AUS DEM LEHRSTUHL FÜR IMMUNOLOGIE
LEHRSTUHLINHABERIN PROF. DR. DANIELA MÄNNEL
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

Binding Specificity of Mouse Ficolin to Different Bacterial Strains

---

Inaugural – Dissertation zur Erlangung des Doktorgrades der Medizin

der Fakultät der Medizin
der Universität Regensburg

vorgelegt von
Liudmila Muraveika

2012

AUS DEM LEHRSTUHL FÜR IMMUNOLOGIE
LEHRSTUHLINHABERIN PROF. DR. DANIELA MÄNNEL
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

Binding Specificity of Mouse Ficolin to Different Bacterial Strains

Inaugural – Dissertation zur Erlangung des Doktorgrades der Medizin

der Fakultät der Medizin
der Universität Regensburg

vorgelegt von
Liudmila Muraveika

2012

Dekan:	Prof. Dr. Dr. Torsten E. Reichert
1. Berichterstatter	Prof. Dr. D. N. Männel
2. Berichterstatter	Prof. Dr. B. Salzberger
Tag der mündlichen Prüfung:	16.11.2012

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt und keine anderen als die hier angegebenen Quellen als Hilfsmittel verwendet habe.

X

Liudmila Muraveika

Table of Contents

Abbreviations

#	Number
Δ	heat aggregated
AATGal	2-acetamido-4-amino-2,4,6-trideoxy-D-galactose
Ac	acetyl-group
AP	alternative pathway
APP5	Actinobacillus pleuropneumoniae serotype 5B
A_x	absorbance at a wavelength of x nm
biot	Biotinylated
cDNA	complementary DNA
CP	classical pathway
CRD	carbohydrate recognition domain
DES	*Drosophila melanogaster* expression system
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF	embryonic fibroblasts
ELISA	enzyme linked immunosorbant assay
ES cells	embryonic stem cells
FACS	fluorescence activated cell sorter
fbg	Fibrinogen
Fig.	Figure
FITC	fluorescein isothiocyanate
FucNAc	N-acetylfucosamine
g	Grams
Gal	Galactose
GalA	galactouronic acid
Gal*p*NAc	pyranosidic 2-acetamido-2-deoxyglucose
GlcA	Glucuronic
GlcNAc	N-acetyl-D-glucosamine
GPC	gel permeation chromatography
H_2O_2	hydrogen peroxide ion
H_2O_d	destilled water

HAT medium	hypoxanthine-aminopterin-thymidine medium
His	Histidin
HSA	human serum albumin
HT medium	hypoxanthine-thymidine medium
IDA	iminodiacetic acid
Ig	immunoglobulin
IMAC	ion-metal affinity chromatography
LP	lectin pathway
LPS	lipopolysaccharyde
LTA	lipothaicoic acid
mAb	monoclonal antibody
MAC	membrane attack complex
ManNAc	*N*-acetylmannosamineuronic
ManNAcA	*N*-acetylmannosamineuronic
MASP	MBL associated serine proteases
MurNAc	N-acetomuramic acid
NAManAc	*N*-acetylmannosamineuronic acid
NO	nitric oxide
O_2-	oxide anion
OAc	*O*-acetyl
OBr-	hypobromide ion
OCl-	hypochlorite ion
$OH^{\cdot}$	hydroxyl radical ion
P	phosphate residue
p	Pyranosidic
PCho	phosphorylcholine
Rha	Rhamnose
SDS	sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TAE buffer	Tris acetate EDTA electrophoresis buffer
TBE buffer	Tris borate EDTA electrophoresis buffer
TBS	Tris buffered saline
TE buffer	Tris EDTA buffer

TEMED	N,N,N’,N’-Tetramethylethylendiamin
TGF	transforming growth factor
TK	thymidine kinase
TLRs	Toll-like receptors
TOPO	tri-o-octylphosphine oxide
Tween	Tween 20
U	Units
vol	volume(s)
WB	Western Blot

Zusammenfassung

Fikoline gehören zur Gruppe der Triggerproteine, die den Lektinweg des Komplementensystems aktivieren und eine Klasse der Rezeptoren darstellen, die spezifisch an Zuckermoleküle der mikrobiellen Oberflächen binden und dadurch zur Aktivierung des Immunsystems führen.

Das Ziel dieser Arbeit ist das Bindungpotenzial der Mausfikoline A und B an unterschiedlichen Bakterien in Screeningsassays zu erforschen. Die Erkennungsmoleküle Ficolin-A und -B der Maus binden an Staphylokokken und Streptokokken mit unterschiedlicher Affinität. Sie erkennen dabei definierte Zuckerstrukturen auf bakteriellen Oberflächen sowohl bekapselter als auch unbekapselter Stämme. Um den Einfluss der Bakterienkapsel auf die Fikolin-bakterielle Bindung zu zeigen, wurden Screeningsassays an den siebzehn verbreitetsten bekapselten Stämmen des *Str. pneumonia,* zwölf bekapselten Stämmen von *S. aureus* und an relevanten unbekapselten Stämmen von *S. aureus* (Wood) and *Str. pneumonia* (SCR2 and TIGR4) durchgeführt. Es wurde festgestellt, dass unterschiedliche Bakterienstämme mit einer unterschiedlichen Affinität an die Mausfikoline binden. Fikolin-A bindet mit einer hohen Affinität an *Str. pneumonia* 7A und 32F, wogleich Fikolin-B mit den ähnlichen Ergebnissen an *Str. pneumonia* 6A and 11F bindet, wobei keins der Mausfikoline an Pneumokokkenstämme 19C, 9L und 9V eine Bindung gezeigt hat.

Somit wurde in dieser Doktorarbeit nachgewiesen, dass die Fikoline an bekapselte Bakterienstämme als auch an unbekapselte binden können und komplexes Bindungsmuster erkennen können, dass sich auch von Humanfikolinen unterscheiden könnte. Beide Maus- und Humanfikoline können N-acetylierte Zuckerreste erkennen. Allerdings war es nicht möglich, bindungsessenzielle Kohlenhydrate zu bestimmen. Es scheint aber möglich zu sein, dass Mausfikoline ein komplexes Bindungmuster erkennen und viele Oligosaccharide mit unterschiedlichen Interaktionsseiten nachweisen können.

Diese Arbeit untersuchte auch Fikolin-B und sein Bindungpotenzial in Anwesenheit der Calciumionen. Die Bindung war nicht möglich, wenn Calzium der Lösung entzogen war. Um die Tatsache zu beweisen, dass Mausfikoline an unterschiedliche Liganden binden können, haben wir Kompetitivassays durchgeführt. Sie ergaben, dass Fikolin-A und -B unterschiedlich überlappende Bindungsstellen haben.

I. Introduction

I.1 Innate immune system

The immune system is divided into innate and adaptive. The innate immune response is also referred to as the first line of host defense, because it protects the host from the microorganisms, which could cause a disease (Janeway Jr. et al., 2005). Most of the pathogens are detected and destroyed within a short period of time by mechanisms of the innate immunity or kept under control until the adaptive immune response is ready to fight the infection. The mechanisms of the non-adaptive immunity act immediately and the early induced responses follow them; however, in contrast to the adaptive immune response, innate immunity does not contain any immunological memory.

The body epithelia make up the first physical line of defense against infection. In case the microorganism manages to cross the epithelium, it is immediately recognized, ingested and destroyed by macrophages or neutrophils in most cases. Using their cell surface receptors, macrophages and neutrophils can discriminate between molecules present on pathogens from those on host cells. The macrophage mannose receptor binds to mannose and the scavenger receptor binds negatively charged ligands (for example, lipoteichoic acid or bacterial lipopolisaccharids). Macrophage mannose receptor is a cell-bound Ca^{++}-dependent lectin, while mannan-like binding lectin (MBL) binds the mannose or fucose residues of bacterial or viral surfaces. Ligation of many of the receptors on the surface of the pathogen leads to phagocytosis of the pathogen. Macrophages and neutrophils possess also lysosome vesicles, which contain enzymes and peptides, that can mediate the intracellular antimicrobial response. The phagosome fuses with one or more lysosomes to generate a phagolysosome in which the lysosomal contents are released to destroy the pathogen (Figure 1) (Janeway Jr. et al., 2005).

In addition macrophages and neutrophils also produce nitric oxide (NO), the superoxide anion (O_2-) and hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), the hypochlorite (OCl-) and the hypobromide (OBr-) ions which are directly toxic to bacteria.

Neutrophils are short-lived compared to the macrophages which continue to produce new lysosomes and get activated by the pathogens to produce cytokines, chemokines, and other inflammatory mediators. Macrophages are able to set up an inflammation in the tissue and attract more neutrophils and plasma proteins to the site of infection. Figure 1 displays the phagocytosis by macrophages.

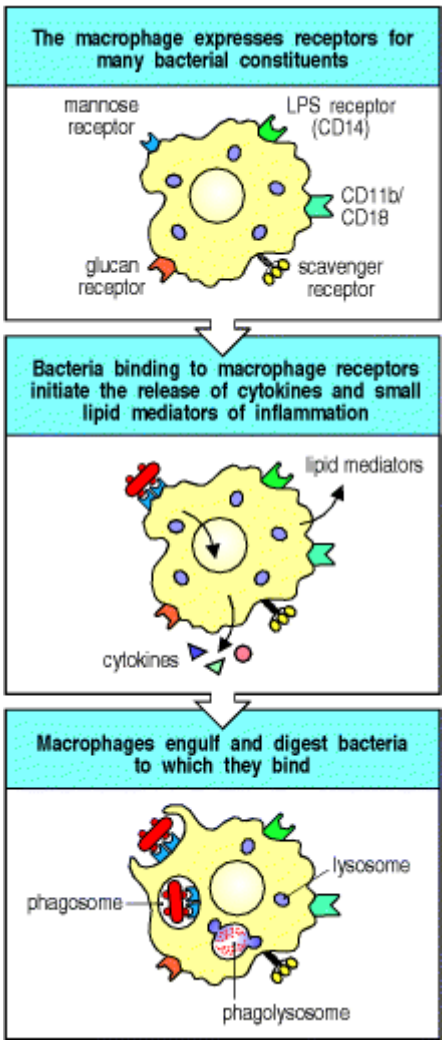


Figure 1. **Phagocytes bear several different receptors that recognize microbial components and induce phagocytosis.** The figure illustrates five such receptors on macrophages—CD14, CD11b/CD18 (CR3), the macrophage mannose receptor, the scavenger receptor, and the glucan receptor, all of which bind bacterial carbohydrates. CD14 and CR3 are specific for bacterial lipopolysaccharide (LPS). (Janeway, Jr. *et al.*, 2005).

The complement system has an important role as part of the innate immunity. The antibacterial responses in the human body begin with complement activation, which promotes recruitment and activation of neutrophils and macrophages. The mechanism of complement activation is as follows: neutrophils stimulate Toll-like receptors (TLRs) on dendritic cells and macrophages. Their products are cytokines which stimulate the innate immune responses. The Figure 2 explains the connection between the innate immunity and complement system.

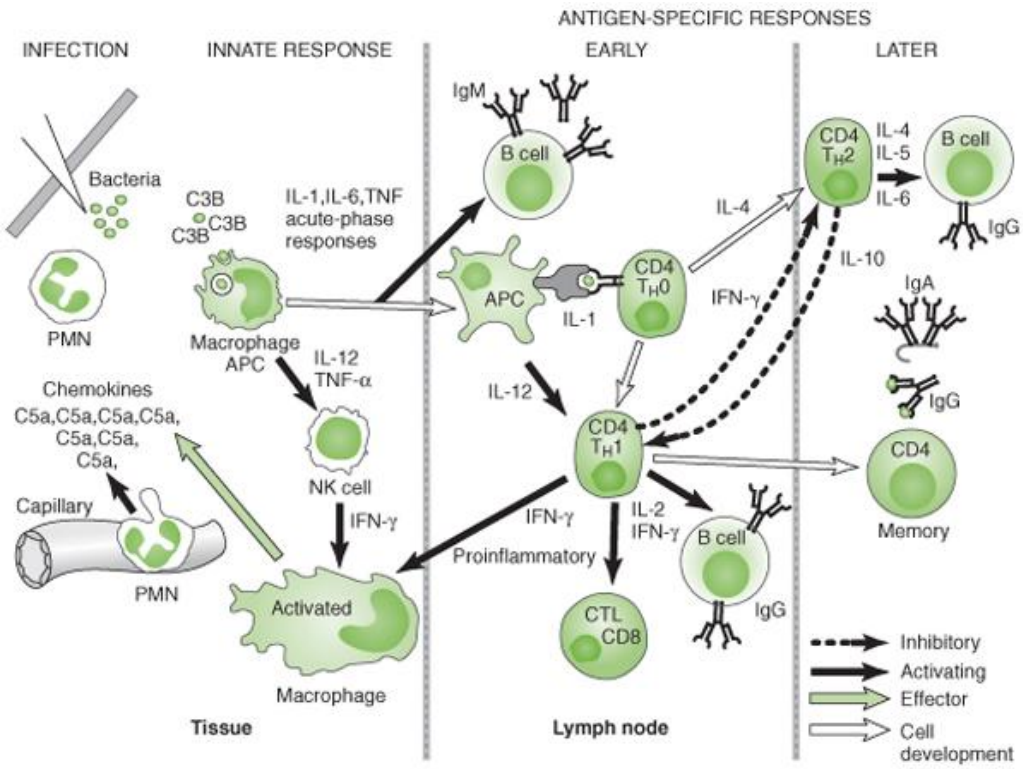


Figure 2. Overview of bacterial immune responses (Rosenthal et al, 2007)

The components of the complement system, distinct plasma proteases, induce the opsonization, phagocytosis and lysis of bacteria and pathogens in a series of inflammatory responses. There are three pathways of complement activation: the classical pathway (CP), which is triggered by binding of the complement components C1q to antibody-complexed antigen by direct binding of C1q to the pathogen surface, or by binding of C1q to C reactive protein bound to the pathogen; the lectin pathway (LP), which is triggered by mannose-binding lectin and ficolins, normal serum constituents, that bind to the carbohydrate molecules on the bacterial surfaces; and the alternative pathway (AP), which is triggered directly on the pathogen surfaces (Janeway et al., 2005). All of these pathways generate a crucial enzymatic activity that, in its turn, generates the effector molecules of the complement.

The formation of C3-convertases it is a converging point of all three pathways of complement activation. The C3-convertase of the alternative pathway is C3bBb and the C3-convertase of the classical pathway is C4b2a (Löffler, et al., 2005). C3bBb and C4b2a bind to the surface of pathogen and cleave C3 into C3a and C3b. In the LP and AP multiple C3b-molecules bind to the complex of C4b2a (in the AP) or of C3bBb (in the LP). These molecules are then able to capture C5 through binding to an acceptor site on C3b. This binding makes C5 susceptible to cleavage by the serine protease activity of

C2a or Bb, generating the products C5a and C5b, and initiating the terminal pathway which leads to the formation of the membrane attack complex (MAC) (Janeway Jr. et al., 2005). The next step in the generation of MAC is the consecutive binding of C6, C7, C8 and C9. MAC integrated in the bacterial surface looks like a pore through which the water enters into the bacterial cell and makes it burst.

I.2 Proteins of the lectin pathway of complement activation

According to today's knowledge there are two groups of trigger-proteins to activate the lectin pathway: mannose-binding lectin (MBL) and ficolins.

MBL circulates in plasma as a free receptor. In humans, the MBL gene encodes for a 32 kDa glycoprotein, showing the typical collectin structure consisting of an N-terminal cysteine-rich region, a collagen-like domain followed by a neck region and a C-terminal carbohydrate recognition domain (CRD) (Turner T. et al., 2000). MBL forms homotrimers composed of a collagenous triple helix subunit and several of these homotrimers assemble to form higher order oligomers. In this way, the lectin domains of the MBL (as in every collectin) undergo two grades of clustering during assembly. The effect of this clustering probably ensures that these molecules only bind with high affinity to dense sugar arrays, typically found on the surface of microbes. There is evidence that full biological function requires assembly to at least the tetrameric level (Yokota Y. et al., 1995).

The MBL recognizes certain bacterial surfaces that present an arrangement of mannose and fucose residues. MBL binds to monosaccharides such as N-acetyl-D-glucosamine, mannose, N-acetyl-D-mannosamine, L-fucose and glucose (Hansen M. et al., 2000) in a Ca^{++}-dependent manner. But the only correct spacing of the mannose and fucose residues ensures the MBL-binding. Ligand binding to one single CRD, however, is very weak, and multiple contacts are necessary for activation. These repetitive carbohydrate structures are found on a wide range of microorganisms, including bacteria, viruses and fungi (Jack B. et al., 2001) (Townsend et al., 2001) (Jack B. et al., 2003), but not on mammalian cells, because of the prevalent termination of self-glycoproteins with sialic acid or galactose (Ezekowitz N. et al., 1998), (Wallis S. et al., 2002). Some bacteria protect themselves from MBL-mediated complement attack by sialylating their surface structures (Jack *et al.*, 2001).

The binding of MBL to bacterial surfaces induces phagocytosis and activates the lectin pathway of the complement system. To date three MBL associated serine proteases (MASP-1, MASP-2, MASP-3) have been identified in a complex with MBL.

Ficolins were originally identified by scientists from Fukushima Medical University School of Medicine in 1991 as a transforming growth factor (TGF)-ß-binding protein (Ichijo et al., 1991). This is a group of proteins which possesses a collagen-like stem structure with a fibrinogen-like domain at the C-terminal end.

Ficolins are built of structural subunits (34-40 kDa) of three identical polypeptide chains. Each subunit includes a short N-terminal region with a cysteine residue, a middle collagen-like domain, a short neck domain and as last follows a globular fibrinogen-like domain (Yokota et al., 1995). Although ficolins do not have a coiled-coil structure acting as the neck region like MBL (Holmskov et al., 2003), they form active oligomers where normally four subunits join together at the N-terminal regions (Holmskow et al., 2003). Ficolins do not contain a Carbohydrate Recognition Particle (CRP) as do other lectins.

Figure 3 shows the best known collectins and ficolins in their trimeric subunits.

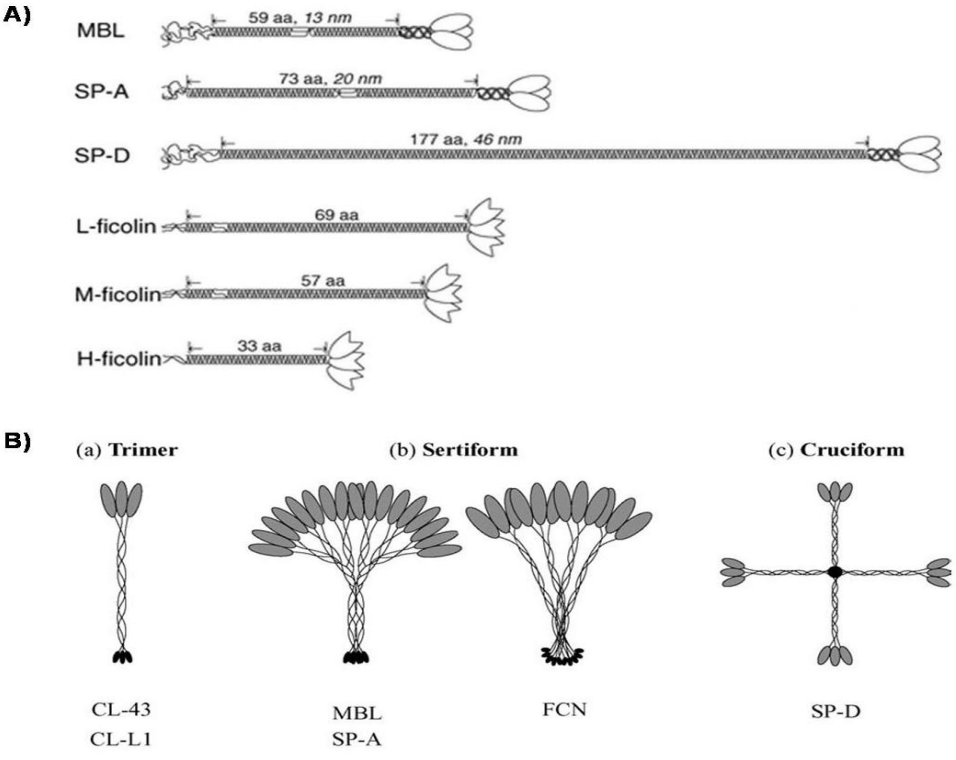


Figure 3. **The structures of collagenous lectins in animals.** A) Trimeric subunit structures of human collectins and ficolins. The molecules are drawn approximately to scale. The number of amino acids spanning the collagen like domains, including interruptions, is indicated. Fibrinogen-like domains are represented as globular heads. Modified from (Holmskov *et al.*, 2003). B) Multimeric structures of a) CL-L1 and CL-43 (trimeric native form), b) MBL, SP-A and FCN ("bundle of tulips" or sertiform oligomers of varying numbers of trimers), and c) SP-D (cruciform oligomers comprised of four trimers). (Lillie *et al.*, 2005).

Ficolins recognize a common carbohydrate N-Acetyl-D-Glucosamin (GlcNAc). The carbohydrate-binding activity of ficolins is executed by the fibrinogen-like domain which has a Ca^{++}-dependent lectin activity. The fibrinogen-like domain shows similarity to the fibrinogen α and γ chains (Endo Y. et al., 2005).

I.3 Pig, human and mouse ficolins in comparison to each other and their role in the bacterial recognition

Pigs contain two closely related ficolin genes. Ficolin α is expressed in liver, bone marrow, spleen and lung (Ohashi H. et al., 1998), while Ficolin β is expressed in bone marrow and neutrophiles (Brooks S. et al, 2003). α- and β-Ficolins share about 82% identity at the amino acid level.

Pig ficolin-α posseses N-glycosylated subunits of about 35 kDa (Ohashi and Erickson, 1998) and its binding activity was shown in experiments with bacterial microorganisms. In a N-acetyl-D-glucosamine (GlcNAc) dependent manner ficolin-α can bind to Actinobacillus pleuropneumoniae serotype 5B (APP5) (Books S. et al., 2003), to LPS from Gram-negative bacteria of both the rough and the smooth types such as *Escherichia coli, Salmonella typhimurium, S. enteriditis, S. abortus equi, Shigella flexeneri, Pseudomonas aeruginosa, Serratia marcescens* (Nahid and Sungii, 2006).

Ficolin β has a molecular weight of 39 kDa. It seems that ficolin β might have a local function as a secreted lectin at sites of inflammation where neutrophils are activated and are able to release ficolin β. That constitutes a bactericidal role in tissue. Both of pig ficolins also can activate the complement system.

In the human body three types of ficolin can be described: L-ficolin, H-ficolin and M-ficolin. L- and M-ficolins share 79% identity at the amino acid level, the H- and M-ficolins are identical only to 45%.

L-ficolin is a multimeric plasma protein with the molecular weight of 35 kDa. It is expressed in the liver. Matsushita and colleagues showed that the L-ficolin binds to the GlcNAc residue to galactose at the non-reducing end of the complex-type oligosaccharides and that does not bind to mannose.

Many scientists showed in their experiments that L-ficolin can bind to bacterial surfaces. Matsushita and co-workers described the binding of L-ficolin to the bacterial strain of *Salmonella typhimurium* TV119 (this strain exposes GlcNAc). This Ca^{++}-dependent binding increases the phagocytosis by neutrophils and monocytes.

L-ficolin also binds to *Escherichia coli* and can be eluted with a mixture of monosaccharides (Lu and Le, 1998).

L-ficolin and MASPs complexes from sera specifically bind to LTA from *Staphyloccoccus aureus, pyogenes and agalastiae* and initiate a C4 turnover (Lynch et al., 2004).

Krarup and co-workers reported that L-ficolin binds to capsulated *S. aureus and S. pneumoniae*, but does not bind to the non-capsulated strains. This is different from H-ficolin and MBL binding properties. The results indicated that the binding of each lectin are directed toward different PAMPs and are specific.

