways. *J Immunol* **196**, 385-394
71. Woehl, J. L., Stapels, D. A. C., Garcia, B. L., Ramyar, K. X., Keightley, A., Ruyken, M., Syriga, M., Sfyroera, G., Weber, A. B., Zolkiewski, M., Ricklin, D., Lambris, J. D., Rooijakkers, S. H. M., and Geisbrecht, B. V. (2014) The extracellular adherence protein from Staphylococcus aureus inhibits the classical and lectin pathways of complement by blocking formation of the C3 proconvertase. *J Immunol* **193**, 6161-6171
72. Woehl, J. L., Ramyar, K. X., Katz, B. B., Walker, J. K., and Geisbrecht, B. V. (2017) The Structural Basis for Inhibition of the Classical and Lectin Complement Pathways by *S. aureus* Extracellular Adherence Protein. *Protein Science 2017* **26**, 1595—1608
73. Rosini, R., Campisi, E., De Chiara, M., Tettelin, H., Rinaudo, D., Toniolo, C., Metruccio, M., Guidotti, S., Sorensen, U. B., Kilian, M., Consortium, D., Ramirez, M., Janulczyk, R., Donati, C., Grandi, G., and Margarit, I. (2015) Genomic analysis reveals the molecular basis for capsule loss in the group B Streptococcus population. *PLoS One* **10**, e0125985
74. Marques, M. B., Kasper, D. L., Pangburn, M. K., and Wessels, M. R. (1992) Prevention of C3 Deposition by Capsular Polysaccharide Is a Virulence Mechanism of Type III Group B Streptococci. *Infection and Immunity* **60**, 3986-3993
75. Aminoff, D., Bell, W. C., Fulton, I., and Ingebrigtsen, N. (1976) Effects of sialidase on the viability of erythrocytes in circulation. *American Journal of Hematology* **1**, 419-432
76. Edwards, M. S., Nicholson-Weller, A., Baker, C. J., and Kasper, D. L. (1980) The role of specific antibody in alternative complement pathway- mediated opsonophagocytosis of type III, group B *Streptococcus*. *Journal of Experimental Medicine* **151**, 1275-1287
77. Pangburn, M. K., and Moller-Eberhard, H. J. (1978) Complement C3 Convertase: Cell Surface Restriction of β–1H Control and Generation of Restriction on Neuraminidase-treated Cells. *Proc. Natl. Acad. Sci. USA* **75**, 2416-2420
78. Baker C.J., E. M. S., Webb B.J., and Kasper D.L. (1982) Antibody-independent Classical Pathway-mediated Opsonophagocytosis of Type Ia, Group B *Streptococcus*. *J. Clin. Invest.* **69**
79. Aoyagi, Y., Adderson, E. E., Rubens, C. E., Bohnsack, J. F., Min, J. G., Matsushita, M., Fujita, T., Okuwaki, Y., and Takahashi, S. (2008) L-Ficolin/mannose-binding lectin-associated serine protease complexes bind to group B streptococci primarily through N-acetylneuraminic acid of capsular polysaccharide and activate the complement pathway. *Infect Immun* **76**, 179-188
80. Fujita, T., Matsushita, M., and Endo, Y. (2004) The lectin-complement pathway: its role in innate immunity and evolution. *Immunol. Rev.* **198**, 185-202
81. Aoyagi, Y., Adderson, E. E., Min, J. G., Matsushita, M., Fujita, T., Takahashi, S., Okuwaki, Y., and Bohnsack, J. F. (2004) Role of L-Ficolin/Mannose-Binding Lectin-Associated Serine Protease Complexes in the Opsonophagocytosis of Type III Group B Streptococci. *The Journal of Immunology* **174**, 418-425
82. Romero-Steiner, S., Libutti, D., Pais, L. B., Dykes, J., Anderson, P., Whitin, J. C., Keyserling, H. L., and Carlone, G. M. (1997) Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. *Clin. Diagn. Lab. Immunol.* **4**, 415-422