L-ficolin binds to *Streptococcus pneumoniae* 11F and this interaction can be inhibited by N-acetylated compounds, either sugars (GlcNAc, ManNAc, GalNAc) or other molecules like CysNAc, GlyNAc, and acetylcholine (Krarup *et al.,* 2004). This finding shed some doubt on the lectin feature of L-ficolin and suggests that it might be considered as an acetyl-binding protein instead.

H-ficolin was initially identified as a serum-antigen detected by auto-antibodies found in some patients with systemic lupus erythematosus (Inaba *et al.,* 1990). The gene encoding H-ficolin (*FCN3*) is located on chromosome 1 and the open reading frame encodes for 299 amino acids that reveal a domain organization similar to L-ficolin (Sugimoto *et al.,* 1998).

H-ficolin is expressed by hepatocytes, bile duct epithelial cells, and in the lung by ciliated bronchial and type II alveolar epithelial cells (Akaiwa *et al.,* 1999).

H-ficolin is found in circulation at a median concentration of 18.4 µg/ml (Krarup *et al.,* 2005) as higher order oligomers whose 35 kDa subunits are linked by disulfide bonding (Yae *et al.,* 1991). Hexamers of trimeric subunits were visualized by electron microscopy (Sugimoto *et al.,* 1998) and it was also reported that H-ficolin shows a Ca^{++}-independent lectin activity which can be inhibited by GlcNAc and GalNAc.

The biological significance of H-ficolin as a lectin has been investigated by studying its binding potential to different strains and serotype forms of bacteria including *S. pneumoniae, E.coli, S.aureus* and *Aerococcus viridans.* Only *A. viridans* was found to be recognized and the binding specificity was assigned to a particular polysaccharide, namely PSA, present on this microorganism (Matsushita *et al.,* 2002).

H-ficolin isolated from serum is associated with MASP-1, MASP-2, MASP-3, and MAp19, and the H-ficolin/MASP complex is able to activate complement by cleavage of C4 upon binding to the PSA ligand (Matsushita *et al.,* 2002).

M-ficolin is expressed in peripheral blood leukocytes and the gene (*FCN1*) has been mapped to chromosome 9 in proximity to the gene encoding L-ficolin (*FCN2*) (Matsushita *et al.,* 1996; Lu *et al.,* 1996). M-ficolin contains a 27 amino acid potential leader peptide, as well as the short N-terminal sequence followed by the collagen-like and the fibrinogen-like domains (Lu *et al.,* 1996). By screening a number of leukocyte cell lines it was shown that M-ficolin mRNA is synthesized in peripheral blood monocytes (PBM) as well as by cells of the monocyte-like cell line U937, and is downregulated when the cells differentiate into macrophages (Lu *et al.,* 1996). M-ficolin was found on the surface of PBMs (Teh *et al.,* 2000). In the same report M-ficolin showed GlcNAc affinity. Furthermore, it was shown that phagocytosis of *Escherichia coli* K12 by U937 cells could be inhibited by anti-M-ficolin-fibrinogen antibodies (Teh *et al.,* 2000). Due to these findings, Teh and co-workers suggested a putative role for M-ficolin in innate immunity by acting as a phagocytic receptor for pathogens (Teh *et al.,* 2000). In contrast, M-ficolin protein was recently localized in secretory granules in the cytoplasm of neutrophils, monocytes, and type II alveolar epithelial cells in lung (Liu *et al.,* 2005b). However, M-ficolin could not be detected in normal serum and has recently been secreted from monocytes and macrophages and also from granules of neutrohiles (Liu *et al.,* 2005; Honore *et al.,* 2008). These facts led to the hypothesis that M-ficolin might act as an acute phase protein that is temporarily stored in the secretory granules of the leukocytes to be secreted into local areas where it could execute its functions in host defense upon the right stimuli, similar to ficolin-β in pigs.

In addition, M-ficolin coprecipitated with MASP-1 and -2, and the complexes were able to cleave C4 on GlcNAc-coated microplates. Regarding its binding specificities, Liu and co-workers found positive binding of M-ficolin to several neoglycoproteins bearing GlcNAc, GalNAc and syalil-LacNAc (Liu *et al.,* 2005b). Interestingly, M-ficolin was found to interact with a rough-type of *Staphylococcus aureus* (LT2) but not with the smooth-type strain TV119, whereas just the opposite is true for L-ficolin (Matsushita *et al.,* 1996), indicating that the spectrum of bacterial recognition might be different among ficolins.

Mice, as well as rats, have two ficolin forms, termed ficolin-A and –B. The ficolin-A gene was first isolated by Fujimori and co-workers in 1998 from a mouse liver library (Fujimori *et al.,* 1998). Ficolin-A is a plasma protein with a molecular weight of 37 kDa, highly expressed in liver and spleen with binding affinity for elastin and GlcNAc (Fujimori *et al.,* 1998). Under the electron microscope, ficolin-A displayed the typical

parachute-like structure composed of four trimers of fibrinogen domains (12-mers) (Ohashi and Erickson, 1998).

Liu and co-workers showed that ficolin-A mRNA is expressed as early as on embryonic day (E) 12.5, displaying an increase during development, peaking around birth, and slightly declining in the adult stages (Liu *et al.*, 2005a). In addition, *in situ* hybridisation studies indicated that ficolin-A mRNA was mainly localized in the liver between two hepatic cords and in the red pulp of the spleen. These observations, together with further immunohistochemical analysis revealing a distribution pattern of ficolin-A comparable to the Kupffer cells in liver, suggest that ficolin-A mRNA is expressed by macrophages (Liu *et al.*, 2005a).

Ficolin-B was first characterized by Ohashi and Erickson in 1998 as a mouse ficolin different from the plasma ficolin (ficolin-A), with a strong mRNA expression in bone marrow and a weak expression in spleen (Ohashi and Erickson, 1998). Ficolin-B mRNA was detected in the spleen at all time points examined after birth, indicating a complementary expression of ficolin-A and –B in spleen (Liu *et al.*, 2005a). Regarding the specific cell types expressing ficolin-B, distinct cell lineages of sorted bone marrow-derived cells showed different expression patterns with high levels in myeloid cells (Gr-1^+ and Mac-1^+) and no expression in the Ter119^+ erythroid, the T-cell (CD$3e^+$), or the B-cell (B220^+) lineages (Liu *et al.*, 2005a).

I.4 General characteristics of bacteria and their interaction with the immune system of mammalians

Bacteria are unicellular microorganisms and they can have a wide range of shapes such as spheres, rods and spirals. The majority of the bacteria are rendered harmless or beneficial by the protective effects of the immune system. A few pathogenic bacteria can also cause infectious diseases and shut down the immune system which tries to defeat them.

Bacteria are prokaryotes, which do not contain a nucleus. Bacterial cells are about 10 times smaller than eukaryotic cells, are typically 0,5 – 5 µm in length and surrounded by a lipid membrane which encompasses the contents of the cell and acts as a barrier to hold nutrition, proteins and other essential components of the cytoplasm within the cells. Many important biochemical reactions occur due to concentration gradients across membranes.

I.5 Bacterial surface layers and their roles in the immunological evasion of bacteria

I.5.1 Cell wall and peptidoglycan

Around the outside of the membrane is the bacterial cell wall, its primary function is to protect a bacterial cell from internal turgor pressure caused by the much higher concentrations of proteins and other molecules inside of the cell compared to its external environment. Bacterial cell wall contains peptidoglycan (poly-N-acetylglucosamine and N-acetylmuramic acid), called murein, which is made of polysaccharide chains crosslinked by unusual peptides of D-amino-acids (von Heijenoort et al., 2001). Peptidoglycan is responsible for the rigidity of the bacterial call wall and for the determination of the cell shape.

The primary chemical structures of peptidoglycans of both Gram-positive and Gram-negative bacteria have been established; they consist of a glycan backbone of repeating groups of β1, 4-linked disaccharides of β1,4-N-acetylmuramyl-N-acetylglucosamine. Tetrapeptides of L-alanine-D-isoglutamic acid-L-lysine (or diaminopimelic acid)-n-alanine are linked through the carboxyl group by amide linkage of muramic acid residues of the glycan chains; the D-alanine residues are directly cross-linked to the ε-amino group of lysine or diaminopimelic acid on a neighboring tetrapeptide, or they are linked by a peptide bridge (Baron S. et al., 2004). In *S. aureus* peptidoglycan, a glycine pentapeptide bridge links the two adjacent peptide structures. The extent of direct or peptide-bridge cross-linking varies from one peptidoglycan to another. The staphylococcal peptidoglycan is highly cross-linked, whereas that of *E coli* is much less so, and has a more open peptidoglycan mesh (Baron S. et al., 2004). The diamino acid providing the ε-amino group for cross-linking is lysine or diaminopimelic acid, the latter being uniformly present in Gram-negative peptidoglycans.

The ß-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine is specifically cleaved by the bacteriolytic enzyme lysozyme. Widely distributed in nature, this enzyme is present in human tissues and secretions and can cause complete digestion of the peptidoglycan walls of sensitive organisms. When lysozyme is allowed to digest the cell wall of Gram-positive bacteria suspended in an osmotic stabilizer (such as sucrose), protoplasts are formed. These protoplasts are able to survive and continue to grow on suitable media in the wall-less state. Gram-negative bacteria treated similarly

produce spheroplasts, which retain much of the outer membrane structure. The dependence of bacterial shape on the peptidoglycan is shown by the transformation of rod-shaped bacteria to spherical protoplasts (spheroplasts) after enzymatic breakdown of the peptidoglycan. The mechanical protection afforded by the wall peptidoglycan layer is evident in the osmotic fragility of both protoplasts and spheroplasts.

I.5.2 Gram-positive and Gram-negative bacteria

According to the comparison of their cell wall, bacteria can be classified as Gram-positive and Gram-negative. Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acids, which are polyalcohols imbedded in the cell wall. The teichoic acids charge negatively the Gram-positive cell wall by the presence of phosphodiester bonds.

In contrast, gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides (LPS) and lipoproteins, which face the external environment and are responsible for many antigenic properties (Hugenholz P. et al., 2002). Table 1 shows the structure of a typical LPS molecule.

Lipid A	Core	O Antigen
Glucosamine β-hydroxymyristate Fatty acids	Ketodeoxyoctonate Phosphoethanolamine Heptose Glucose, galactose, *N*-acetylglucosamine	Polysaccharide chains: repeating units of species-specific monosaccharides, e.g., galactose, rhamnose, mannose and abequose in *S typhimurium* LPS

Table 1. The three major, covalently linked regions that form the typical LPS (Baron S., et al., 2004).

The highly charged nature of the lipopolysaccharides confers an overall negative charge to the Gram-negative cell wall. As a phospholipid bilayer, the lipid portion of the outer membrane is largely impermeable to all charged molecules.

The LPS of all Gram-negative species are also called endotoxins, thereby distinguishing these cell-bound, heat-stable toxins from heat-labile, protein exotoxins secreted into

culture media. Endotoxins possess an array of powerful biologic activities and play an important role in the pathogenesis of many Gram-negative bacterial infections. In addition to causing endotoxic shock, LPS is pyrogenic, can activate macrophages and complement, is mitogenic for B lymphocytes, induces interferon production, causes tissue necrosis and tumor regression, and has adjuvant properties (Baron S., et al., 2004). The endotoxic properties of LPS reside largely in the lipid A components. Usually LPS molecules have three regions: the lipid A structure required for insertion in the outer leaflet of the outer membrane bilayer; a covalently attached core composed of 2-keto-3deoxyoctonic acid (KDO), heptose, ethanolamine, N-acetylglucosamine, glucose, and galactose; and polysaccharide chains linked to the core. Fig 1.4 shows the structure of bacterial surfaces. The polysaccharide chains constitute the O-antigens of Gram-negative bacteria, and the individual monosaccharide constituents confer serologic specificity on these components. Figure 4 shows the main differences between gram-positive and gram negative bacteria.

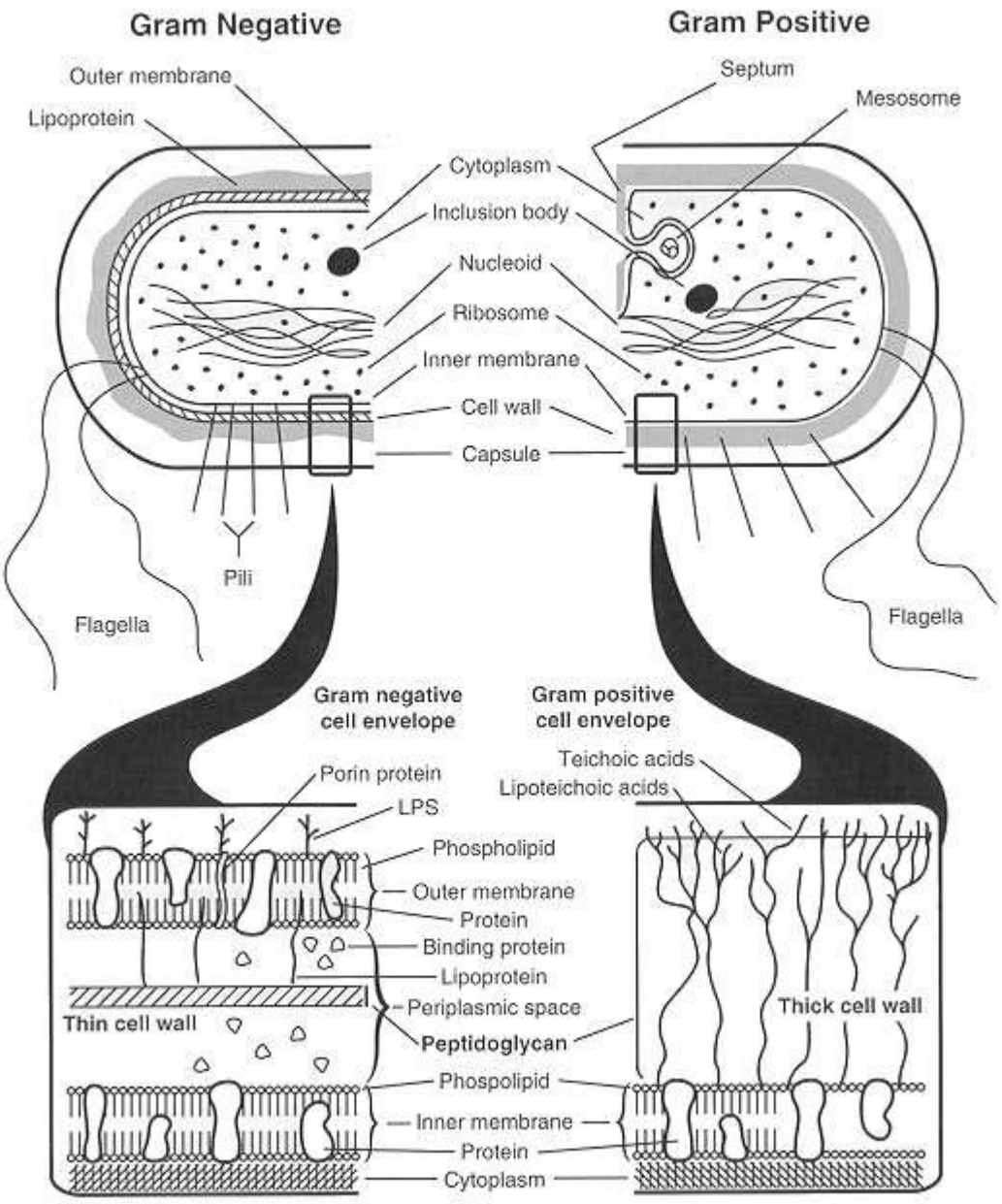


Figure 4. Comparison of the thick cell wall of Gram-positive bacteria with the comparatively thin cell wall of Gram-negative bacteria. Note the complexity of the Gram-negative cell envelope (outer membrane, its hydrophobic lipoprotein anchor; periplasmic space) (Baron S., et al., 2004).

I.5.3 Capsule and slime layer

Capsules or slime layers are produced by many bacteria to surround their cells with relatively thick layer of the viscous gel, and vary in structural complexity: ranging from a disorganised slime layer of extra-cellular polymer, to a highly structured capsule or glycocalyx. Capsules may be up to 10 μm thick and can protect cells from engulfment by eukaryotic cells, such as macrophages (Stokes R., et al, 2004). They can also act as

antigens and be involved in cell recognition, as well as aiding attachment to surfaces and the formation of biofilms (Daffe M., et al., 1999). Not all bacterial species produce capsules; however, the capsules of encapsulated pathogens are often important determinants of virulence. Encapsulated species are found among both Gram-positive and Gram-negative bacteria. In both groups, most capsules are composed of high molecular-weight viscous polysaccharides that are retained as a thick gel outside the cell wall or envelope. Cell viability is not affected when capsular polysaccharides are removed enzymatically from the cell surface. However capsules confer resistance to phagocytosis and hence provide the bacterial cell with protection against host defenses to invasion. (Baron S., et al., 2004).

I.5.4 Surface-layer and endospores

A surface-layer (S-layer) is a part of the cell envelope in the bacteria and it consists of a monomolecular layer composed of identical proteins or glycoprotein and enclosing the whole cell surface. S-layer proteins are poorly or not at all conserved and can differ even between related species. Depending on species S-layers have a thickness between 5-25 nm in diameter (Sleytr U., et al., 2007).

Depending on the type of the cell wall the S-layers are fixed differently. In Gram-negative bacteria S-layers are associated to the LPS via ionic, carbohydrate-carbohydrate, protein-carbohydrate interactions or/and protein-protein interactions. In the Gram-positive bacteria whose S-layers contain a surface layer homology domain the binding occurs to the peptidoglycan and to a secondary cell wall polymer.

The biological functions of the S-layer are protection against bacteriophages and phagocytosis, resistance against low pH, barrier against lytic enzymes, adhesion, stabilization of the membrane.

Some bacteria are able to adapt to stress and form endospores. The endospores are the bacterial survival structures which are resistant to many types of different chemical and environmental stresses

The assembly of these extracellular structures is dependent on bacterial secretion systems. These transfer proteins from the cytoplasm into the periplasm or into the environment around the cell. Many types of secretion systems are known and these structures are often essential for the virulence of pathogens, and, therefore, intensively studied.

Table 2 displays the structures of the bacterial cell envelope, it's functions and chemical constituents.

Structure	Primary functions	Chemical Constituents
Cytoplasmic membrane	Energy production, metabolite transport, synthesis of cell wall and capsule, support	Phospholipid bilayer, transport proteins, enzymes
Gram-positive cell wall		
Peptidoglycan	Osmotic stability, structural integrity, cell shape, permeable to antibiotics	Thick meshwork of peptide crosslinked polysaccharide chains
Teichoic and lipoteichoic acids	Adhesion to the host cells, weak endotoxin activity, antigenic	Polymers of substituted ribitol or glycerol phosphate
Proteins	Adhesion to the host cells, antyphagocytic, antygenic	
Gram-cell negativ cell wall		
Peptidoglycan	as in Gram-positive cell wall	Thinner version of that found in Gram-positive bacteria; linked to lipoproteins, that are anchored in outer membrane
Periplasmic space	Transport of nutrients, degradation of the macromolecules	Between cytoplasmic outer membranes; carrier proteins and hydrolytic enzymes
Outer membrane	Structural support, uptake of metabolites, permeability barrier, protection, antigenic	Phospholipid bilayer, porins, transport and other proteins, lipopolysaccharide
Lipopolisaccharide	Endotoxin activity, anticomplement activity	lipid A, core polysaccharide, O antigen
Porin channel	Allow small and hydrophilic molecules to pass outer membrane	Porin proteins

Capsule	Antiphagocytic	Layers of polysaccharides and polypeptides

Table 2. Bacterial envelope and associated structures. The bold marked structures lay on outer surface layer and have a direct contact with ficolins.

I.6 Some microbial organisms and their biology

I.6.1 Staphylococcus aureus

S. aureus belongs to the Gram-positive cocci and grows in grape-like clusters. The bacteria are between 0,8 – 1,2 μm in diameter. Major contributors to the virulence of *S. aureus* are the capsule, protein A, lipoteichoic and teichoic acids on the bacterial surface. Encapsulated bacteria are better protected from phagocytosis. Protein A inhibits complement fixation and opsonization and is also a part of antibody dependent cellular cytotoxity by binding to antibodies. Lipoteichoic and teichoic acids promote adherence to mucosal surfaces and persistence in tissues by binding to fibronectin.

The members of the genus *Staphylococcus* are Gram-positive cocci (0.5-1.5 μm) and contain unsaturated polyisoprenoid side chains.

S. aureus is catalase-positive and is able to convert H_20_2 to water and oxygen that reduces phagocytic killing. Coagulase helps localize infection by forming fibrin layer around abscesses.

S. aureus produces different toxins as leukocidins, enterotoxins A, B, C, D and E, exfoliative toxins A and B and toxic shock syndrome toxin 1. Toxic shock syndrome toxin 1 acts as super-antigen and can cause toxic shock syndrome and death. *S. aureus* is commonly present on skin, which can cause nosocomial wound infections. Among the 13 known serotypes, T-5 and T-8 account for approximately 75% of *S. aureus* infections (Kraup A. et al, 2005).

S. aureus usually causes a variety of diseases either by toxin production or invasion, such as erythema, food poisoning and abscess.

I.6.2 Streptococcus pneumonia

Str. pneumoniae, or *pneumococcus*, is the most common streptococcal pathogen in mammalians. *Str. pneumoniae* is Gram-positive, alpha-hemolytic diplococcus. These

bacteria grow in pairs or chains. The serotypes of *Str. pneumoniae* are divided in groups based on the serologic identification of group-specific C-carbohydrates on the cell-wall. *Str. pneumoniae* is catalase negative and can be encapsulated.

Its virulence is based on the polysaccharide capsule, pneumolysins, pneumococcal IgA protease and neuraminidase. The polysaccharide capsule prevents phagocytosis by host immune cells by inhibiting C3b opsonization of the pneumococcal cells. Pneumolysins lyse blood cells and platelets and stimulate release of lysosomal enzymes. Pneumococcal IgA protease cleaves secretory IgA and increases adherence to mucosal surfaces, while neuraminidase promotes bacterial spread into tissue**.**

Those bacteria usually cause the variety of diseases of the respiratory system, inflammations of the upper skin layers and mucous membranes.

I.6.3 Escherichia coli

E. coli is facultative anaerobe, Gram-negative strain, permitting survival in the gastrointestinal tract of mammalians. All strains produce endotoxin that is responsible for many of the systemic manifestations of infection such as high fever, hypotension, shock or disseminated intracellular coagulation or urinary tract infections**.**

I.6.4 Candida albicans

Candida albicans is part of the normal flora in mucous membranes. *C. albicans* represents a diploid dimorphic filamentous fungus, that is composed of a mass of branching threadlike tubular filaments (hyphae), that elongate at their tips.

Fungi are eukaryotic organisms whose cells possess a membrane-enclosed nucleus and various organelles. Fungal membranes contain ergosterol rather than cholesterol found in other eukaryotic membranes. The cell wall surrounding fungal cells, which differs in composition from bacterial cell walls, contains chitin, glucans, and protein.

II. Materials and Methods

II.1 Materials

II.1.1 Chemicals, solutions and media

Ampicillin, >98%	Sigma-Aldrich
all other chemicals and solutions of analytical grade	Sigma-Aldrich or Merck
Chelating Sepharose Fast Flow	GE Healthcare
Coomassie Brilliant Blue R250	Fluka
H_2O (deionized)	Milli Q UF Plus system
Heparin	Sigma-Aldrich
Hygromycin-B	Invitrogen
Insekt Express Medium	Cambrex
Kanamycin	Invitrogen
Methanol, technical grade	Merck
Nowa Solution A+B (ECL)	MoBiTec
SDS-PAGE Molecular weight standard, broad range	Biorad
TEMED, Tetramethylethylendiamin	Biorad
Triton X-100	GE Healthcare
Tween 20	Fluka

Table 3. Chemicals, solutions and media

II.1.2 Kits

BCA™ Protein Assay Kit	Pierce

II.1.3 Bacterial strains

Short name of a bacterial strain	Whole name of the bacterial strain
Sa1	Staphylococcus aureus T-1
Sa2	Staphylococcus aureus T-2
Sa3	*Staphylococcus aureus* T-3
Sa4	*Staphylococcus aureus* T-4
Sa5	*Staphylococcus aureus* T-5
Sa6	*Staphylococcus aureus* T-6
Sa7	*Staphylococcus aureus* T-7
Sa8	*Staphylococcus aureus* T-8
Sa9	*Staphylococcus aureus* T-9
Sa10	*Staphylococcus aureus* T-10
Sa11	*Staphylococcus aureus* T-11
Sa12	*Staphylococcus aureus* T-12
Sa Wood	*Staphylococcus aureus* Wood
Sp1	*Streptococcus pneumonia* 14
Sp2	*Streptococcus pneumonia* SCR
Sp3	*Streptococcus pneumonia* 7A
Sp4	*Streptococcus pneumonia* 27
Sp5	*Streptococcus pneumonia* 6A
Sp6	*Streptococcus pneumonia* TIGR4
Sp7	*Streptococcus pneumonia* 9L
Sp8	*Streptococcus pneumonia* 6B
Sp9	*Streptococcus pneumonia* 19C
Sp10	*Streptococcus pneumonia* 19F
Sp11	*Streptococcus pneumonia* 32F
Sp12	*Streptococcus pneumonia* 23F
Sp13	*Streptococcus pneumonia* 7F
Sp15	*Streptococcus pneumonia* 11F
Sp16	*Streptococcus pneumonia* 1
Sp18	*Streptococcus pneumonia* 9V

Sp22	*Streptococcus pneumonia* 11D
E. coli	*Escherichia coli*
Ca	*Candida albicans*

Table 4. Bacterial strains

II.1.4 Proteins

To study the binding affinities of murine ficolins to microorganisms, mouse recombinant ficolin A and B were expressed in Drosophila Schneider 2 cell line, purified by ion metal affinity chtomatography and stored frozen in a concentration of 1 – 1,6 mg/ml.

II.1.5 Eukaryotic cell lines

Drosophila Schneider 2 (S2) cell line: (Invitrogen) derived from a primary culture of late stage (20-24 hours old) *Drosophila melanogaster* embryos (Schneider, 1972). The S2 cell line and the DES® system are used specially for the high yield expression of heterologous proteins which are secreted into the culture medium, thus avoiding cell lysis steps and facilitating the purification of the recombinant protein from the cell supernatant. S2 cells were grown as semi-adherent monolayers at 28°C without CO_2 supply in insect media (Insect X-press, Cambrex) containing 100 mg/l kanamycin, and were regularly split at a 1:2 to 1:5 ratio when they were 90-100% confluent.

II.1.6 Buffers and mediums

TBS/Tween/Ca^{2+}-buffer:

1L of buffer contains 25 mM of Tris Base, 140 mM of NaCl and 2 mM KCl and 0.05% of Tween 20 and 5 mM CaCl, pH was adjusted to 7,4. The buffer was stored at the 4˚C.

TBS/Tween/EDTA-buffer:

1L of buffer contains 10mM EDTA, 25mM Tris Base and 0,005% of Tween20. pH was adjusted to 7,4. The buffer was stored at the 4˚C.

TBS/Tween/NaCl-buffer:

1L of buffer contains 2M NaCl, 25mM Tris Base and 0,005% of Tween20. pH was adjusted to 7,4. The buffer was stored at the 4˚C.

PBS:

1L of buffer contains 137 mM NaCl, 2.7 mM KCl, 2 mM K_2HPO_4 and 10 mM Na_2HPO_4. pH was adjusted to 7.4. The buffer was stored at room temperature.

4 x Laemli buffer:

1L of buffer contains 120 mM Tris Base, 0.95 M glycine and 0.5% SDS.

Basic Medium:

1 L of basic medium contains 10 g of casein hydrolysate (Peptone), 5 g of yeast extract, 5 g of NaCl, 1 g of Glucose, 1g of $K_2HPO_4 \cdot 3H_2O$. pH was adjusted to 7,2.

II.1.7 Software and databases

Peptidoglycan structures of bacteria (at molecular level) were obtained from PubMed publications and databases. Screening analysis of bacterial and ficolin binding was performed using the BD FACSDiva™ software (2006, flow cytometry acquisition and analysis software) and WinMDI Software (Version 2.9). The curves for competitive assays were plotted with Microsoft Excel 2002 for Windows XP.

II.2 Methods

II.2.1 Cell culture techniques

II.2.1.1 Culture of Drosophila Schneider-2 (S2) cells in the mini PERM Bioreactor (Greiner bio-one)

Ficolin-A/-B-transfected DS-2 cells (Runza, V., "Cloning and Characterization of Mouse Ficolins-A and -B", doctoral thesis, 2006) were used to produce recombinant ficolin A and B. Cells were grown in suspension in a mini bioreactor containing with with 10 µg/ml of heparin in the production module to avoid cell adherence or clumping.

3 x 10^6 cells/ml in 40 ml total volume (20ml fresh medium + 20ml conditioned medium) were inoculated into the production module. The nutrition module was filled with 400 ml

complete medium. Cells were cultivated at room temperature at a turning speed of 5 rpm. The growing medium was changed once a week.

II.2.1.2 Induction of protein expression

Cells were harvested every seventh day after inoculation, by removing 2 x 10 ml cell suspension from the production module. Each 10 ml were poured into a 50 ml tube and topped up with fresh medium. To induce ficolin expression the cell suspension was supplemented with $CuSO_4$ (at a final concentration of 500 μM). The cells were incubated under rotation for 3 days. Afterwards, cells were spun by 3000 *g* for 10 min and the supernatant collected and purified by ion-metal affinity chromatography (see sec. II.II.2.2.1).
To check for a positive protein expression, supernatant aliquots were taken 2-3 days after induction and analysed by Western Blot.

II.2.2 Protein-biochemical techniques

II.2.2.1 Purification of recombinant ficolins by ion-metal affinity chromatography

Due to the features of the pMT/BiP/V5-His A expression vector in which the ficolin genes were cloned (Runza, V., 2005: Cloning and Charakterization of Mouse Ficolins A and B, doctoral Thesis), the recombinant ficolins (rfcn) were fused to a C-terminal V5- and His- tags, and secreted into the culture medium, enabling the (i) purification of the protein from the insect medium by His-tag specific ion-metal affinity chromatography (IMAC) and (ii) the detection by immunoblotting with an anti-V5 antibody.
In addition to rapid, one-step purification, IMAC also offers the advantage of high capacity. However, one limitation of standard IMAC methods is the inability to purify His-tagged proteins directly from a source containing free metal ions, which interfere with the binding of the protein to immobilized metal-ion resins such as Cu^{2+}. This is the case in the copper-inducible Drosophila S2 system where the recombinant protein accumulates in the conditioned medium which still contains free copper ions or, even worse, some copper remains bound to the His-tag, resulting in a low yield of purified protein.

One method that overcomes with this disadvantage is the use of the Chelating Sepharose Fast Flow resin (GE Healthcare). This resin consists of iminodiacetic groups coupled to sepharose able to form complexes with transition metal ions such as Cu^{2+}, therefore, selectively retaining proteins with exposed histidine residues present in the medium (Lehr et al., 2000).

Three days after induction, the conditioned medium was collected and cleared by centrifugation at 3000xg for 10 minutes at 4°C. Binding to the resin was performed batchwise (1 ml resin/L medium, enough to bind approximately 5 mg His-tagged protein) overnight at 4°C under rotation. The resin-Cu^{2+}-protein slurry was then poured into a column and attached to the BioRad Econo System device (BioRad) to facilitate the forthcoming steps. Washing was performed sequentially at a rate of 0.5 ml/min with PBS until baseline UV absorbance monitored at 280 nm and then again with 10 mM imidazole in 0.5 M/PBS to remove non-specifically bound proteins. Competitive elution of the desired protein was carried out with 250 mM imidazole in 50 mM Tris pH 8.0. Elution fractions were collected in 0.5 ml aliquots and analysed by SDS-PAGE (see section II.2.2.2). Finally, the column was stripped with 20 mM EDTA in PBS to remove any metal bound to the resin and re-equilibrated with 50 vol of deionized water.

Positive elution fractions were pooled, dialysed overnight against PBS at 4°C and stored in aliquots at -20°C. Freezing/thawing cycles were always avoided. Protein concentration of the samples was assessed by a modified Lowry method (see section II.2.2.4)

II.2.2.2 SDS-PAGE

Protein purification and characterization was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The strongly anionic detergent SDS is used in combination with a reducing agent (e.g. β-mercaptoethanol) and heat to dissociate the proteins before they are loaded onto the gel. The denatured polypeptides bind SDS and all become negatively charged in a sequence-independent fashion, thus allowing the proteins to migrate according to their size. Therefore, by using markers of known molecular weight, it is possible to estimate the molecular size of the polypeptide of interest.

The most common SDS-PAGE is carried out with a discontinuous buffer system (Ornstein and Davis, 1964) in which the buffer in the reservoir is of a pH and ionic strength different from that of the buffer used to cast the gel, and all the components of

the system contain 0,1% SDS (Laemmli, 1970). The SDS-polypeptide complexes are swept along by a moving boundary created when an electric current is passed between the electrodes. After migrating through a "stacking gel" of high porosity, the complexes are deposited in a very thin zone on the surface of the "resolving gel", through which they will be resolved according to their size.

In this work, 30 µl of each sample were diluted in 2x SDS gel loading buffer (with or without a reducing agent) and denatured by heating at 95°C for 5 minutes before being loaded onto a polyacrylamide (PAA) gel. Gels were run in Laemmli buffer at 25 mA in the stacking gel and 45 mA in the resolving gel.

	Stacking (5%)	Resolving (12,5%)
Rotiphorese Gel 30 (30% Acrylamide, 0.8% Bisacrylamide)	0.85 ml	6.25 ml
1.5 M Tris buffer pH 8.8	---	3.75 ml
0.5 M Tris buffer pH 6.8	1.5 ml	---
Deionized water	3.75 ml	5 ml
10% SDS	60 µl	150 µl
N,N,N',N'-Tetramethylethylendiamin (TEMEI	5 µl	10 µl
10% Ammoniumpersulphate (APS)	50 µl	100 µl

Table 5. Composition of a 12.5% PAA-gel

II.2.2.3 Coomassie staining and drying of PAA-gels

In order to visualise protein bands on the polyacrylamide gel or a Western Blot membrane, they were stained with coomassie blue. For this, membranes or gels were soaked in the staining solution for some minutes and further decoloured in destaining solution until the background was clear enough and the protein bands sharp visible. For long term storage of the gels, they were intensively washed with water and dried in the BIO RAD SLAB DRYER (Model 483).

II.2.2.4 Determination of protein concentration

The amount of protein in the elution fractions was measured with the BCA™ Protein Assay Kit (Pierce). This is a colorimetric assay for protein concentration following detergent solubilization. As with the known Lowry assay, there are two steps which lead to colour development: the reaction of the protein and copper in an alkaline medium and

the subsequent reduction of Cu^{2+} to Cu^{1+} reagent by the copper-treated protein. Colour development is primarily due to the amino acids tyrosine and tryptophan. Proteins induce a reduction of reagent by containing bicinchonicic acid (BCA). The purple-coloured reaction product of this assay is a formed by chelation of two molecules of BCA with the cuprous ion. This water soluble complex exhibits a strong absorbance at 562 nm, respectively.
Ficolin concentration was determined according to the manufacture's instructions.

II.2.3 Labelling of Ficolins

Recombinant ficolins used for the bacterial screening were directly labelled either with biotin or with Cy5 dye.

II.2.3.1 Biotinylation

The biotinylation of recombinant ficolins was performed according to the manufacture's instructions of the Pierce EZ-Link® NHS-PEO Solid Phase Biotinylation Kit.
Briefly, this method uses SwellGel® Nickel Chelated Disks composed of a dehydrated nickel-chelated agarose resin to first immobilize purified ficolins. The proteins are then biotinylated by adding a solution of NHS-PEO_4-Biotin. Excess biotin is washed from the column, and the ficolins are eluted in the buffered imidazole solution. NHS-PEO_4-Biotinreacts with primary amines, primary ε-amine groups on available lysine residues. Afterwards SwellGel® Disk must be placed in the bottom of a 1.5 ml microcentrifuge tube and Ficolin A or B Binding Solution must be added to the tube. After 30 min of incubation the column was centrifuged and the pellet was washed three times with 1 ml PBS. Other 0.3 ml of PBS was added to the pellet and the resin was resuspended gently. The entire volume was pipetted into resin column. After the centrifugation of the column the flow-through was discarded, the column was plugged from the bottom. The contents of a One No-Weigh™NHS-PEO_4-Biotin Microtube was diluted in 0.2 ml of PBS and added to the amount of ficolin directly to the column. The biotinylation reaction was incubated for 30 min. After the column was washed with 0.4 ml of PBS three times, Elution Buffer was added to elute the bound biotinylated ficolins from the column. The biotinylated ficolins were stored at 4°C.

II.2.3.2 Cy5-labelling

The Cy5 Ab Labelling Kit (Amersham Biosciences) was used according to the manufacturing instructions. Cy5 is a cyanine reagent and has been shown to be useful as a fluorescent label, which produces an intense signal in the far red region of the spectrum. 1 mg of purified recombinant ficolins was dissolved at 1mg/ml in 50mM of PBS and mixed with coupling buffer and tranfered to the vial of reactive dye. The reaction was incubated at the room temperature for 30 minutes with additional mixing every 10 minutes. The ficolin-labelling mixture then was transferred to the top of a mini-spin column and allowed to enter the packet. The addition of 2 ml of elution buffer allowed a faster moving blue band of labelled ficolin to be separated from the free dye. Labelled ficolins were collected in clean tubes and stored at 4˚C avoiding direct light contact.

II.2.4 Bacteriological procedures

II.2.4.1 Fixation of bacteria

The bacterial strains used for the binding screening were kindly provided by Dr. Stefen Thiel co-workers (University of Aarhus, Denmark). *S. pneumoniae* serotypes 1, 4, 14, 6A, 6B, 7A, 7F, 9L, 9V, 11A, 11B, 11C, 11D, 11F, 19C, 19F, 23F, 27, 32F, and 45 and the non-capsulated variant strain SCR2 (Statens Serum Institut, Copenhagen, Denmark) were grown in Todd-Hewitt broth medium (Oxoid, Basingstoke, England) overnight at 37°C in 5% CO_2. *S. aureus* serotypes 1 to 13 (T1 to T13) and the non-capsulated variant strain Wood (National Institutes of Health, Bethesda, Md.) were cultured on Columbia agar plates (Difco, Kansas City, Kans) supplemented with 1% (wt/vol) yeast extract and 0.1% (wt/vol) glucose at 37°C overnight to ensure maximum production of capsules (4, 9, 16, 29). *E. coli* was grown in Luria-Bertani broth (Q-Biogene, Carlsbad, Calif.) overnight at 37°C. In order to fix the cells formaldehyde (Sigma-Aldrich, St. Louis, Mo.) was added to the broth cultures to a final concentration of 1% (wt/vol), and the cultures were kept at room temperature until the next day. This treatment stabilizes the cells but does not alter the polysaccharide antigens. *S. aureus* organisms were washed off the agar plates, resuspended in 5 ml of PBS, and fixed with formaldehyde as described above. Residual reactive aldehyde groups were blocked by incubation with a 1/10 volume of 1M ethanolamine (pH 9.0) for 1 hour. The bacterial cells were then washed three times with TBS and stored at 4°C. The bacterial concentration (BC) was estimated by reading the

optical density (OD) at 600 nm (Eppendorf Bio Photometer 6131) and considering that an optical density of 1.0 corresponds to approximately 1.8 $\times 10^9$ bacteria/ml.
$BC = OD \times DF \times 1.8x10^8$ *CFU/mL*.

II.2.4.2 Bacterial screening for ficolin A and B binding by flow cytometry

Stabilized bacterial cells (1.5×10^8) were incubated with 6 µl of biotinylated or Cy5 conjugated ficolin in a total volume of 400 µl of TBS/Tw/Ca (see section II.1.6) for 2 h at room temperature with end-over-end rotation.
Samples were centrifuged, and the pellets were washed three times with 1 ml of TBS/Tw/Ca, resuspended in the same buffer. Whenever biotinylated ficolins were used, the cells were then incubated at room temperature for 1 hour with 6 µg of fluorescein isothiocyanate (FITC)-labeled streptavidin. Bacterial cells were washed three times, resuspended in 200 µl of TBS/Tw/Ca, and subtected to flow cytometry using a FACS LSRII flow cytometer (BD Biosciences, San Jose, California).

II.2.4.3 Competitive assay

II.2.4.3.1 Competitive assay between labelled and unlabelled ficolins

In order to confirm that the screening results were not due to artefact and that the bacteria-ficolin binding was specific, competitive assays with unlabeled ficolins were performed.
For this the bacterial strains which showed positive results in the binding screening were pre-incubated with unlabeled ficolin A or B for two hours at room temperature and intensively washed before being subjected to the binding screening as described above (see section II.2.4.2) and analysed by flow cytometry.

II.2.4.3.2 Competitive assays between ficolin A and B

In order to test if ficolin A and B compete for binding to the same cell, competitive assays with both mouse ficolins were performed. For this the bacterial strains *Str. pneumoniae* serotype 23F and serotype 1were pre-incubated with unlabelled 1) ficolin A or 2) ficolin B for two hours at room temperature. After intensive washing the cells were

further subjected to the binding screening procedure (see section II.2.4.2) with either labelled 1) ficolin B or 2) ficolin A, and analysed by flow cytometry.

II.2.4.4 Calcium and Sodium requirement

In order to test if the binding of mouse ficolins to bacterial strains requires the presence of calcium ions to stabilize the complexes, the binding screening was repeated with the staphylococcal strain (serotype 5) and streptococcal strains (serotype 21) under different salt conditions. Cells were incubated with labelled ficolins either in the presence or absence (EDTA-TBS) of calcium and increasing concentrations of sodium chloride (NaCl) raging from 31,25 mM to 1M.

III. Results

Ficolin-A and -B are pattern recognition molecules of the mouse innate immune system. Assuming that these recognition molecules bind to different microorganisms, we compared the reactivities of ficolin-A and -B with the opportunistic mammalian pathogens *Staphylococcus aureus*, *Streptococcus pneumoniae* as well as with strains of *Escherichia coli* and with the fungus *Candida albicans*. By testing different strains we investigated the ability of ficolins to bind to capsulated and non-capsulated bacterial cells. The various bacterial serotypes were incubated with purified biotinylated ficolin-A or -B followed by FITC-labeled streptavidin. Screening by flow cytometry of *S. aureus, Str. pneumoniae, E. coli* and *Candida albicans* revealed an overlapping but not identical binding of ficolin-A and -B.

In this work the binding affinity of a ficolin-A or -B to a bacterial strain less than 20% is considered as negative. Figures show the binding affinities of ficolins. "Not-binding" means that the flow cytometry of the the ficolin after incubation with bacteria is similar to the autofluorescence curve or shifted to the left. "Binding" means that the flow cytometry of the the ficolin after incubation with bacteria is similar shifted to the right from the autofluorescence curve. The more is the shift to the right the stronger is the binding.

III.1 Staphylococcus aureus

We investigated the binding ability of murine ficolins to 13 *S. aureus* strains and detected that ficolin-A and -B bind to different strains of *S. aureus* with variable affinity. *S. aureus* serotypes 1 – 12 are capsulated strains, while *S. aureus* Wood is non-capsulated. Figures 5 – 17 show the results of the binding screening with *S. aureus*. Ficolin-A and -B bound to some *S. aureus* strains T4, T5, Wood. However, the most efficient binding was detected to the non-capsulated variant (Wood). But neither ficolin A nor ficolin B bound to the staphylococcal serotypes T6, T8, T9 and T10. Table 5 summarizes the results obtained from the screening.

These results indicate that mouse ficolins recognize structures present in both capsulated and non-capsulated bacteria, suggesting that ficolins bind either to molecule present in both serotypes or that they recognize different antigens on these strains. The observed

binding to strain Wood may be caused by peptidoglycan in the staphylococcal cell wall consisting of alternating 1,4-beta-linked subunits of GlcNAc and N-acetylmuramic acid.

S. aureus serotype or strain	Binding (%)*	
	ficolin-A, %	ficolin-B, %
T-1	30	38
T-2	15	30
T-3	39	35
T-4	80	72
T-5	60,5	71
T-6	**8**	**5**
T-7	28	30
T-8	**13**	**8.9**
T-9	**8**	**15**
T-10	**10**	**9**
T-11	20	10
T-12	20	30
Wood	74.5	77

Table 6. Binding of ficolin-A and –B to different *S. aureus* strains. (*) Binding percentage of capsulated as non-capsulated bacterial cells.