83. Morley, B. J., and Walport, M. J. (2000) The Complement Facts Book *Academic Press, London.*
84. Wallis, R., Mitchell, D. A., Schmid, R., Schwaeble, W. J., and Keeble, A. H. (2010) Paths reunited: Initiation of the classical and lectin pathways of complement activation. *Immunobiology* **215**, 1-11

85. Raju, T. S. (2008) Terminal sugars of Fc glycans influence antibody effector functions of IgGs. *Current Opinion in Immunology* **20**, 471-478
86. Berends, E. T., Kuipers, A., Ravesloot, M. M., Urbanus, R. T., and Rooijakkers, S. H. (2014) Bacteria under stress by complement and coagulation. *FEMS Microbiol Rev* **38**, 1146-1171
87. Rooijakkers, S. H., and van Strijp, J. A. (2007) Bacterial complement evasion. *Mol Immunol* **44**, 23-32
88. Hammel, M., Sfyroera, G., Pyrpassopoulos, S., Ricklin, D., Ramyar, K. X., Pop, M., Jin, Z., Lambris, J. D., and Geisbrecht, B. V. (2007) Characterization of Ehp, a Secreted Complement Inhibitory Protein fromStaphylococcus aureus. *Journal of Biological Chemistry* **282**, 30051-30061
89. Domina, M., Lanza Cariccio, V., Benfatto, S., Venza, M., Venza, I., Donnarumma, D., Bartolini, E., Borgogni, E., Bruttini, M., Santini, L., Midiri, A., Galbo, R., Romeo, L., Patane, F., Biondo, C., Norais, N., Masignani, V., Teti, G., Felici, F., and Beninati, C. (2016) Epitope Mapping of a Monoclonal Antibody Directed against Neisserial Heparin Binding Antigen Using Next Generation Sequencing of Antigen-Specific Libraries. *PLoS One* **11**, e0160702
90. Diefenbach, R. J., and Isenman, D. E. (1995) Mutation of residues in the C3dg region of human complement component C3 corresponding to a proposed binding site for complement receptor type 2 (CR2, CD21) does not abolish binding of iC3b or C3dg to CR2. *J Immunol* **154**, 2303-2320
91. Van Den Elsen, J. M. H., and Isenman, D. E. (2011) A Crystal Structure of the Complex Between Human Complement Receptor 2 and its Ligand C3d. *Science* **332**, 608-611
92. Upadhyay, A., Burman, J. D., Clark, E. A., Leung, E., Isenman, D. E., van den Elsen, J. M. H., and Bagby, S. (2008) Structure-Function Analysis of the C3 Binding Region of *Staphylococcus aureus* Immune Subversion Protein Sbi. *Journal of Biological Chemistry* **283**, 22113-22120
93. Malito, E., Faleri, A., Lo Surdo, P., Veggi, D., Maruggi, G., Grassi, E., Cartocci, E., Bertoldi, I., Genovese, A., Santini, L., Romagnoli, G., Borgogni, E., Brier, S., Lo Passo, C., Domina, M., Castellino, F., Felici, F., van der Veen, S., Johnson, S., Lea, S. M., Tang, C. M., Pizza, M., Savino, S., Norais, N., Rappuoli, R., Bottomley, M. J., and Masignani, V. (2013) Defining a protective epitope on factor H binding protein, a key meningococcal virulence factor and vaccine antigen. *Proc Natl Acad Sci U S A* **110**, 3304-3309
94. Hammel, M., Sfyroera, G., Ricklin, D., Magotti, P., Lambris, J. D., and Geisbrecht, B. V. (2007) A structural basis for complement inhibition by *Staphylococcus aureus*. *Nat Immunol* **8**, 430-437
95. Ricklin, D., Ricklin-Lichtsteiner, S. K., Markiewski, M. M., Geisbrecht, B. V., and Lambris, J. D. (2008) Cutting Edge: Members of the Staphylococcus aureus Extracellular Fibrinogen-Binding Protein Family Inhibit the Interaction of C3d with Complement Receptor 2. *The Journal of Immunology* **181**, 7463-7467