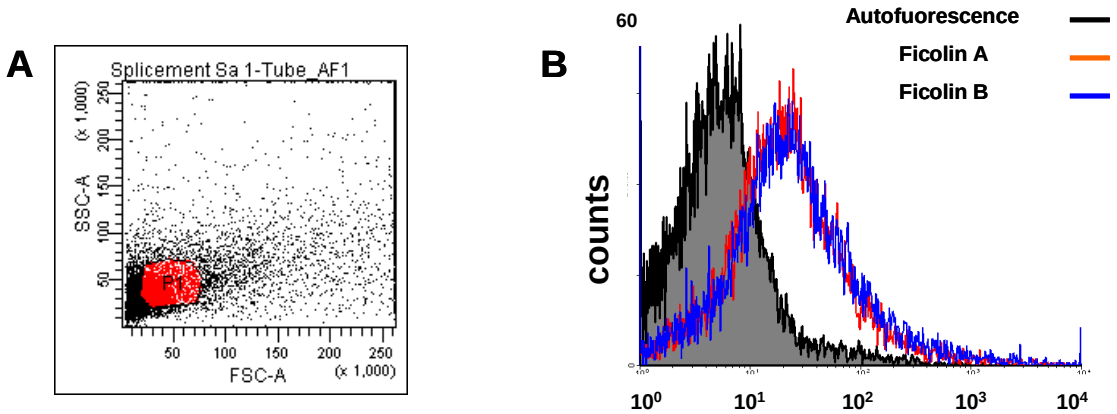


Figure 6. Both ficolin A and B bind to *S. aureus* serotype T-1 with the same affinity. **A)** Dot plot or the gated population of *S. aureus* serotype T-1 and **B)** Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

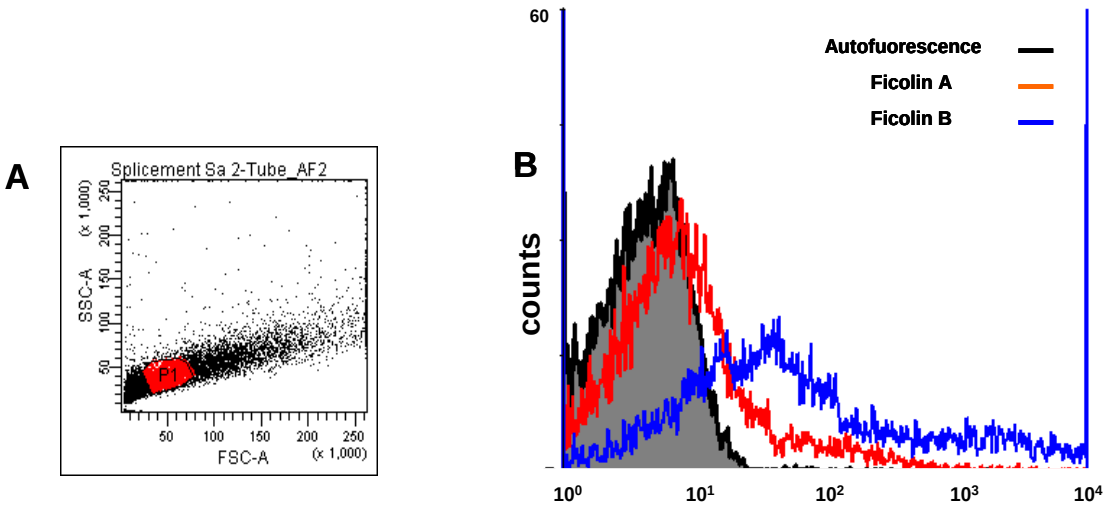


Figure 5. Ficolin B but not ficolin A binds to *S. aureus* serotype T-2. Dot plot or the gated population of *S. aureus* serotype T-2 and B) Binding of ficolin A and ficolin B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

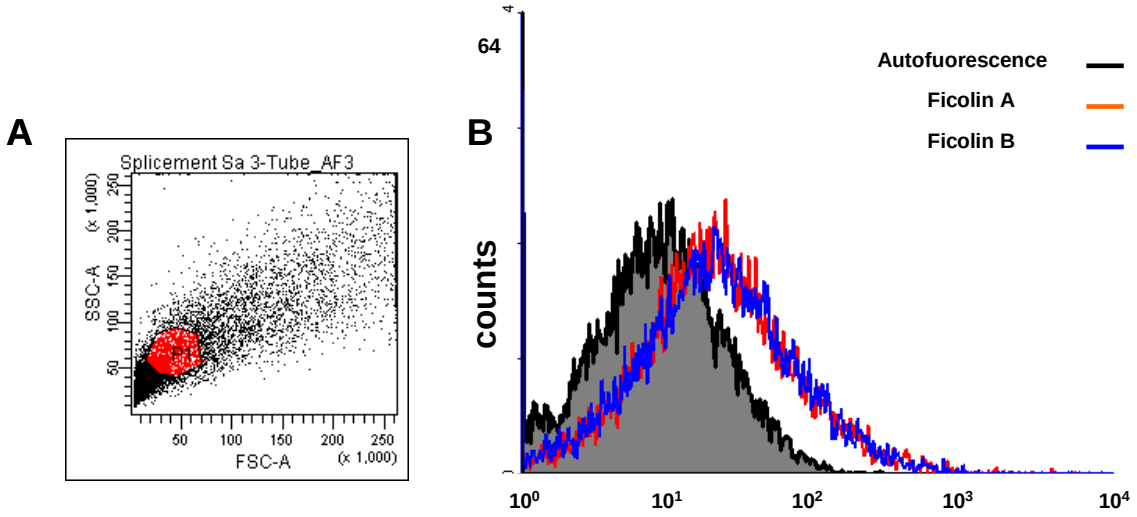


Figure 6. Both ficolins A or B show minimal binding to *S. aureus* serotype T 3. Dot plot or the gated population of *S. aureus* serotype T-3 and B) Binding of ficolin A and ficolin B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

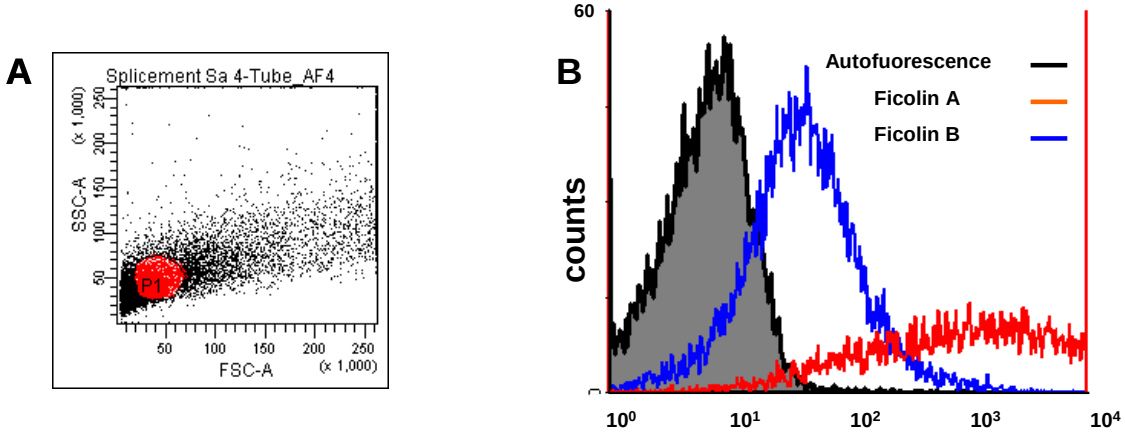


Figure 7. Ficolins A or B bind to *S. aureus* serotype T-4 with different affinity. While ficolin B shows almost total recognition (96%), ficolin A binding was achieved only to a certain extent (15%). Ficolin B does not bind equally to *S. aureus* serotype 4. Ficolin A (15%) binds with a lower intensity than ficolin B (96%). Dot plot or the gated population of *S. aureus* serotype T-4 and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

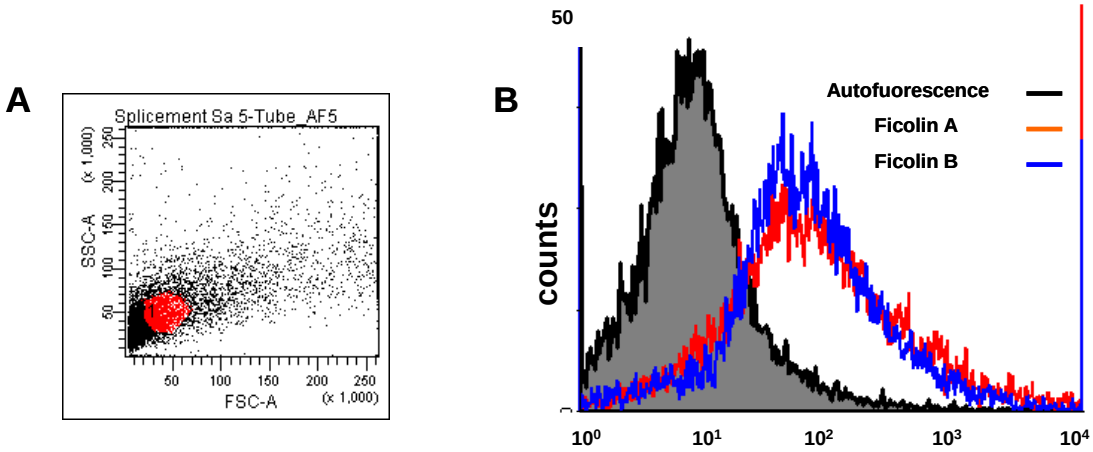


Figure 8. Both ficolin A and B bind to *S. aureus* serotype T-5 with same affinity. A) Dot plot or the gated population of *S. aureus* serotype T-5 and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

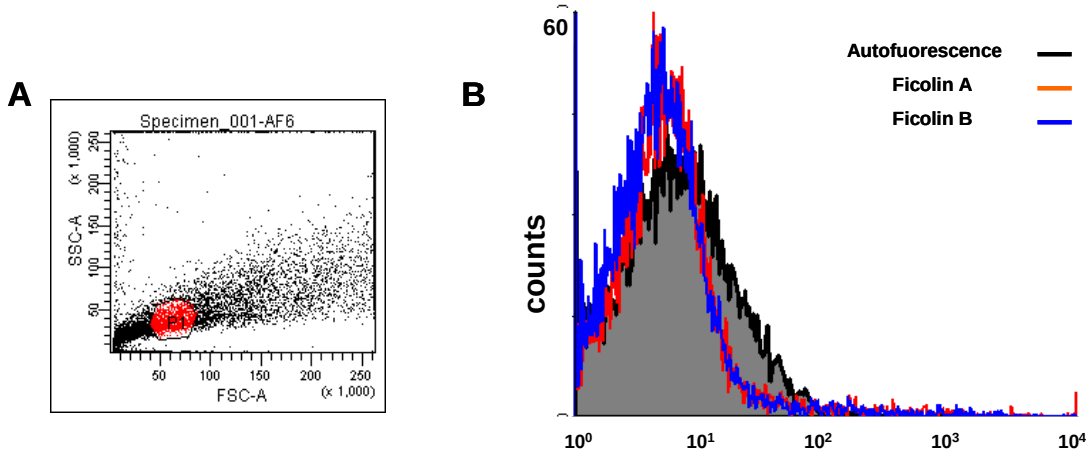


Figure 9. Neither ficolin A nor ficolin B bind to *S. aureus* serotype T-6. Dot plot or the gated population of *S. aureus* serotype T-6 and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

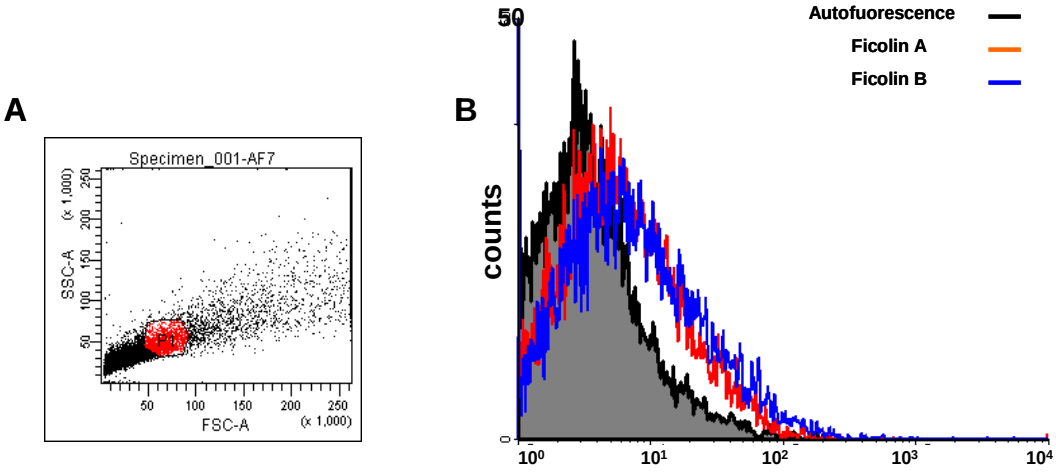


Figure 10. Neither ficolin A nor ficolin B bind to *S. aureus* serotype T-7. Dot plot or the gated population of *S. aureus* serotype T-7 and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

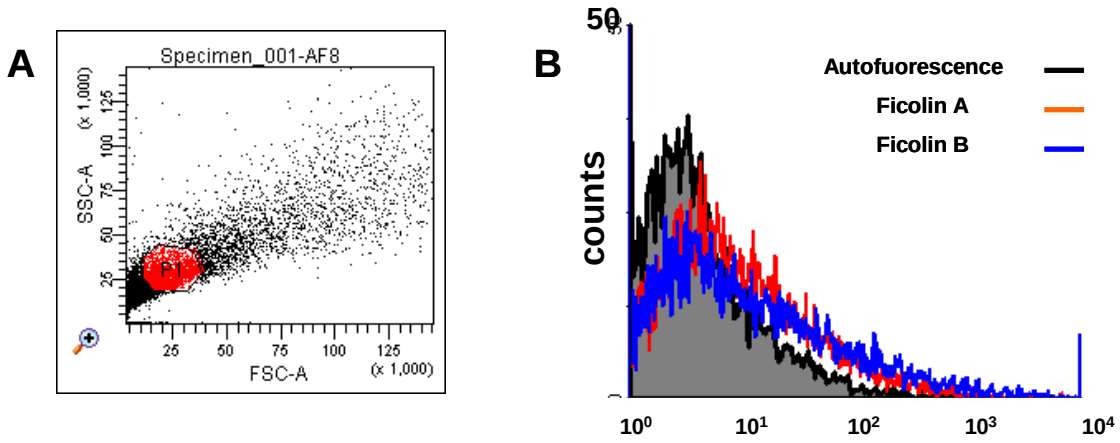


Figure 11. Neither ficolin A nor ficolin B bind to *S. aureus* serotype T-8. Dot plot or the gated population of *S. aureus* serotype T-8 and **B)** Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

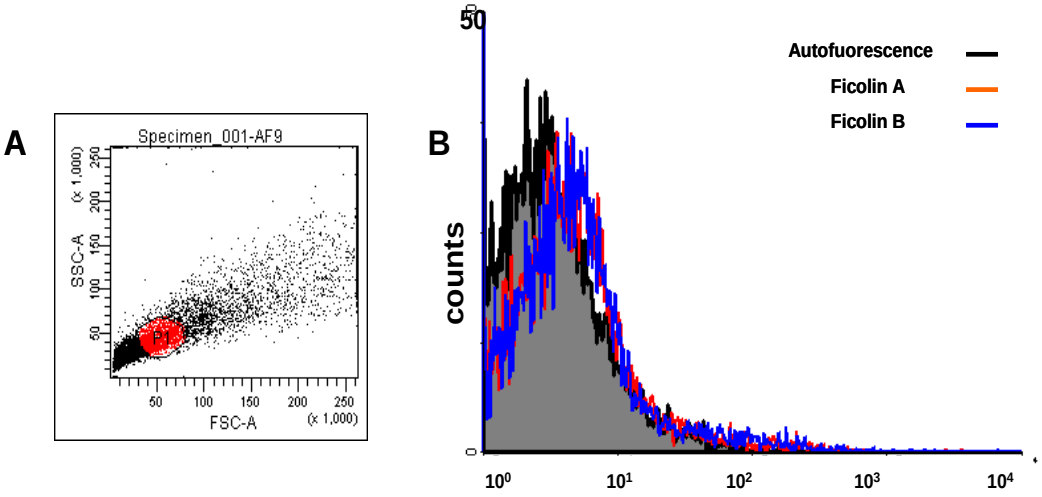


Figure 12. Neither ficolin A nor ficolin B bind to *S. aureus* serotype T-9. Dot plot or the gated population of *S. aureus* serotype T-9 and **B)** Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

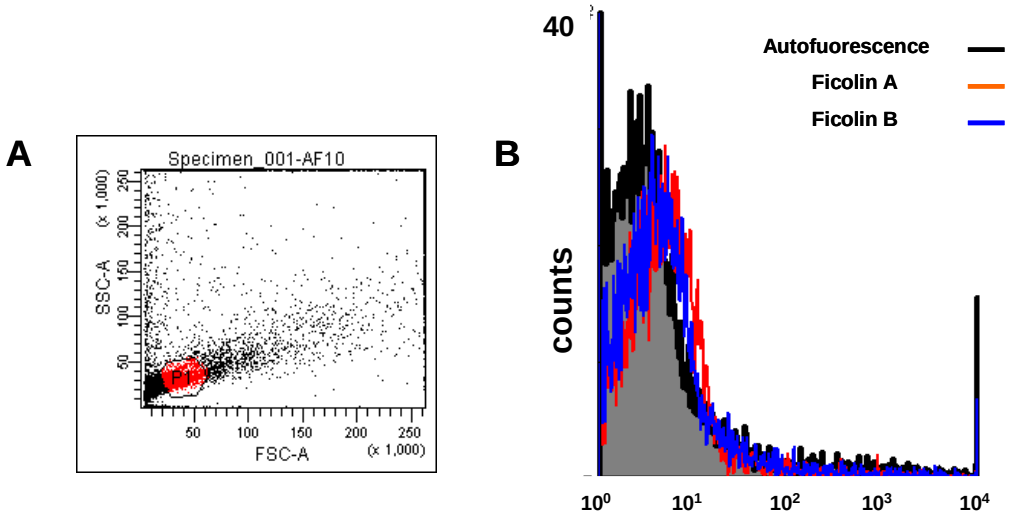


Figure 13. Neither ficolin A nor ficolin B bind to *S. aureus* serotype T-10. Dot plot or the gated population of *S. aureus* serotype T-10 and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

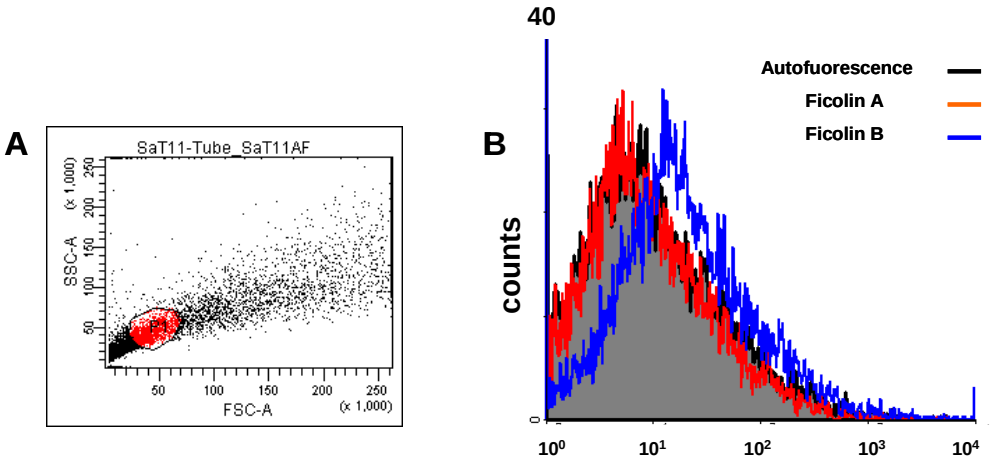


Figure 14. Neither ficolin A nor ficolin B bind to *S. aureus* serotype T-11. Dot plot or the gated population of *S. aureus* serotype T-11 and **B)** Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

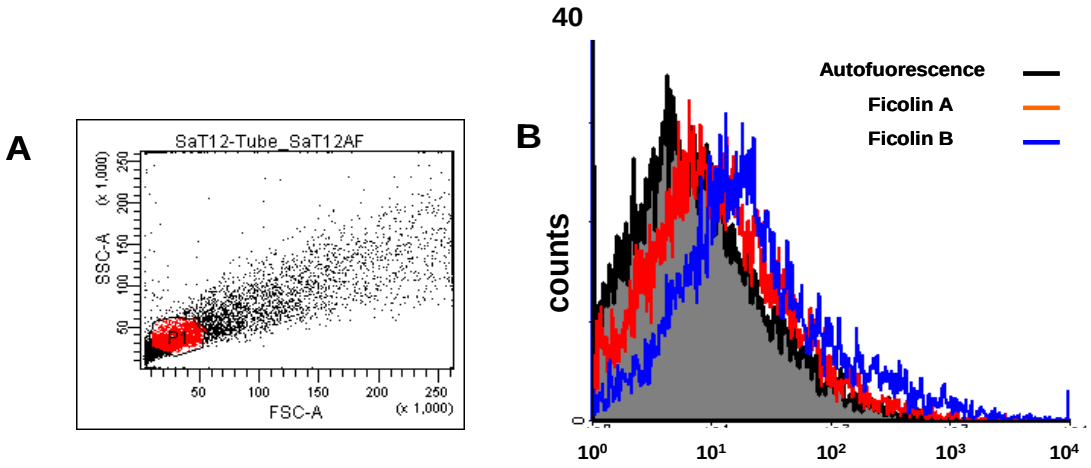


Figure 15. Neither ficolin A nor ficolin B bind to *S. aureus* serotype T-12. Dot plot or the gated population of *S. aureus* serotype T-12 and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

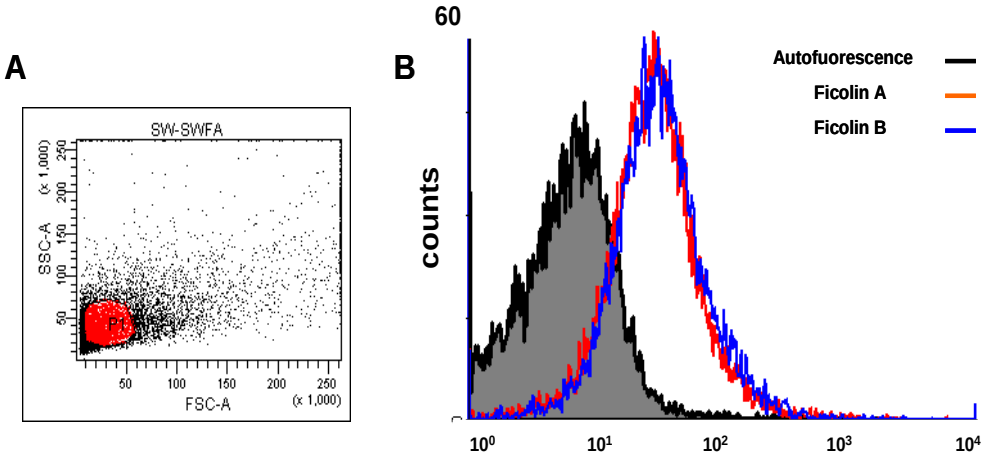


Figure 16. Both ficolin A and B bind to *S. aureus* serotype Wood with the same affinity. Dot plot or the gated population of *S. aureus* serotype Wood and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

III.2 Streptococcus pneumonia

We tested the binding ability of murine ficolins to 18 *Str. pneumoniae* strains and found that ficolin-A and -B bind to different strains with variable affinity.

Figures 18 – 34 show the results of the binding screening with *Str. pneumoniae*. Ficolin A bound to *Str. pneumoniae* strains SCR2, 7A, 27, 1, 11D with affinities over 70%. The most efficient binding of ficolin B (over 70%) was detected to *Str. pneumoniae* strains

14, SCR2, 27, 6A, 1, 11F, 11D. Very low binding was observed by the strains 45, 9VL, and 19C. Table 2 summarizes the results obtained from the screening.

Str. pneumoniae serotype	% of binding*	
	Ficolin-A	Ficolin-B
SCR2	75	73
TIGR4	13	14
1	85.5	81
6A	56	70.5
6B	53	53
7A	79	20
7F	44	40
9F	**2.5**	**15**
9L	**18**	**19**
9V	**17**	**13**
11D	77	78
11F	30	76
14	69	72
19C	**8**	**6**
23F	55	50
27	81	82
32F	43	11
45	**Neg.**	**12.3**

Table 7. Binding of ficolin-A and –B to different *Str. pneumoniae* strains. (*) Binding percentage of capsulated as non-capsulated bacterial cells.

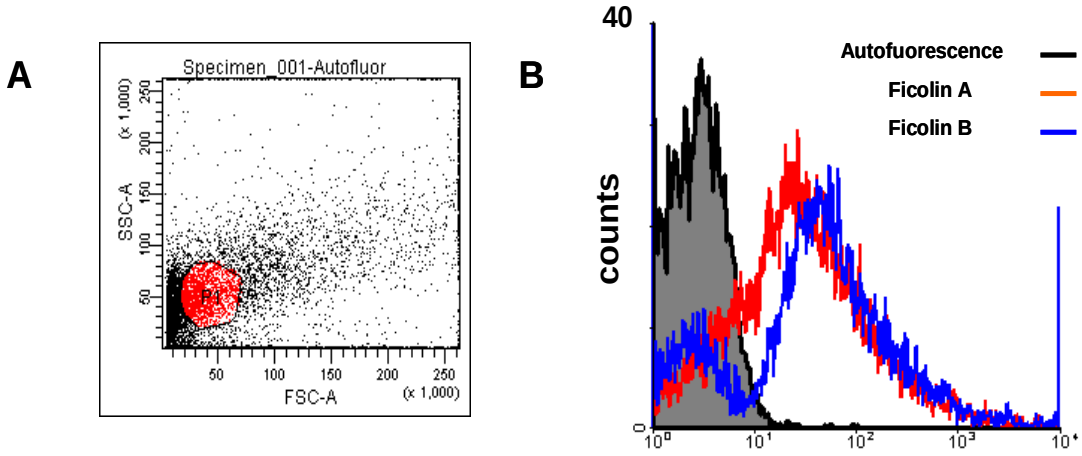


Figure 17. Both ficolin A and B bind to *Str. pneumoniae* serotype 14 with comparable affinity. A) Dot plot and gated population of *Str. pneumoniae* serotype 14. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

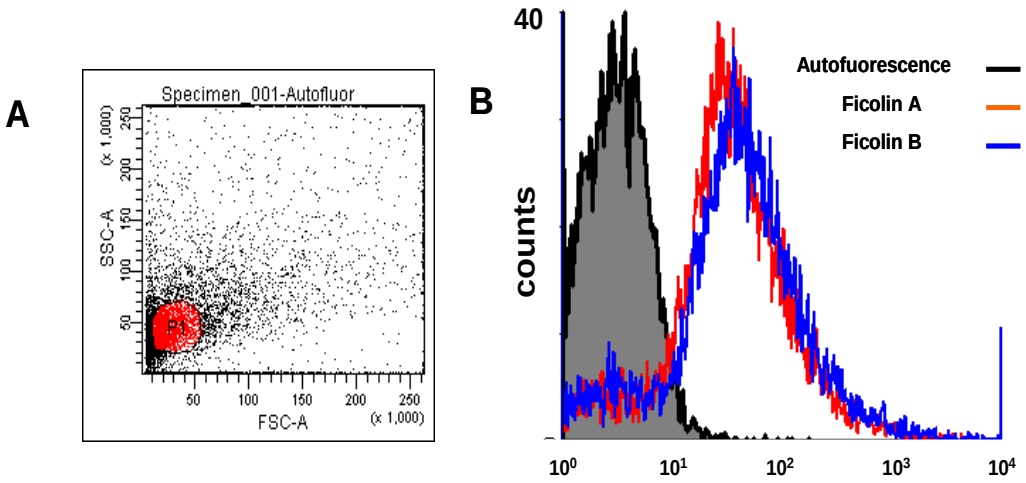


Figure 18. Both ficolin A and B bind to *Str. pneumoniae* serotype SCR2 with same affinity. A) Dot plot and the gated population of *Str. pneumoniae* serotype. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

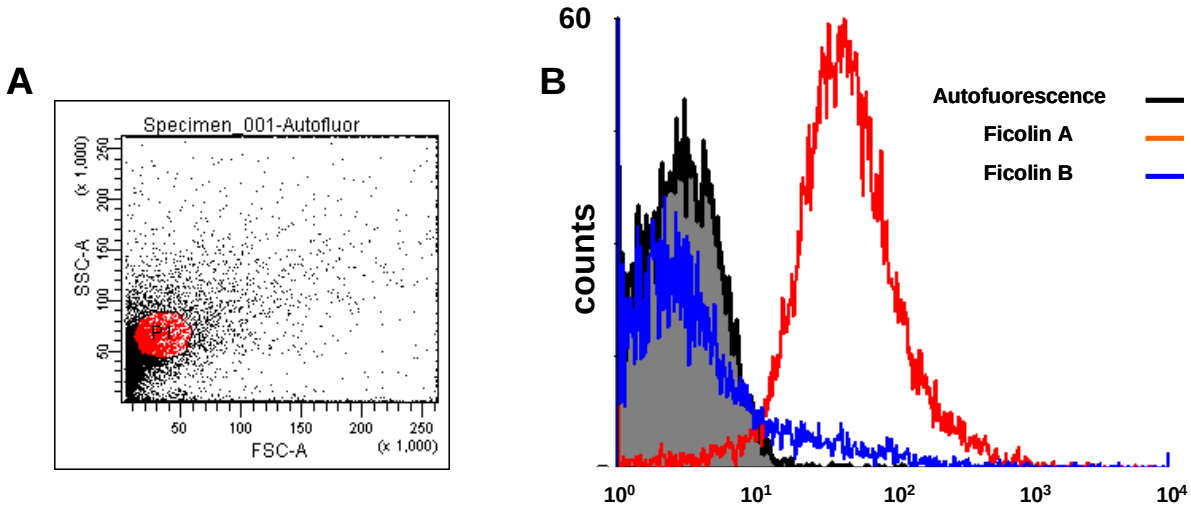


Figure 19. Ficolin A but not ficolin B bind to *Str. pneumoniae* serotype 7a. While ficolin A shows high affinity (80%) to *Str. pneumoniae* serotype 7a, ficolin B binding was not achieved to signicant extent (less than 20%). A) Dot blot and gated population of *Str. pneumoniae* serotype 7. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

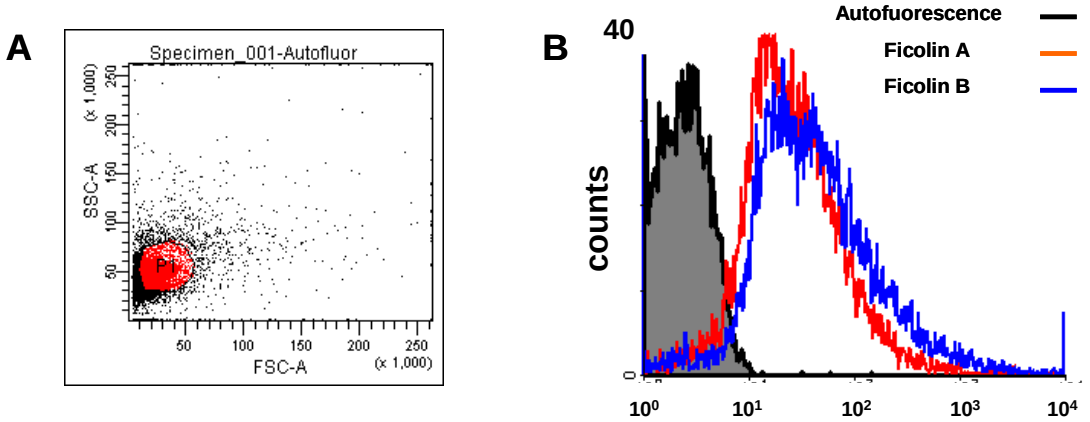


Figure 20. Both ficolin A and B bind to *Str. pneumoniae* serotype 27 with same affinity. **A)** Dot plot and the gated population of *Str. pneumoniae* serotype. **B)** Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

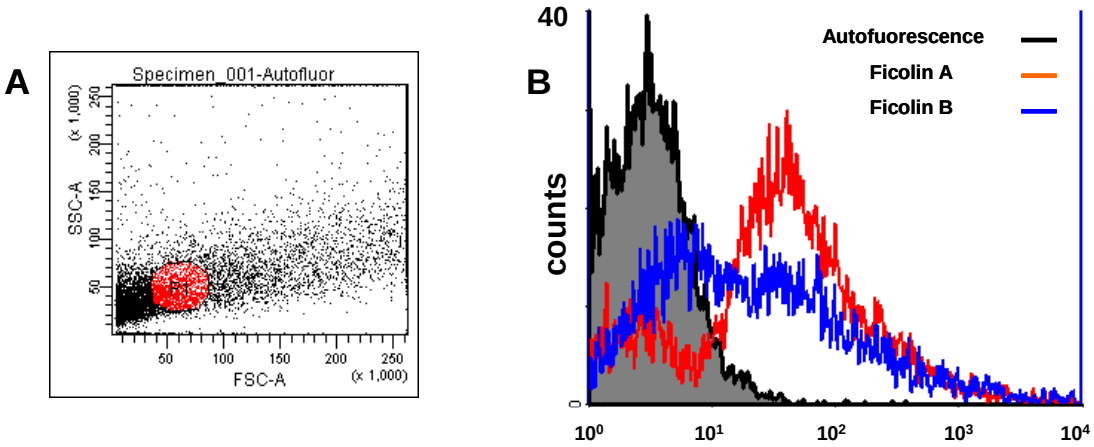


Figure 21. Both **ficolin A and B bind to *Str. pneumoniae* serotype 6A.** A) Dot plot and the gated population of *Str. pneumoniae* serotype 6A. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

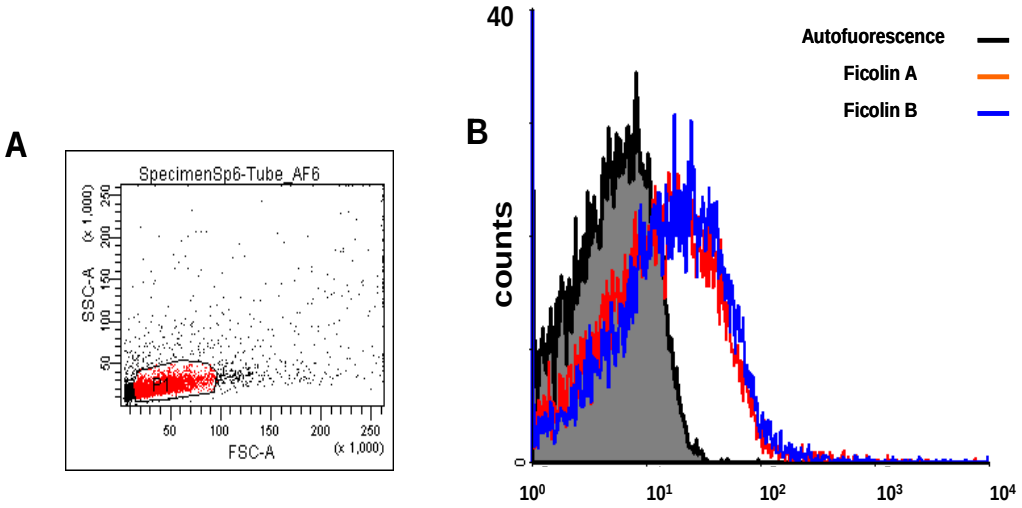


Figure 22. Both ficolins A or B show partial binding to *Str. pneumoniae* serotype TIGR4. A) Dot plot or the gated population of *Str. pneumoniae* serotype TIGR4 and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

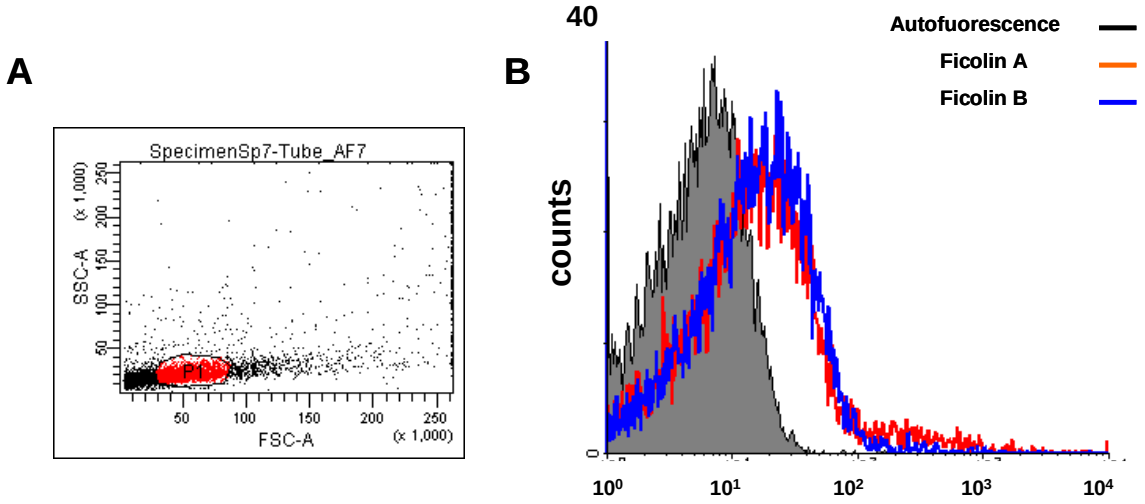


Figure 23. Both ficolins A or B show minimal binding to *Str. pneumoniae* serotype 9L. Dot plot and the gated population of *S. aureus* serotype 1. **B)** Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

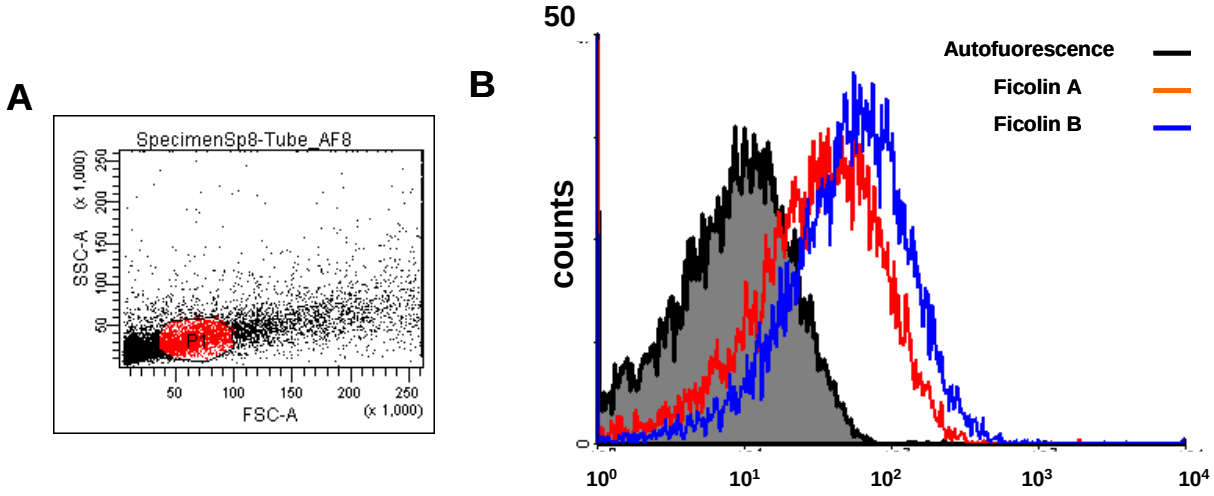


Figure 24. Both ficolin A and B bind to *Str. pneumoniae* serotype 6B with comparable affinity. A) Dot plot and the gated population of *Str. pneumoniae* serotype 6B. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

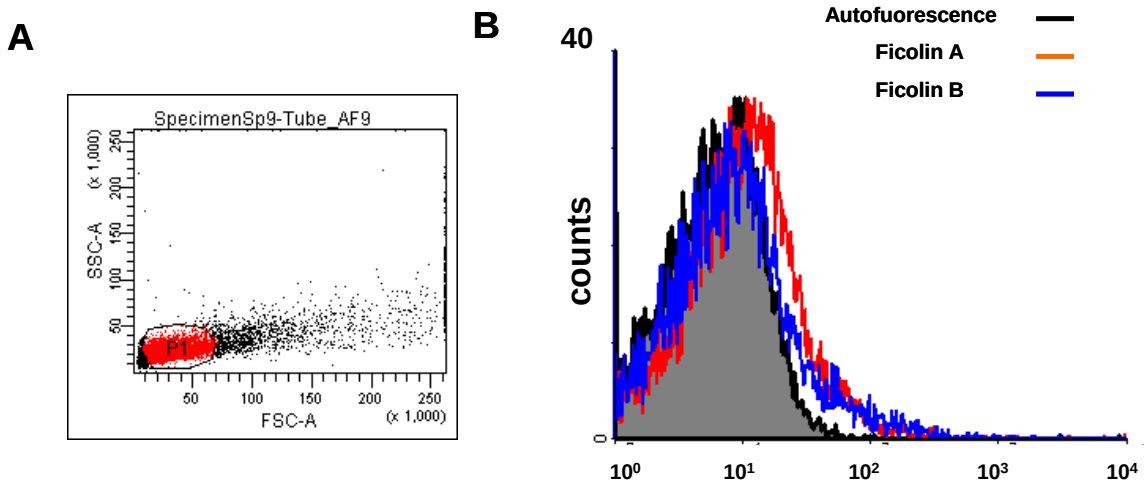


Figure 25. Neither ficolin A nor ficolin B bind to *Str. pneumoniae* serotype 19C. A) Dot plot and the gated population of *Str. pneumoniae* serotype 19C. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

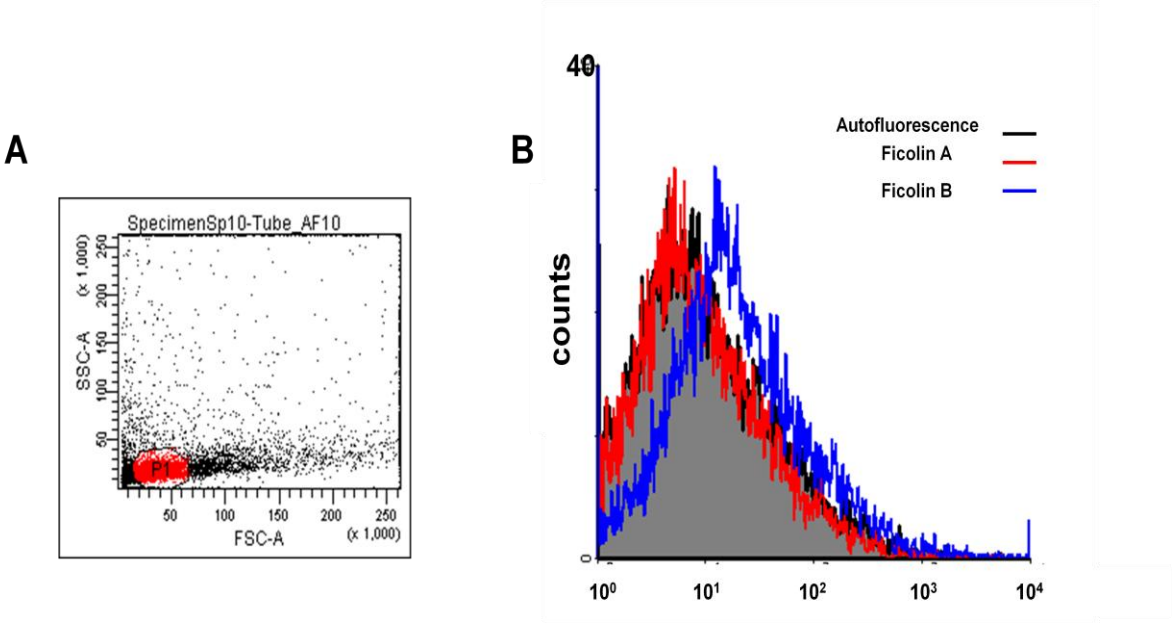


Figure 26. **Neither ficolin A nor ficolin B bind significantly to *Str. pneumoniae* serotype 9F. A)** Dot plot or the gated population of *Str. pneumoniae* serotype 19F and **B)** Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

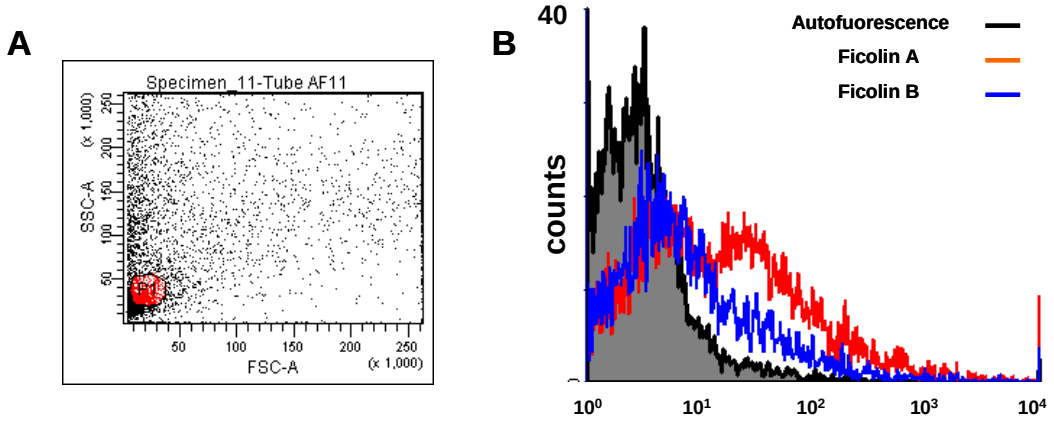


Figure 27. Ficolin A and B bind to *Str. pneumoniae* serotype 32F with different affinity While ficolin B does not show significant binding to *Str. pneumoniae* serotype 32F, ficolin A binds to a certain extent (43 %). A) Dot plot and the gated population of *Str. pneumoniae* serotype 32F and. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

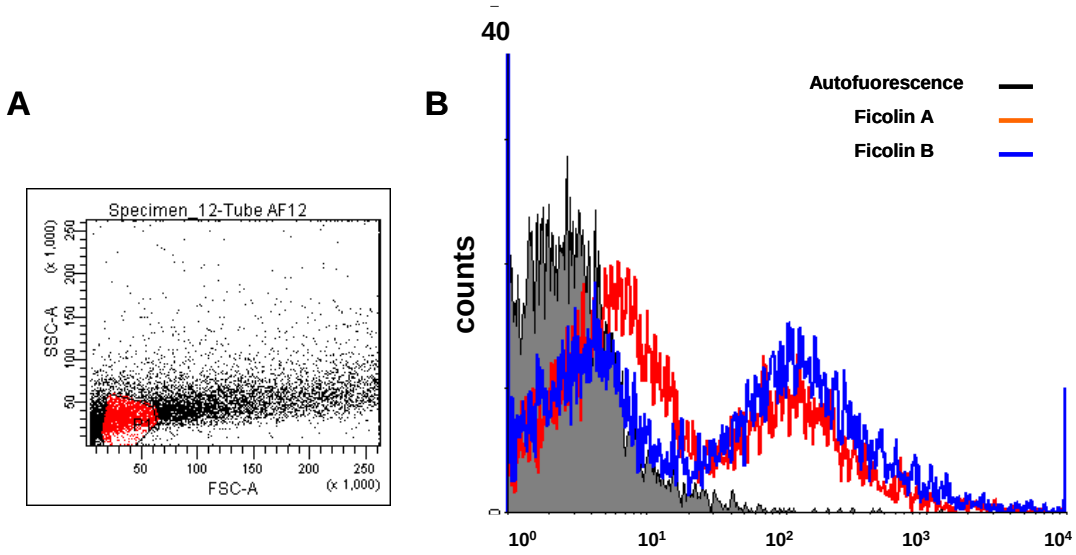


Figure 28. Both ficolin A and B bind to *Str. pneumoniae* serotype 23F with the same affinity. A) Dot plot and the gated population of *Str. pneumoniae* serotype 23F. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

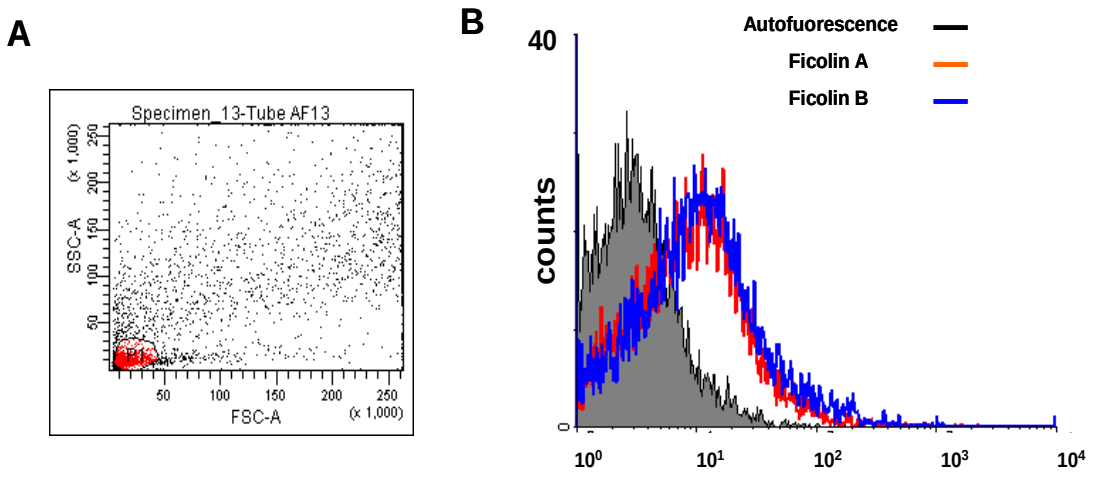


Figure 29. Both ficolin A and B bind to *Str. pneumoniae* serotype 7F with the same affinity. A) Dot plot and the gated population of *Str. pneumoniae* serotype 7F. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