96. Margarit, I., Bonacci, S., Pietrocola, G., Rindi, S., Ghezzo, C., Bombaci, M., Nardi-Dei, V., Grifantini, R., Speziale, P., and Grandi, G. (2009) Capturing host-pathogen interactions by protein microarrays: identification of novel streptococcal proteins binding to human fibronectin, fibrinogen, and C4BP. *FASEB J* **23**, 3100-3112
97. Buhlmann, D., Eberhardt, H. U., Medyukhina, A., Prodinger, W. M., Figge, M. T., Zipfel, P. F., and Skerka, C. (2016) FHR3 Blocks C3d-Mediated Coactivation of Human B Cells. *J Immunol* **197**, 620-629
98. Haupt, K., Reuter, M., van den Elsen, J., Burman, J., Halbich, S., Richter, J., Skerka, C., and Zipfel, P. F. (2008) The Staphylococcus aureus protein Sbi acts as a complement inhibitor and forms a tripartite complex with host complement Factor H and C3b. *PLoS Pathog* **4**, e1000250
99. Haspel, N., Ricklin, D., Geisbrecht, B. V., Kavraki, L. E., and Lambris, J. D. (2008) Electrostatic contributions drive the interaction between Staphylococcus aureus protein Efb-C and its complement target C3d. *Protein Sci* **17**, 1894-1906

100. Gonzalez, S. F., Degn, S. E., Pitcher, L. A., Woodruff, M., Heesters, B. A., and Carroll, M. C. (2011) Trafficking of B cell antigen in lymph nodes. *Annu Rev Immunol* **29**, 215-233
101. Dempsey, P. W., Allison, M. E., Akkaraju, S., Goodnow, C. C., and Fearon, D. T. (1996) C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* **271**, 348-350
102. Cyster, J. G., Ansel, K. M., Reif, K., Ekland, E. H., Hyman, P. L., Tang, H. L., Luther, S. A., and Ngo, V. N. (2000) Follicular stromal cells and lymphocyte homing to follicles. *Immunological Reviews* **176**, 181–193
103. Roozendaal, R., and Carroll, M. C. (2007) Complement Receptors CD21 and CD35 in humoral immunity. *Immunological Reviews* **219**, 157–166
104. Glaser, P., Rusniok, C., Buchrieser, C., Chevalier, F., Frangeul, L., Msadek, T., Zouine, M., Elisabeth, C., Lalioui, L., Poyart, C., Trieu-Cuot, P., and F., K. (2002) Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Molecular Microbiology* **45**, 1499–1513
105. Chuzeville, S., Dramsi, S., Madec, J. Y., Haenni, M., and Payot, S. (2015) Antigen I/II encoded by integrative and conjugative elements of Streptococcus agalactiae and role in biofilm formation. *Microb Pathog* **88**, 1-9
106. Rego, S., Heal, T. J., Pidwill, G. R., Till, M., Robson, A., Lamont, R. J., Sessions, R. B., Jenkinson, H. F., Race, P. R., and Nobbs, A. H. (2016) Structural and Functional Analysis of Cell Wall-anchored Polypeptide Adhesin BspA in Streptococcus agalactiae. *J Biol Chem* **291**, 15985-16000
107. Nagar, B. J., R.G.; Diefenbach, R.J.; Isenman, D.E.; Rini, J.M. (1998) X-ray Crystal Structure of C3d: A C3 Fragment and Ligand for Complement Receptor 2. *Science* **280**, 1277-1281
108. Suzuki, K., Grigorova, I., Phan, T. G., Kelly, L. M., and Cyster, J. G. (2009) Visualizing B cell capture of cognate antigen from follicular dendritic cells. *J Exp Med* **206**, 1485-1493
109. Bajic G., Y. L., Sim R.B., Vorup-Jensen T., Andersen G.R. (2013) Structural insight on the recognition of surface-bound opsonins by the integrin I domain of complement receptor 3. *PNAS* **110**, 16426–16431