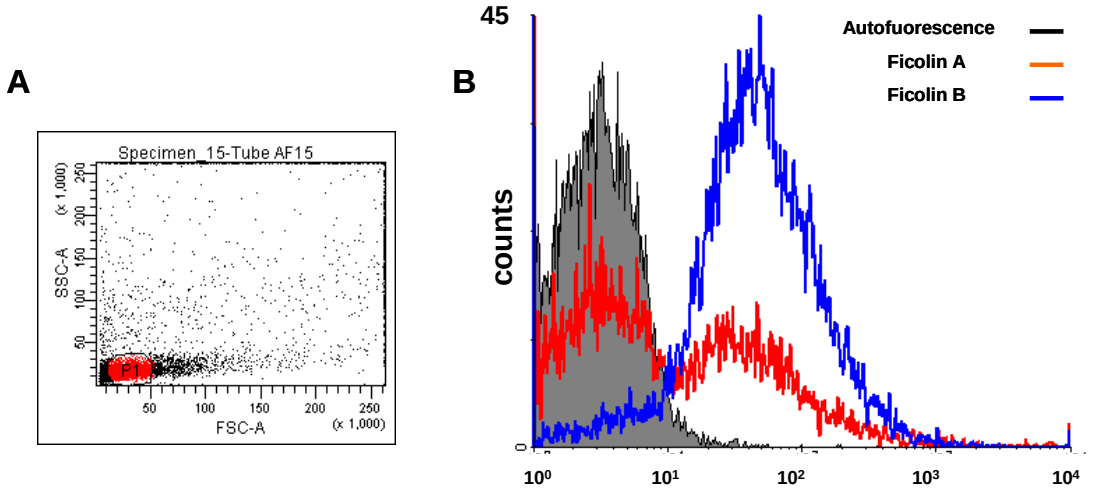


Figure 30. Both Ficolin A and B bind to *Str. pneumoniae* serotype 11F with different avidity. While ficolin B shows high recognition (76%) to *Str. pneumoniae* serotype 11F, ficolin B binding was achieved only to 30%. A) Dot plot and the gated population of *S. aureus* serotype 1. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

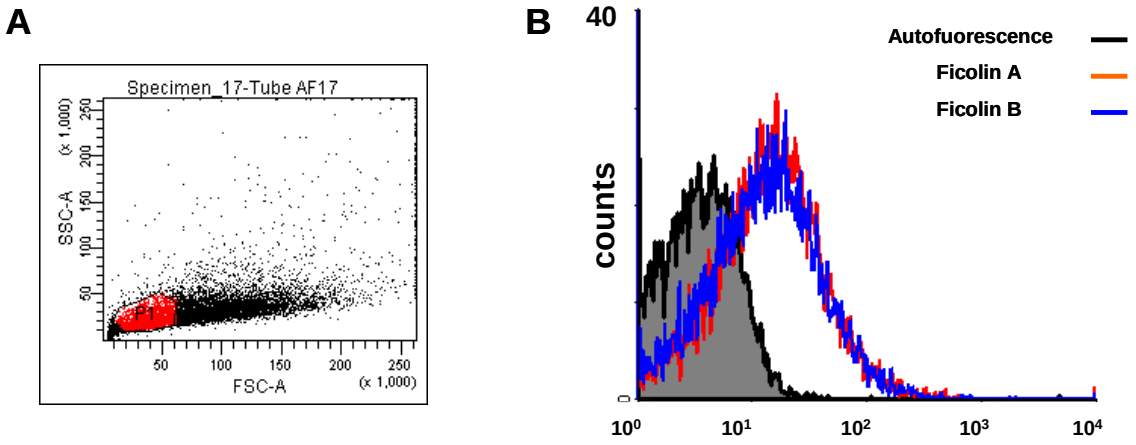


Figure 31. Both ficolin A and B bind to *Str. pneumoniae* serotype 1 with comparable affinity. A) Dot plot and the gated population of *Str. pneumoniae* serotype 1. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

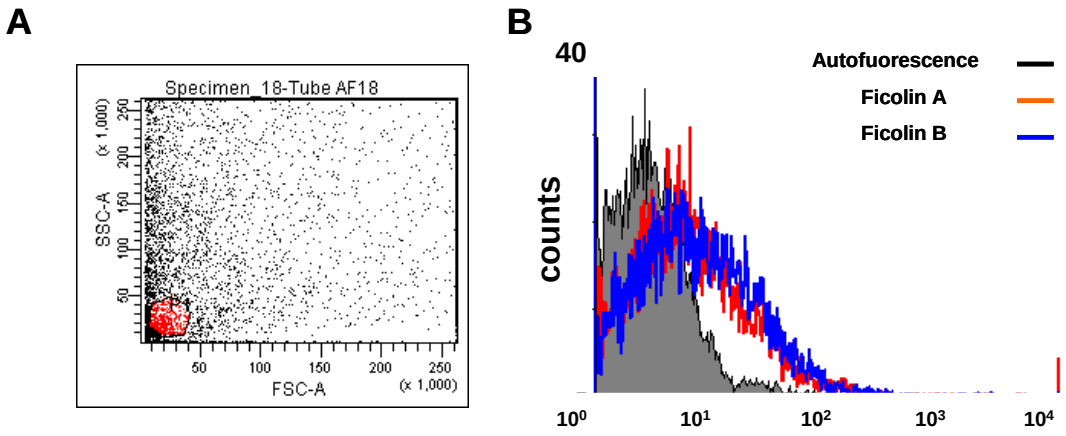


Figure 32. Minimal binding to *Str. pneumoniae* serotype 9V. A) Dot plot and the gated population of *Str. pneumoniae* serotype 19V. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

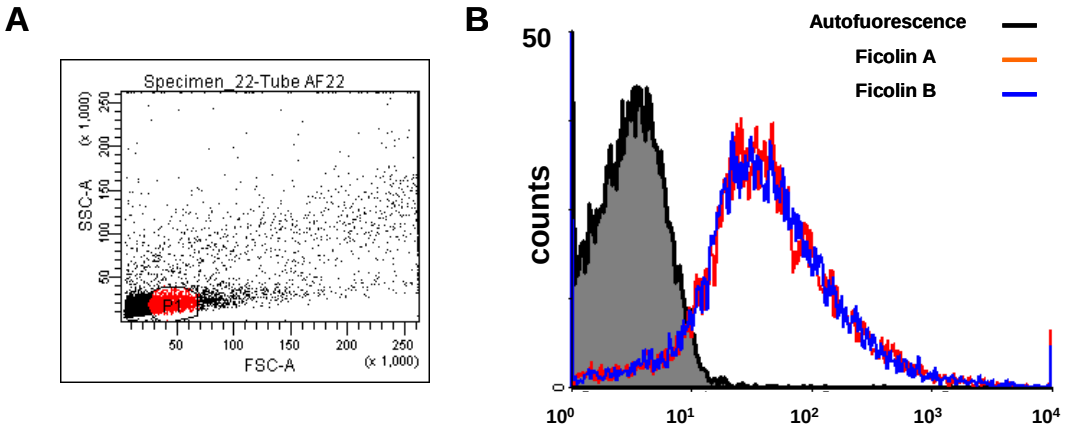


Figure 33. Both ficolin A and B bind to *Str. pneumoniae* serotype 11D with the same affinity. A) Dot plot and the gated population of *Str. pneumoniae* serotype 11D. B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed at the mean fluorescent intensity. The results shown in 3 to 5 independent experiments.

III.3 Escherichia coli

E.coli belongs to the group of enterobacteriaceae. Enterobacteriaceae are all gram-negative and encapsulated, which contain lipopolisaccharide (LPS) in their envelope. In order to test whether immune ficolins are specific pattern recognition molecules for Gram-positive bacteria or are also able to recognize Gram-negative cells. Test of binding to *E.coli* was also included to this study and found to be negative.

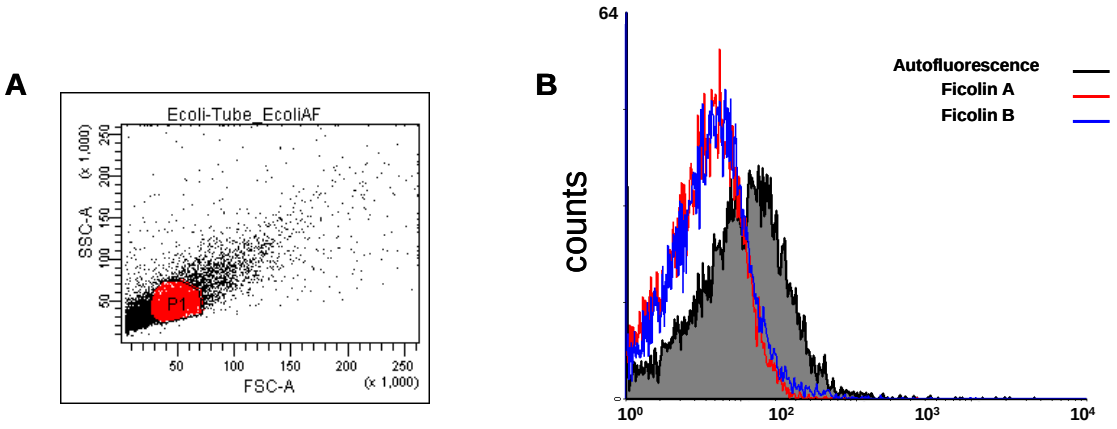
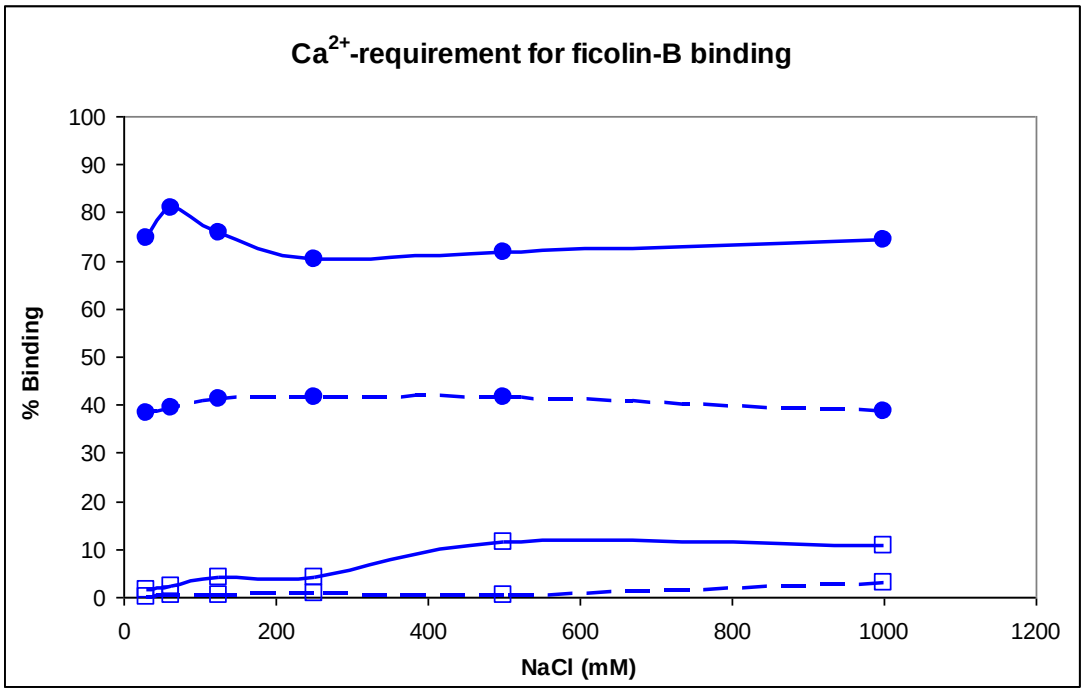


Figure 34. Neither ficolin A nor ficolin B bind to *E. coli*. A) Dot plot or the gated population of *E. coli* and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

III.4 Calcium requirement of mouse ficolin B

In this work it was investigated whether ficolins bind to bacterial surfaces in a calcium dependent manner. In particular, the ability of ficolin B to bind to *S. aureus* serotype 5 and *S. pneumonia* serotype 21 was examined at increasing salt concentrations in the presence or absence of calcium. Equation 1 shows that ficolin B binding to both bacterial strains is absolutely calcium dependent since no binding was detected in the presence of EDTA. Furthermore, this calcium requirement was not affected by increasing salt conditions.



Equation 1. Ficolin B binding to bacterial cells is calcium dependent. Ficolin B binds to *S. aureus* serotype 5 (bold lines) in the presence (filled dots) but not in its absence of calcium (open squares) independently of the NaCl concentration. The same is true for *Str. pneumoniae* (dotted lines).

III.5 Competitive Assay

In order to confirm that the screening procedure shows specific ficolin binding, competitive assays were performed where first unlabelled ficolin molecule blocks the binding sites and consequently can not be recognized by the same Cy5-labeled ficolin any more (the graphic results are not shown). All the bacterial strains which were positive for ficolin binding were tested in this way.

One strain of *S. aureus* and one strain of *Str. pneumonia* positive for both ficolin A and B binding, did not bind anymore after the bacteria had been pre-incubated with unlabeled ficolins.

In order to test whether ficolin A and B compete for the binding on the same bacterial strains, competitive assays were also performed by pre-incubating the bacteria with unlabelled ficolin A or B followed by incubation with Cy5-labelled ficolin B and A. Several bacterial strains were tested in this way and the results indicate that competition between mouse ficolins for the binding to the same ligand does occur only with certain bacterial strains. For example, the binding of labeled ficolins A and B to *Str. pneumonia* serotype 27F is strongly affected by pre-incubating the cells with the other unlabeled ficolin. However this seems not to be the case of *Str. pneumonia* serotype 1, where the competition is only partial.

III.6 Candida albicans

In our experiments we wanted to test whether ficolins bind to *C. albicans*. Like in the bacterial screening, fungal cells were incubated with fluorescently labeled ficolin A or B and binding measured by flow cytometry. Fluorescent intensities were measured by flow cytometry and no binding was found (Figure 1).

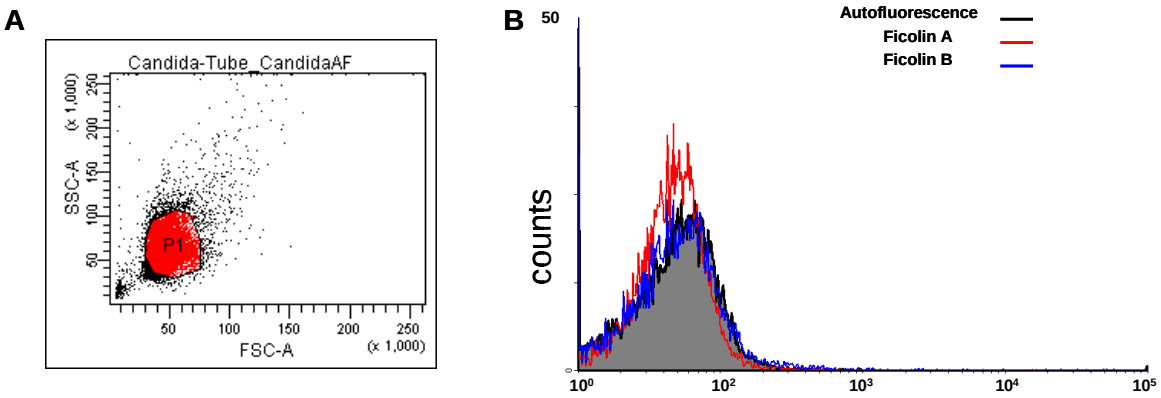


Figure 35. Neither ficolin A nor ficolin B bind to *C. albicans*. A) Dot plot or the gated population of *C. albicans* and B) Binding of ficolin A and B was measured by flow cytometry after incubation with bacteria and expressed the results shown in 3 to 5 independent experiments.

IV. Discussion

IV.1 Binding studies

The efficient elimination of bacteria requires the cooperation of multiple mechanisms from the innate and the adaptive immune system. The complement system is a protein cascade, capable of neutralizing invading pathogens. One of its activation pathways is the lectin pathway which is dependent on the binding of MBL or ficolins to the pathogens. Ficolins form a class of pattern recognition receptors that bind specifically to carbohydrate moieties on microbial surfaces and provide the front line defense against infection. However, the specificity of mouse ficolin recognition remains uncertain despite studies which showed these proteins may have a common carbohydrate binding specificity to N-acetylglucosamine (Matsushita *et al.*, 1996). In this work the potential of mouse ficolins A and B to adhere to various strains of capsulated and non-capsulated bacteria was examined in screening assays. Seventeen of the most common serotypes of *Str. pneumonia* and twelve known capsulated serotypes of *S. aureus* (T1 – T12) were used in these experiments. Relevant non-capsulated strains of *S. aureus* (Wood) and *Str. pneumonia* (SCR2 and TIGR4) were also included to examine the influence of capsulation on the binding recognition by mouse ficolins. No binding was detected of either mouse ficolin to *E.coli* and *Candida albicans*.

The screening of *S. aureus* revealed that ficolin A and B bind to different strains with variable affinity: ficolin A and B bind to *S. aureus* strains T4, T5 and Wood with affinities over 70%. But neither ficolin A nor ficolin B bind to the staphylococcal serotypes T6, T8, T9 and T10. On the other hand, the screening of *Str. pneumonia* showed that both ficolins bind with a high affinity to pneumococcal strains 1, 11D, 14, 27 and SCR2. However, comparing the binding activities of murine ficolins to the serotypes 7A, 6A, 11F and 32F it becomes clear that, while one ficolin shows high bacterial recognition, the other ficolin achieved binding only to a very low extent. Ficolin A binds with a high affinity to the strains 7A and 32F, while ficolin B displays similar results with strains 6A and 11F.

Those differences in bindings are considered statistically significant because we repeated the experiments from 3 to 5 times, our measurements were reproducible in the next setting of experiments and due to p-value of 0,1 % it is highly unlikely that our results occurred by chance.

We suspect that ficolins A and B might not always overlap but also display differential binding preferences to different bacterial surfaces. In addition, no significant binding of ficolin A or B was observed to the pneumococcal serotypes 19C, 9L, 9V, 9F and 45. Interestingly, the results obtained with these staphylococcal and pneumococcal strains also indicate that mouse ficolins recognize structures present in both capsulated and non-capsulated bacteria.

The majority of the staphylococcal capsular polysaccharides is mostly uncharged or possesses negative charges. In this regard it is interesting to compare the similar polysaccharide structures of serotypes T5 (binding) and T8 (non-binding).

Moreau reported the polysaccharide structure of *S. aureus* serotype T5 (binding) to be (Moreau M *et al.*, 1990):

[→4)-3-*O*-Ac-β-D-Man*p*NAcA-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-(→]n[1]

Vann *et al.* haves shown in their studies that the structure of *S. aureus* in serotype T8 (non-binding) is (Vann W *et al.*, 1989):

[→3)-4-O-Ac-β-Man*p*NAcA-(1→3)-α-Fuc*p*NAc-(1→3)-β-Fuc*p*NAc-(1→]n[2]

On the one hand, *S. aureus* serotype T5 (binding) capsular polysaccharide is structurally similar to the one found on serotype T8 (non-binding). The two polysaccharides differ only in the kind of linkages between the sugars (1→4 in T5 and 1→3 in T8) and the sites of O-acetylation of the ManNAcA residues (on the third or fourth position in T5 and T8, respectively). However, only *S. aureus* serotype T5 was recognized by both mouse ficolins.

On the other hand, structural studies on both *S. aureus* serotype T5 and T8 revealed that serotype T8 (non-binding) has a zwitterionic charged motif conferred by the negatively charged carboxyl group of N-acetylmannosaminuronic acid and free amino groups available on partially N-acetylated fucosamine residues, which not only provides resistance to phagocytosis but also directly modulates the host immune response to bacterial infection (Tzianabos *et al.*, 2001).

In addition, the impaired ficolin binding to *S. aureus* serotype T8 compared to serotype T5 could also be the result of steric hindrance: two linkages between the sugars or O-acetylation of the ManNAcA residues (Krarup *et al*., 2008). However, this is unlikely

[1] ManNAc = *N*-acetylmannosamineuronic acid; FucNAc = N-acetylfucosamine; *p* = pyranosidic; Ac = acetylgroup.

[2] ManNAcA = *N*-acetylmannosamineuronic acid; FucNAc = N-acetylfucosamine; *p* = pyranosidic; Ac = acetylgroup.

since the saccharides are bound to the surface through a spacer; but the possibility could not be eliminated in the present work.

Mouse ficolins show high binding affinity to the capsulated streptococcal strains 1, 11D, 14, 27.

The capsular polysaccharide of *Str. pneumonia* serotype 1 (binding) consists of the following repeating trisaccharide units (Jennings H.I. et al., 1980), (Stroop C. et al., 2002):

→4)-α-Gal*p*A-(1→3)-α-Gal*p*A-(1→3)-α-6dGal*p*NAc4N-(1→[3]

Schule and Ziegler describe the repeating unit of the immunodominant capsular polysaccharide of *Str. pneumoniae* serotype 27 (binding) as the synthesis of the 5-aminopentyl glycoside tetrasaccharide:

β-D-Glc*p*-(1→3)-4,6-carboxyethylidene-β-D-GlcNAcP-(1→3)-α-D-Gal*p*-(1→4)-β-L-Rha*p* (Szu S.C. et al., 2005).[4]

Marc Kolkman and colleagues reported the chemical structure of the capsular polysaccharide of *Str. pneumoniae* serotype 14 (binding). It is composed of repeating units, with monosaccharide side chains of β-D-Galp-(1→ linked to C4 of each N-acetylglucosamine residue.

→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ (Kolkman M.A. et al., 1997).[5]

All these serotypes include similar molecular structures in their capsules: N-acetylgroups and 1,3-β-D-glucans. Previous studies have shown that mouse ficolins have an affinity for N-acetylated compounds (Matsushita *et al.*, 1996). This motif likely plays an important part, but the presence of such N-acetylated carbohydrates in the capsules of *Str. pneumonia* serotypes did not always lead to binding. It could be possible that ficolins A and B recognize any oligosaccharides containing N-acetylated compounds where the N-acetyl groups may have to be in a specific conformation that has to be fulfilled to maximize the ficolin binding. The oligosaccharide of the capsule of *Str. pneumonia*

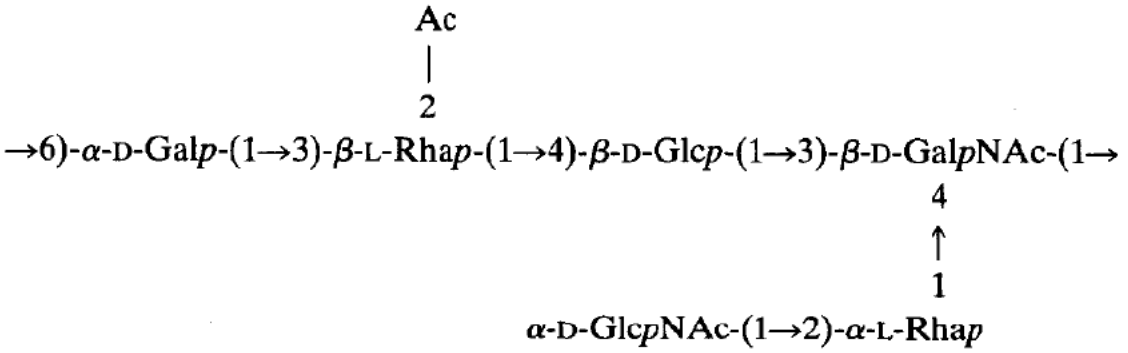
[3] GalA = galactouronic acid; Gal = galactose; 6dGalNAc4N = 2-acetamido-4-amino-2,4,6-tridexo-D-galactose; *p* = pyranosidic. The polysaccharide also contains a non-stichometric amount of O-acetyl-groups, the position of which has not been reported.

[4] Gal = galactose; GlcNAc = N-Acetylglucosamin; Rha = rhamnose; *p* = pyranosidic; P = phosphate residue.

[5] Glc = glucose; Gal = galactose; Rha = rhamnose; *p* = pyranosidic.

serotype 1 (binding) consists of 3 molecules, one of those is N-acetylated and has a 1,3-β-D-conformation. Likely ficolins A and B bind to every third molecule in the polysaccharide capsule. The oligosaccharide present in *Str. pneumonia* serotype 27 consists of a phosphorylated saccharide with one N-acetylated 1,3-β-D-glucan molecule. In the oligosaccharide of *Str. pneumonia* serotype 14 there are 3 pyranosidic sugar molecules, one of them is N-acetylated and involved in a 1,3-β-D-linkage. The high binding potential may arise from the high density of N-acetyl groups, so that some of them are found in the same conformation to enable ficolins to bind.

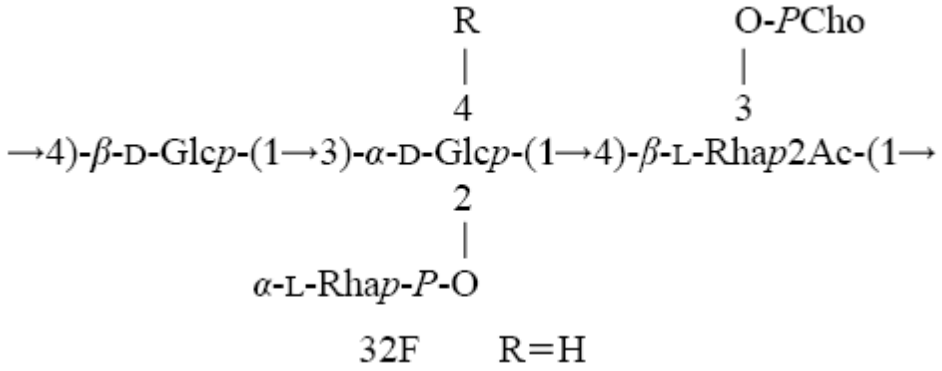
As expected, mouse ficolins A and B not always overlap but also display differential binding preferences. For example ficolin A binds with a high affinity to the strains 7A and 32F and ficolin B does so with strains 6A and 11F, while the opposite does not occur. *Str. pneumoniae 7A* (binding ficolin A) possesses a neutral polysaccharide whose hexasaccharide repeating unit contains two L-rhamnose residues and one residue each of D-glucose, D-galactose, 2-amino-2-desoxy-D-glucose and 2-amino-2-desoxy-D-galactose. The amino sugar residues are N-acetylated and every such polysaccharide residue contains one O-acetyl group (Backman-Marklund I et al., 1990). As mentioned above, ficolin A binds to *Str. pneumoniae 7A* with an affinity of 79%, while Ficolin B does not (Backman-Marklund I et al., 1990), which is statistically significant and might speak for a fact that ficolin A tend to bind to N-acatylated sugar residues, which are also reach on additional O-acatyl group.



Where: Glc = glucose; GlcA = glucuronic acid; Gal = galactose; Rha = rhamnose; Ac = acetyl-group; *p* = pyranosidic; Gal*p*NAc = pyranosidic 2-acetamido-2-deoxygalactose; Gal*p*NAc = pyranosidic 2-acetamido-2-deoxyglucose.

Likewise, while ficolin B shows very low binding (11%) to *Str. pneumoniae* serotype 32F, ficolin A binds to this strain with an affinity of 43%. The specific capsular polysaccharide of *Str. pneumoniae* serotype 32F is composed of tetrasaccharide repeating units with a phosphorylcholine group linked to the 3-position of the 4-substituted β-L-

rhamnose residue. The same rhamnose molecule is, in addition, O-acetylated at the 2-position (Karlsson C et al., 1998): [6]



32F R=H

The capsular polysaccharide units of both of *Str. pneumoniae* 7A and 32F possess β-D-glucose and β-L-rhamnose, which is O-acetylated on the second C-atom. Interestingly, the oligosaccharide molecule of *Str. pneumoniae* serotype 32A possesses two phosphate molecules, which charge the capsular polysaccharide negatively, which is not the case of the capsule molecule of *Str. pneumonia* serotype 7A, where instead some sugar residues are N-acetylated. This observation might suggest that the binding potential of ficolin A may not always arise from the high density of N-acetyl groups but that it can depend on the overall charge of the oligosaccharide. The presence of N-acetyl groups may affect the binding of ficolins by simply neutralizing the charge of the sugar amine group which, without the acetyl substitution, would appear protonated (Runza *et al.*, 2008). In the same way, the presence of phosphate could also neutralize a positive charge or confer a negative charge to the oligosaccharides.

On the other hand *Str. pneumoniae* serotypes 11F and 6A display different binding results: ficolin B binds significantly over 70% to both strains in comparison to ficolin A, which only binds to a certain extent (20-30%).

The capsular polysaccharide of *Str. pneumoniae* serotype 11F is an unbranched linear polymer of a ribitol-phosphate substituted repeating tetrasaccharide unit composed of 2-acetamino-2-deoxy-D-glucose (one part), D-glucose (one part), D-galactose (two parts), ribitol (one part), phosphate (one part), and O-acetyl (two parts) (Richards JC *et al.*, 1985).

The capsular polysaccharide of serotype 6A (binding ficolin B) is a linear polymer with a repeating unit containing four monosaccharides: rhamnose, ribitol-phosphate, galactose

[6] Glc = glucose; Rha = rhamnose; *p* = pyranosidic; P = phosphate; PCho = phosphorylcholine

and glucose (Kamerling et al., 2000) with the following chemical structure (Ho Park *et al.*, 2000):

→2) α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-β-D-Rha*p*-(1→3)-ribitol (5→P [7]

Comparing the capsular polysaccharides of *Str. pneumonia* serotype 6A and serotype 11F both of them include ribitol-phosphate, 1,3-β and 1,3-α-D-glucans and phosphate groups, while only serotype 11 F (binding ficolin B) includes one N-acatylated residue in its capsule.

However the presence or absence of the acetyl group is not likely to determine binding of ficolin A, although its confirmation and/or density might.

In general, both ficolins bind to strains that rich of α- and β-D-glucose, pyranosidic α-and β-D-N-acetylglucosamin or α-D-galactose residues in their capsides. Ficolin B binding was also observed with strains which possess ribitol-phosphate in their capsule structure and ficolin A with strains bearing pyranosidic β-D-glucose and acetylated β-L-rhamnose. This indicates, that the ficolin binding to bacterial surfaces can depend on different polysaccharide structures such as sugar molecules, their configuration and acetylation, phosphate groups and amount of particular polysaccharides in the polymer chain.

No significant binding of mouse ficolins was observed to the capsulated pneumococcal serotypes 19C, 9L, 9V and 9F. The specific capsular polysaccharide produced by *Str. pneumoniae* type 9V is composed of D-glucuronic acid (1 part), 2-acetamido-2-deoxy-D-mannose (1 part), D-glucose (2 parts), and O-acetyl (1.6 parts) in the following configuration (Perry *et al.*, 1981; Rutherford *et al.*, 1991)[8]:

→4)-α-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→

2 OAc (3%)	2 OAc (17%)	4 OAc (6%)
3 OAc (4%)	3 OAc (25%)	6 OAc (55%)

The specific capsular polysaccharide produced by *Streptococcus pneumonia* serotype 9L (American type 49) is composed of D-galactose (one part), D-glucose (one part), D-glucuronic acid (one part), 2-acetamido-2-deoxy-D-mannose (one part), and 2-acetamido-2-deoxy-D-glucose (one part) in the following configuration (Rutherford *et al.*, 1991)[9]:

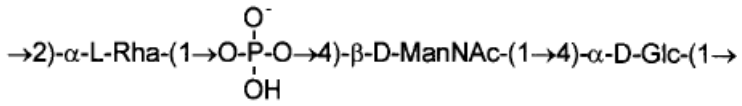
[7] Glc = glucose; Rha = rhamnose; *p* = pyranosidic; P = phosphate.

[8] Glc = glucose; GlcA = glucuronic acid; Gal = galactose; ManNAc = 2-acetamido-2-deoxymannose; OAc = *O*-acetyl.

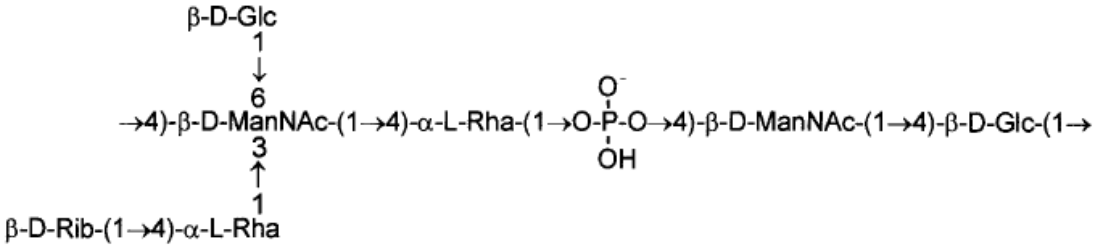
[9] Glc = glucose; GlcA = glucuronic acid; Gal = galactose; ManNAc, 2-acetamido-2-deoxymannose; OAc = *O*-acetyl; *p* = pyranosidic.

→4)-*α*-Glc*p*NAc-(1→4)-*α*-Glc*p*A-(1→3)-*α*-Gal*p*-(1→3)-*β*-Man*p*NAc-(1→4)-*β*-Glc*p*-(1→

The polysaccharide structure of *Str. pneumonia* serotype 9F is composed of the following oligosaccharide (Beynon LM et al., 1991 and Morona JK et al., 1999)[10]:



The serotype 19C is composed of repeating tetrasaccharide units as it follows pyranosidic (Beynon LM et al., 1991 and Morona JK et al., 1999)[11]:



Interestingly, all these structures of capsular polysaccharide possess β-D-ManNAc. Capsular polysaccharides of serotypes 9V and 9L also include glucuronic acid, which imparts a positive charge to the polysaccharide chain. Due to the phosphate molecules the serotypes 19C and 19F is negatively charged. It seems that β-D-ManNAc molecules do not play a role in the binding of mouse ficolins to streptococcal polysaccharides, suggesting that the repertoire of microbial organisms recognized by mouse ficolin does not necessarily overlap to the one recognized by MBL (which binds to the mannan residues). At the same time although the strains 9V, 19C, 19F do not bear a GlcNAc molecules in their capsule, the strain 9L does, which could only be an evidence, that ficolins recognize GlcNAc residues only in a specific configuration (9L posses a GlcNAc molecule).

Mouse ficolins also bind to non-capsulated *S. aureus* Wood and *Str. pneumonia* strains SCR2 (a non-capsulated variant of serotype 2) and TIGR2.

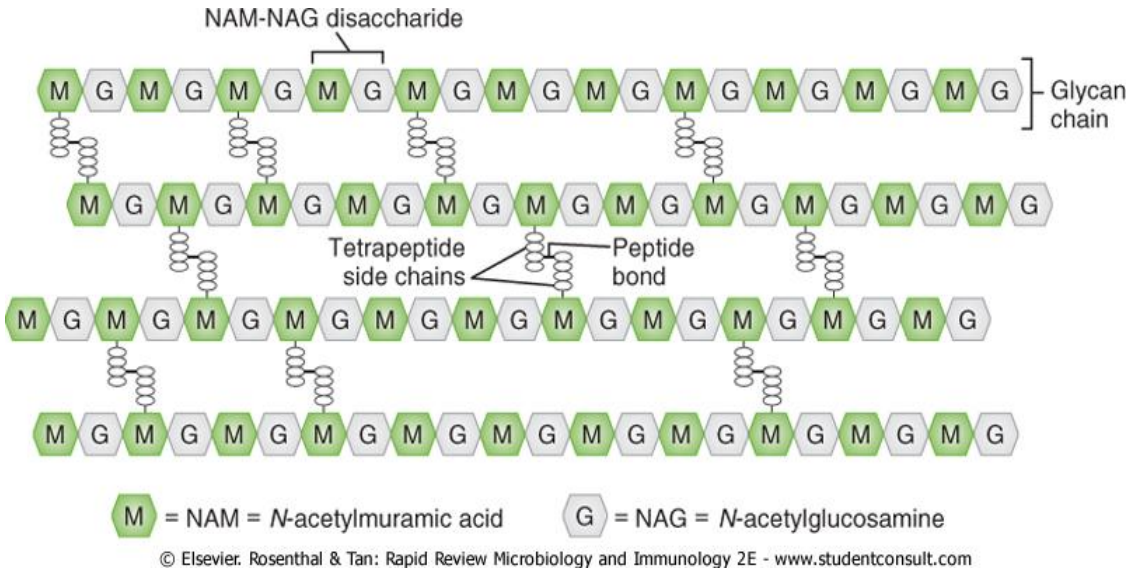
Non-capsulated strains consist of phospholipid membrane, peptidoglycan, teichoic and lipoteichoic acids. The cell wall of gram positive bacteria includes a thick peptidoglycan

[10] D-Glc = glucose; D-ManNAc = *N*-acetylmannosamine; L-Rha = rhamnose and all sugar molecules are pyranosidic.

[11] D-Glc = glucose; D-ManNAc = *N*-acetylmannosamine; L-Rha = rhamnose; D-Rib = ribose and all sugar molecules are.

layer, which consists of three structural parts, such as glycan chains of a repeating disaccharide composed of *N*-acetylglucosamine and N-acetylmuramic acid, tetrapeptide chains and peptide bonds (Fig. A and B).

A.



B.

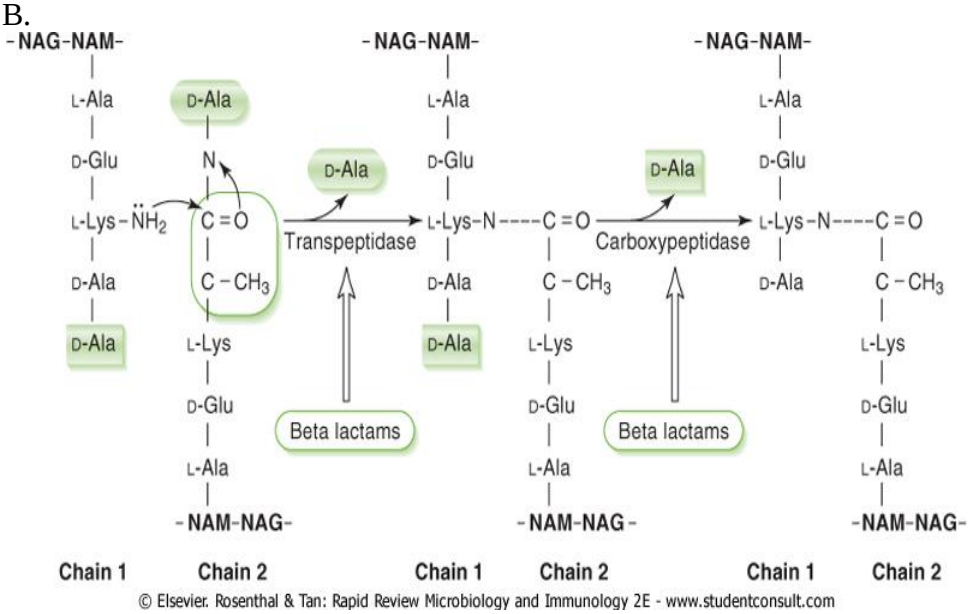


Figure 36. A. Peptidoglycanstructure of gram-positive bacteria. B. Transpeptidation reaction as a final step of peptidoglycan synthesis (Rosenthal *et al.*, 2007).

The cell wall-associated teichoic and lipoteichoic acids of *S. aureus* Wood are composed of a linear backbone structure of 4-O-β- and 4-O-α-N-acetyl-D-glucosaminyl bridged by 1,5-phosphodiester linkages. Approximately 50% of the ribitol residues are esterified at the C-2 position with D-alanine (Tzianabos *et al.*, 2001).

According to the structure of pneumococcal teichoic and lipoteichoic acids, the polysaccharide is comprised of several repeating units, each of which starts with glucose and ends with ribitol, with the lipid anchor predicted to be Glc(β1→3)AATGal(β1→3)Glc(α1→3)-acyl$_2$Gro, where AATGal is 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (Ho Seong Seo *et al.,* 2008). The peptidoglycan scaffold of the pneumococcal cell wall is a repeating GlcNAc-*N*-acetylmuramic (MurNAc) disaccharide (GlcNAc-(β-1,4)-MurNAc) unit having a pentapeptide attached to the D-lactyl moiety of each MurNAc unit (Loeffler *et al.,* 2001). Another usual component of the pneumococcal cell wall is phosphorylcholine, which anchors choline-binding proteins non-covalently to the cell wall (Bergmann S *et al.,* 2006).

Since the results obtained with capsulated bacterial strains suggest that mouse ficolins have an affinity towards α-configurated N-acetylated compounds this motif might also play an important role in the ficolin binding to peptidoglycan of non-capsulated gram-positive bacterial strains.

The experiments of the present work were done from three to five times independently and the measurements were reproducible in up to 92,3%. The results of the present work indicate with a significant difference that mouse ficolins recognize structures present in both capsulated and non-capsulated bacteria, suggesting that ficolins either bind to molecules exclusively present in both serotypes or that they recognize different antigens on these strains. The possibility that the bacterial cells of the same strain is always glycosylated in an identical fashion can be not completely excluded due to high possibility of the surface antigen mutation. However ficolins A and B might have a common binding affinity for 1,4-β-GlcNAc, α-N-acetylmuramic acid, α-D-Glcp, α-D-GalNAc, β-D-Gal, because those sugar molecules are present on the bacterial strains recognized by mouse ficolins. Likewise β-D-glucose and β-L-rhamnose could be ligands for the binding of the ficolin A while ribitol-phosphate, α-D-Glucose and β-D-Galactose could be the target of mouse ficolin B.

Mouse ficolins A and B are the orthologues of L- and M-ficolins in humans, respectively. However, they seem to recognize different pattern. The ability of human L-ficolin to bind to different serotypes and non-capsulated variants of *S. aureus* was investigated by other research groups. L-ficolin binds to some capsulated *S. aureus* serotypes (T1, T8, T9, T11 and T12) but not to the non-capsulated strain Wood (Krarup *et al., 2005*). According to the results of this doctoral thesis none of the mouse ficolins binds with an affinity > 20% to any of these capsulated serotypes. The screening results of Krarup's research group

show that the L-ficolin binds significantly to serotype T8 and does not show a high binding affinity to the serotype T5 (Krarup *et al., 2005*), which is exactly the opposite of the results obtained in the present work.

In addition, Krarup and co-workers suggested the binding of L-ficolin to be directed towards the capsule (Krarup, *et al.,* 2005), which is in opposition to another report that L-ficolin binds to teichoic and lipoteichoic acids and protein A which are present on the non-capsulated bacterial surface of *E. coli* (Lynch *et al.,* 2004).

Krarup and colleagues also investigated the ability of human L-ficolin to bind to different serotypes and non-capsulated variants of *Str. pneumoniae,* which were also included in this doctoral thesis. Like with *S.aureus,* L-ficolin binds to some capsulated *Str. pneumoniae* serotypes (serotypes 11A, 11D and 11F), but not to non-capsulated strains TIGR and SCR2 (Krarup *et al.,* 2005). Again, mouse recombinant ficolins A and B showed a high binding affinity to both capsulated and non-capsulated streptococcal serotypes (serotypes 1, 11D, 14, 27, SCR2 and TIGR) (present work).

This might indicate that ficolins of different species recognize different structures on bacteria. This is not surprising, given the fact that each different pathogen interacts with a different host species in different ways depending on the ability whenever it causes a disease in thiese organisms or not.

The results obtained in this doctoral thesis indicate that mouse ficolins have complex binding requirements to particular bacterial strains which are not necessarily the same for human ficolins for particular bacterial strains. Both human and mouse ficolins, recognize N-acetylated sugar patterns, however, the actual conformation and amount of particular sugar molecules in a polysaccharide unit might also play an important role in this recognition. It was not possible to determine which residues are essential for binding to bacterial cells by mouse ficolins but it seems likely that a single carbohydrate residue is not enough to allow for the mouse ficolin to bind. The screening indicates that mouse ficolins recognize binding motifs by multiple interaction sites per oligosaccharide. The fibrinogen-like domain of mouse ficolins might also coordinate and at the same time be affected by the N-acetyl and negative charged groups (Krarup et al., 2008). By having complex conformational requirements, mouse and human ficolins might be capable of recognizing microorganisms, which attempt to mask themselves with carbohydrates to in order to escape the immune systems. By doing so this might lead to opsonization and subsequent complement activation.

IV.2 Calcium requirement of mouse ficolin B

Ficolins belong to the Ca^{2+}-dependent (C-type) lectin super family and are characterized by the presence of a fibrinogen like (fbg) domain. Through this domain they bind to sugar residues on microbial surfaces in a calcium-dependent manner. The fbg-like domain in ficolins seems to be Ca^{2+}-dependent, but there has been some controversy about it: Matsushita and colleagues compared amino acid sequences in the carbohydrate recognition domain (CRD) of MBL, which are responsible for calcium-dependent carbohydrate binding and fibrinogen-like domains of ficolins: there is no sequence homology between the CRD and the fbg-domain, but they discovered potential calcium-binding sites within the fbg-domain (Matsushita *et al.,* 1996).

Le and colleagues investigated the calcium-dependence of ficolin binding and observed that its binding to GlcNAc was independent of activating the complement cascade (Le *et al.,* 1997). In the most recent studies of Gout and colleagues on M-ficolin it was shown to bind PTX3 with high affinity in the presence of calcium ions. The interaction was abolished in the presence of EDTA and inhibited by N-acetyl-D-glucosamine, indicating involvement of the fibrinogen-like domain of M-ficolin (Gout *et al.,* 2011). Considering this controversity it was investigated in this doctoral thesis whether ficolins bind to bacterial surfaces in a calcium dependent manner. As already mentioned in Results this calcium requirement was not affected by increasing salt conditions. In contrast, it was shown by Krarup and colleagues who reported the ability of L-ficolin to bind to bacterial surfaces in the absence of calcium at the high NaCl concentrations (Krarup et all, 2004).

As these results demonstrate ficolin B requires calcium molecules to bind to bacterial surface molecules that ficolin B does not bind to pathogens in the absence of calcium ions. It could be that binding of mouse ficolins to bacterial surfaces has different requirements in presence or absence of calcium depending on the interaction between the ligands on the fbg domain and the bacterial surfaces.

IV.3 Competitive Assay

As we recon from Results they have shown that mouse ficolins are versatile recognition molecules capable of binding to a wide range of opportunistic pathogens. Mouse ficolins

A and B might recognize different ligands on the bacterial surfaces extending and overlapping each other in their range of recognition.

IV.4 Future studies

Ficolin binding to different kinds of organisms was examined in previous studies by the groups of Matsushita, Krarup, and Lynch, but the exact structures involved in this interaction were not identified. The results of this work show no influence of encapsulation on the binding of mouse ficolins A and B to bacteria although no defined carbohydrate pattern could be found to be associated with positive binding.

Due to the complex nature of these molecules the binding specificity of ficolins could be investigated by using the glycan-array technology to screen for different oligosaccharides including acetylated and non-acetylated carbohydrates, non-sugar molecules, etc.. Furthermore, competitive assays with single carbohydrates, non-sugar molecules would give an opportunity to define the exact molecules, which are recognized by mouse ficolins in different conformations and with different substitutions.

Thus in future studies, homologues of non-capsulated and capsulated mutants may be included in control experiments. By having a *Streptococcus pneumonia* wild type strain, which could bind to ficolins and act as a positive control, it would be possible to use mutants of this particular strain which lacking one structure in the cell wall such as D-alanine in teichoic and lipoteichoic acids in the mutant Δ dltA, lipoproteins in the strain Δ Lgt, lipoteichoic acid in the strain TagO and protein A in the mutant Δ Spa or the lack of O-acetylation in Δ OatA strain at peptidoglycan in the polysaccharide chain. By testing these mutants to ficolin binding it would be possible to localize the structure that causes adhesion between ficolins and bacteria.

It remains to be tested to which extent the ficolin binding to acetylated carbohydrate molecules is relevant under physiological conditions *in vivo.* Considering that ficolin B is found in the lysosomes of activated macrophages (Runza *et al.,* 2006) it would be interesting to elucidate the influence of low pH on the ficolin-bacterial interaction. Garlatti and colleagues investigated this interaction between acetylated compounds and M-ficolin at different pH and found that the ligand binding site of M-ficolin was dislocated.

During the past years, expression of recombinant M-ficolin allowed characterization of its recognition specificity for acetylated ligands and revealed a marked preference for N-acetylneuraminic or sialic acid, a property not shared with L- and H-ficolins (Hummelshoj *et al.,* 2008). Kjaer et al. show human serum M-ficolin binds to capsulated isolates of a pathogenic bacterium, namely Group B Streptococcus, and identify sialic acid as the bacterial ligand. Interestingly, this pathogen is recognized neither by L- and H-ficolins nor by MBL. Moreover, they demonstrate that binding of M-ficolin to the bacteria triggers complement activation, which strongly suggests that serum M-ficolin acts as a soluble PRR similar to L-ficolin and MBL. This clearly opens the way to the search for other pathogenic bacteria, fungi, or parasites that express sialic acids on their surfaces (Varki *et al.,* 2008) as potential M-ficolin targets. The possible collaboration of serum M-ficolin with other soluble PRRs, such as PTXs (PTX3, C-reactive protein, serum amyloid protein), should also be investigated.

Mouse ficolin B seems to recognize terminal N-acetylneuraminic acid residues present in molecules like SiaLacNAc and fetuin (Endo *et al.,* 2005), suggesting that the cell associated ficolin B, in addition to bacterial recognition, might have other functions related to cellular host events (Runza *et al.,* 2008) and recognition of other pathogenic bacteria, fungi, or paracytes.

In previous studies it was reported that L-ficolin recognizes malignant cells since they often undergo changes in their glycosylation pattern compared to normal cells (Kim *et al.,* 1997). In this regard, the glycosylated structures found on mouse tumor cells might be susceptible to recognition by ficolins A and B. In a recent studies it was shown that recombinant FcnB binds to late apoptotic cells and to apoptotic bodies as well as to necrotic cells but not to early apoptotic cells (Schmid, Hunold *et al.,* 2011) This binding was calcium-dependent and could be competitively inhibited by acetylated BSA, a classical binding substrate of FcnB. In addition, DNA inhibited binding of FcnB to apoptotic and necrotic cells, indicating that DNA exposed by dying cells could also be a ligand for FcnB. Thus, FcnB may play a role in the removal of damaged host cells and maintenance of tissue homeostasis. Thererefore, it remains to be investigated, whether ficolin A and B are also involved in the immune surveillance of altered self.

Furthermore, Jensen and Honore reported that L-ficolin binds to late apoptotic cells as well as to apoptotic bodies and necrotic cells, but not to early apoptotic cells. They also demonstrated that L-ficolin binds DNA in a calcium dependent manner suggesting that DNA on permeable dying cells act as plausible ficolin ligands (Jensen, Honore *et al.,*

2006). Binding L-ficolin to DNA of permeable late apoptotic and necrotic cells might lead to enhanced uptake by macrophages (Jensen, Honore *et al.*, 2007). Whether in the mouse system, ficolin A and B are involved in the clearance of dead cells raises a highly interesting question and remains to be investigated.

Also, it would be interesting to investigate, whether ficolin B can change its GlcNAc-binding activity in different still physiological pH-changes. Based on work of Yang L. and Zhang J., a detailed understanding of the pH-dependent conformational changes in M-ficolin and pH-mediated discrimination mechanism of GlcNAc-binding activity are crucial to both immune-surveillance and clearance of apoptotic cells (Yang, Zhang *et al.*, 2011).

Finally, up to date it is not known whether mouse ficolins can bind to a variety of viruses, fungal cells (except Candida albicans which were found not be able to bind neither ficolin A or B), or promastigotes and, therefore, still remains as an interesting field to be explored.

In any case there are several promising hypotheses concerning the functions of ficolins A and B that are in need of further investigation which would lead to a better understanding of these innate multifunctional proteins of the immune system.

Literature

Aoyagi Y., Adderson E.E., Min J.G., Matsushita M., Fujita T., Takahashi S., Okuwaki Y., Bohnsack J.F.. 2005. 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.

Arizono T., Umeda A., Amako K.. 1991. Distribution of capsular materials on the cell wall surface of strain smith siffuse of *Staphylococcus aureus*. Journal of Bacteriology. 173, No. 14. p. 4333-4340.

Backman-Marklund I., Jansson P., Lindberg B., Enrichsen J. 1990. Structural studies of the capsular polysaccharide from *Streptococcus pneumoniae* type 7A. Carbohydr Res.. 198(1):67-77.

Backman-Marklund I., Jansson P.-E., Lindberg B.. Structural studies of the capsular polysaccharide from *Streptococcus pneumoniae* type 7A. 1990. Carbohydr Res.. 198: 67-77.

Bergmann S., Hammerschmidt S.. 2006. Versatility of pneumococcal surface protein. Microbiology. 152(Pt 2):295-303.

Beynon L. M., Richards J. C., Perry M.B., Kniskern P. J.. 1991. Antigenic and structural relationships within group 19 *Streptococcus pneumoniae*: chemical characterization of the specific capsular polysaccharides of type 19B and 19C. Can. J. Chem.. 70**:**131–137.

Brooks, A. S., J. Hammermueller, J. P. DeLay, and M. A. Hayes. 2003. Expression and secretion of ficolin beta by porcine neutrophils. Biochim. Biophys. Acta. 1624: 36-45.

Butler K.M., Baker C.J., Edwards M.S...1987. Interaction of soluble fibronectin with Group B *Streptococci*. Infection and Immunity. Vol. 55, No. 10: 2404-2408.

Christensen K., J. Myers, J. Swanson. 2001. pH-dependent regulation of lysosomal calcium in macrophages. J Cell Sci. 115: 599-607.

Christensen K., Myers J., Swanson J.. 2001. pH-dependent regulation of lysosomal calcium in macrophages. J Cell Sci. 115: 599-607.

Dahl M.R., Thiel S., Matsushita M., Fujita T., Willis A.C., Christensen T., Vorup-Jensen T., Jensenius J.C.. 2001. MASP-3 and tts association with distinct complexes of the mannan-binding lectin complement activation pathway. Immunity. 15: 127–135.

Endo Y., Matsushita M., Fujita T.. 2007. Role of ficolin in innate immunity and its molecular basis. Immunobiology. 212(4-5):371-9.

Endo, Y., Nakazawa N., Liu Y., Iwaki D., Takahashi M., Fujita T., Nakata N., Matsushita M.. 2005. Carbohydrate-binding specificities of mouse ficolin A, a splicing variant of ficolin A and ficolin B and their complex formation with MASP-2 and sMAP. Immunogenetics. 57(11):837-44.

Fournier B., Philpott D.J.. 2005. Recognition of *Staphylococcus aureus* by the innate immune system. Clinical Microbiology Reviews. 18 (3): 521–540.

Garlatti V., Belloy N., Martin L., Lacroix M., Matsushita M., Endo Y., Fujita T., Fontecilla-Camps J.C., Arlaud G.J., Thielens N.M., Gaboriaud C.. 2007. Structural insights into the innate immune recognition specificities of L- and H-ficolins. European Molecular Biology Organization. 26: 623–633.

Girija U. V., Mitchell D.A., Roscher S., Russell Wallis R.. 2011. Carbohydrate recognition and complement activation by rat ficolin-B. Eur. J. Immunol. 41: 214–223.

Gokudan S., Muta T., Tsuda R., Koori K., Kawahara T., Seki N., Mizunoe Y., Wai S. N., Iwanaga S., Kawabata S.. 1999. Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are structurally related to fibrinogen. Proc. Natl. Acad. Sci.U.S.A. 96: 10086–10091.

Gout E., Moriscot C., Doni A.. 2011. M-ficolin interacts with the long pentraxin PTX3: a novel case of cross-talk between soluble pattern-recognition molecules. J. Immunology., 186;5815-5822.

Hansen S., Thiel S., Willis A., Holmskov U., Jensenius J.C.. 2000. Purification and characterization of two mannan-binding lectins from mouse serum. J. Immunol. 164: 2610-2618.

Heijenoort v., J.. 2001. Formation of the glycan chains in the synthesis of bacterial peptidoglycan. Glycobiology. 11 (3): 12-16.

Holmskov U., Thiel S., C. Jensenius J.. 2003. Collectins and ficolins: humoral lectins of the innate immune defense. Annu. Rev. Immunol. 21:547–78.

Honore C., Rørvig S, Hummelshøj T, Skjoedt M.-O.. 2011. Tethering of Ficolin-1 to cell surfaces through recognition of sialic acid by the fibrinogen-like domain. J. Leuk. Biol., 90: 445 – 458.

Honore C, Rorvig S, Munthe-Fog L, Hummelshoj T, Madsen HO, Borregaard N, et al. The innate pattern recognition molecule Ficolin-1 is secreted by monocytes/macrophages and is circulating in human plasma. Mol Immunol 2008; 45: 2782-2789.

Huang H., Huang S., Yingcai Yu Y.. 2011. Functional characterization of a ficolin-mediated complement pathway in amphioxus. J. Biol. Chem., 286: 36739–36748.
Hugenholtz P.. 2002. Exploring prokaryotic diversity in the genomic era. Genome Biol., 3(2).

Hummelshoj T., Fog L. M., Madsen H. O., Sim R. B., Garred P. 2008. Comparative study of the human ficolins reveals unique features of ficolin-3 (Hakata antigen). Mol. Immunol. 45, 1623–1632.

Ichijo, H., Ichijo H., Rönnstrand L., Miyagawa K., Ohashi H., Heldin C.H., Miyazono, K. 1991. Purification of transforming growth factor-beta 1 binding proteins from porcine uterus membranes. J. Biol. Chem. 266(33):22459-64.

Inamori K., Saito T., Iwaki D., Nagira T., Iwanaga S., Arisaka F., Kawabata S.. 1999. A newly identified horseshoe crab lectin with specificity for blood group A antigen recognizes specific O-antigens of bacterial lipopolysaccharides. J. Biol. Chem. 274: 3272–3278.

Ishii M., Ohsawa I., Inoshita H.. 2011. Serum concentration of complement components of the lectin pathway in maintenance hemodialysis patients, and relatively higher levels of L-ficolin and MASP-2 in mannose-binding lectin deficiency. Therap. apheresis and dialysis 15(5):441–447.

Jack D. L., Turner M. W.. 2003. Anti-microbial activities of mannose-binding lectin. Biochem. Soc. Trans. 31: 753-757.

Jack, D. L., Klein N.J., Turner N.W.. 2001. Mannose-binding lectin: targeting the microbial world for complement attack and opsonophagocytosis. Immunol. Rev. 180: 86-99.

Janeway Ch. Jr.. Immunobiology. 2005. 28, 25, 302.

Jedrzejas M.J.. 2001. Pneumococcal virulence factors: structure and function. Microbiology and Molecular Biology Reviews. 65 (2): 187–207.

Jennings H.J., Lugowski C., Young N.M.. 1980. Structure of the complex polysaccharide C-substance from Streptococcus pneumoniae type 1. Biochemistry. 19(20):4712-9.

Jensen M.L., Honoré C., Hummelshøj T., Hansen B.E., Madsen H.O., Garred P.. 2007. Ficolin-2 recognizes DNA and participates in the clearance of dying host cells. Mol. Immunology. 44(5): 856-65.

Joosten J.A., Kamerling J.P., Vliegenthart J.F.. 2003. Chemo-enzymatic synthesis of a tetra- and octasaccharide fragment of the capsular polysaccharide of Streptococcus pneumoniae type 14. Carbohydr Res. 338 (23): 2611-27.

Kaji E., Osa Y., Tanaike M., Hosokawa Y., Takayanagi H., Takada A.. 1996. An alternative access to a trisaccharide repeating unit of the capsular polysaccharide of *Streptococcus pneumoniae* serotype 19A. Chem Pharm Bull (Tokyo). 44 (2): 437-40.

Kamerling J. P., Liebert M.A.. 2002. Pneumococcal polysaccharides: a chemical view. *Streptococcus pneumoniae* molecular biology and mechanisms of disease. 81-114.

Karlsson C., Jansson P.E., Sørensen U.B.. 1998. The chemical structures of the capsular polysaccharides from *Streptococcus pneumoniae* types 32F and 32A. Eur J Biochem. 255(1):296-302.

Kawabata S., Tsuda R.. 2002. Molecular basis of non-self recognition by the horseshoe crab tachylectins. Biochim. Biophys. Acta 1572, 414–421.

Kim Y.I., Varki A.. 1997. Perspectives on the significance of alteresd glycosylation of glycoproteins in cancer. Glycoconj J: 14:569-76.

Kjaer T. R., Hansen A.G., Sørensen U. B., Nielsen O., Thiel S., Jensenius J. C.. 2011. Investigations on the pattern recognition molecule M-ficolin: quantitative aspects. J Biological Chemistry. 272(31):14502-6

Kolberg J., Jones C.. 1998. Monoclonal antibodies with specificities for *Streptococcus pneumoniae* group 9 capsular polysaccharides. FEMS Immunol Med Microbiol. 20(4):249-55.

Kolkman M. A. B., van der Zeijst B., Piet J. M.. 1997. Functional analysis of glycosyltransferases encoded by the capsular polysaccharide biosynthesis locus of *Streptococcus pneumoniae* serotype 14. J Biological Chemistry. 272(31):19502-8.

Kolkman M.A.B., van der Zeijst B.A.M., Nuijten P.J.M.. 1997. Functional analysis of glycosyltransferases encoded by the capsular polysaccharide biosynthesis locus of *Streptococcus pneumoniae* Serotype 14. The Journal of Biological Chemistry. 272 (31): 19502–19508.

Krarup A., Mitchell D.A., Sim R.B.. 2008. Recognition of acetylated oligosaccharides by human L-ficolin. Immunology Letters. 118: 152–156.

Krarup A., Sørensen U.B., Matsushita M., Jensenius J.C., Thiel S.. 2005. Effect of capsulation of opportunistic pathogenic bacteria on binding of the pattern recognition molecules mannan-binding lectin, L-ficolin, and H-ficolin.Infect Immun.. 73(2):1052-60.

Krarup A., Thiel S., Fujita S., Jensenius J.C.. 2004. L-ficolin is a pattern recognition molecule specific for acetyl groups.J Bio Chem. 279: 47513-47519.

Krarup A., Thiel S., Hansen A., Fujita J., and Jensenius C. 2004. L-ficolin is a pattern recognition molecule specific for acetyl groups. J Bio Chem. 279: 47513-47519.

Krasko M.Y., Golenser J., Nyska A., Nyska M., Brin Y.S., Domb A.J.. 2006. Gentamicin extended release from an injectable polymeric implant. Journal of Controlled Release 117 90–96.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Le Y., Tan S.M., Lee S.H., Kon O.L., Lu J.. 1997. Purification and binding properties of a human ficolin-like protein. J of Immunol Methods. 204: 43–49.

Lea Y., Lee S.H., Kona O.L., Lu J.. 1998. Human L-ficolin: plasma levels, sugar specificity, and assignment of its lectin activity to the fibrinogen-like (FBG) domain. FEBS Letters 425: 367-370.

Lee C.F., Fraser B.A., Szu S., Lin K.T.. 1981. Chemical structure of and immune response to polysaccharides of Streptococcus pneumoniae. Rev. Infect. Dis.. 3 (2):323-31.

Lehr R. V., Elefante L.C., Kikly K.K., O'Brien K.P., Kirkpatrick K.P.. 2000. A modified metal-ion affinity chromatography procedure for the purification of histidine-tagged recombinant proteins expressed in Drosophila S2 cells. Protein Expr. Purif. 19: 362-368.

Lillie B. N., Brooks A.S., Keirstead N.D., Hayes M.A.. 2005. Comparative genetics and innate immune functions of collagenous lectins in animals. Vet. Immunol. Immunopathol. 108: 97-110.

Liu Y., Endo Y., Homma Y., Kanno K., Yaginuma H., Fujita T.. 2005b. Ficolin A and ficolin B are expressed in distinct ontogenic patterns and cell types in the mouse. Mol. Immunol. 42: 1265-1273.

Liu Y., Endo Y., Iwaki D., Nakata M., Matsushita M., Wada I., Inoue K., Munakata M., and Fujita T.. 2005a. Human M-ficolin is a secretory protein that activates the lectin complement pathway. J. Immunol. 175: 3150-3156.

Loeffler J. M., Nelson D., Fischetti V. A.. 2001. Rapid killing of *Streptococcus pneumoniae* with a bacteriophage cell wall hydrolase. Science 294, 2170-2172.

Lopez R., Garcia E.. 2004. Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. FEMS Microbiology Reviews. 28:553-580.

Lu J., Le L.. 1998. Ficolins and the fibrinogen-like domain. Immunobiology 199: 190-199.

Lu J., Tay T. P., Kon O. L., Reid K. B.. 1996. Human ficolin: cDNA cloning, demonstration of peripheral blood leucocytes as the major site of synthesis and assignment of the gene to chromosome 9. Biochem. J. 313 (Pt 2): 473-478.

Lu J., Teh C., Kishore U., Reid K.M.B.. 2002. Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system. Biochimica et Biophysica Acta. 1572: 387– 400.

Lynch N.J., Roscher S., Hartung T., Morath S., Matsushita M., Maennel D.N., Kuraya M., Fujita T., Schwaeble W.J.. 2004. L-Ficolin specifically binds to lipoteichoic acid, a cell wall constituent of Gram-positive bacteria, and activates the lectin pathway of complement. The Journal of Immunology. 172:1198–1202.

M., Endo Y., Taira S., SatoY., Fujita T., Ichikawa T., Nakata M., Mizuochi T.1995. A Novel Human Serum Lectin with Collagen- and Fibrinogen-like Domains That Functions as an Opsonin. J Biol Chem.; 271(5):2448-54.

Ma Y.G., Cho M.Y., Zhao M., Park J.W., Matsushita M., Fujita T., Lee B.L.. 2004. Human mannose-binding lectin and L-ficolin function as specific pattern recognition proteins in the lectin activation pathway of complement. The Journal of Biological Chemistry. 279 (24) :25307–25312.

Ma Y.G., Cho M.Y., Zhao M., Park J.W., Matsushita M., Fujita T., Lee B. L.. 2004. Human mannose-binding lectin and L-ficolin function as specific pattern recognition proteins in the lectin activation pathway of complement. The Journal of Biological Chemistry. Vol. 279 (24): 25307–25312.

Matsushita M., Endo Y., Fujita T.. 2000. Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease1. The Journal of Immunology. 164: 2281–2284.

Matsushita M., Endo Y., Taira S., Sato Y., Fujita T., Ichikawa N., Nakata M., Mizuochi T.. 1996. A novel human serum lectin with coll. J. Biol. Chem. 271: 2448-2454.

Matsushita M., Kuraya M., Hamasaki N., Tsujimura M., Shiraki H., Fujita T.. 2002. Activation of the lectin complement pathway by H-ficolin (Hakata antigen). J. Immunol. 168: 3502-3506.

Moreau M., Richards J. C., Fournier J. M., Byrd R. A., Karakawa W. W., Vann W. F.. 1990. Structure of the type 5 capsular polysaccharide of Staphylococcus aureus. Carbohydr. Res. 201: 285–297.

Moreau M., Richards J.C., Fournier J.M., Byrd R.A., Karakawa W.W., Vann W.F.. 1990. Structure of the type 5 capsular polysaccharide of Staphylococcus aureus. Carbohydr Res. 1;201(2):285-97.

Moreillon P., Majcherczyk P.A.. 2003. Proinflammatory activity of cell-wall constituents from gram-positive bacteria. Scand J Infect Dis. 35(9):632-41.

Morona J., Morona R., Paton J.C.. 1999. Comparative genetics of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* types belonging to serogroup 19. J Bacteriol. 181(17):5355-64.

Morona J.K., Morona R., Paton J.C.P.. 1999. Comparative genetics of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* types belonging to serogroup 19. J Bacteriol..181(17):5355-64.

Nahid A. M., Sugii S.. 2006. Binding of porcine ficolin-alpha to lipopolysaccharides from Gram-negative bacteria and lipoteichoic acids from Gram-positive bacteria. Dev. Comp Immunol. 30: 335-343.

Ohashi T. Erickson H.P.. 1998. Oligomeric structure and tissue distribution of ficolins from mouse, pig and human. Arch. Biochem. Biophys. 360: 223-232.

Okino N., Kawabata S., Saito T., Hirata M., Takagi T., Iwanaga S.. 1995. Purification, characterization, and cDNA cloning of a 27-kDa lectin (L10) from horseshoe crab hemocytes. J. Biol. Chem. 270**:** 31008–31015.

Park I.H., Pritchard D.G., Catee R., Brandao A.. 2007. Discovery of a new capsular serotype (6C) within Serogroup 6 of Streptococcus pneumoniae. J of clinical microbiology. 45(4):1225-33.

Perez-Dorado I., Campillo N.E., Monterroso B., Hesek D., Lee M., Paez J.A., Garcıa P., Martinez-Ripoll M., Garcia J.L., Mobashery S., Menendez M., Hermoso J.A.. 2007. Elucidation of the molecular recognition of bacterial cell wall by modular pneumococcal phage endolysin CPL-1.The Journal of Biological Chemisry. 282 (34): 24990–24999.

Pérez-Dorado I., Campillo N.E., Monterroso B., Hesek D., Lee M., Páez J.A., García P., Martínez-Ripoll M., García J.L., Mobashery S., Menéndez M., Hermoso J.A. 2007

Elucidation of the Molecular Recognition of Bacterial Cell Wall by Modular Pneumococcal Phage Endolysin CPL-1. J Biol Chem. 282(34):24990-9.

Perry M.B., Daoust V. Carlo D.J.. 1981. The specic capsular polysaccharide of Streptococcus pneumoniae type 9V. Can. J. Biochem. 59: 524-533.

Petersen S.V., Thiel S., Jensenius J.C.. 2001. The mannan-binding lectin pathway of complement activation: biology and disease association. Molecular Immunology. 38:133–149.

Richards J.C., Perry M.B., Kniskern P.J.. 1984. Structural analysis of the specific polysaccharide of *Streptococcus pneumoniae* type 9L (American type 49). Can. J. Biochem. Cell. Biol. 62: 1309-1320.

Richards J.C., Perry M.B.. 1985. The structure of specific capsular polysaccharides of *Streptococcus pneumoniae* type 11F (American type 11). Can. J. Biochem. Cell. Biol.. 61: 1209-1220.

Robbins J.B., Lee C.-J., Rastogi C.S., Schifman G., Henrichsen J.. 1979. Comparative immunogenicity of group 6 pneumococcal type 6A(6) and Type 6B(26) capsular polysaccharides. Infection and Immunity. 26 (3): 1116-1122.

Rosenthal K.S., James S. T.. 2004. Rapid Raview. Microbiology and Immunology. 32, 33, 107.

Runza V. L., Schwaeble W., Männel D.M. 2008. Ficolins: novel pattern recognition molecules of the innate immune response. Immunobiology. 213(3-4):297-306.

Runza V. L.. 2005. Doctoral thesis: Cloning characterisation of mouse ficolins –A and –B. 23-29, 34-38, 40-55.

Runza V., Hehlgans T., Echtenacher B., Zählinger W., Schwaeble W.J., Männel D.N.. 2006. Localization of the diffence lectin ficolin B in activated macrophages. J Endotoxin Res. 12: 120-6

Rutherford T.J., Jones C., Davies D.B. and Elliott A.C. 1991. Location and quantitation of the sites of O-acetylation on the capsular polysaccharide from Streptococcus *pneumoniae*

type 9V by 1H-n.m.r. spectroscopy: comparison with type 9A. Carbohydr. Res. 218: 175-184.

Saito T., Hatada M., Iwanaga S., and Kawabata S.. 1997. A Newly Identified Horseshoe Crab Lectin with Binding Specificity to O-antigen of Bacterial Lipopolysaccharides. J. Biol. Chem. 272: 30703–30708.

Schmid M., Hunold K., Weber-Steffens D., Männel D.N.. Ficolin-B marks apoptotic and necrotic cells. J. Immunol..

Schule G., Ziegler T.. 1996. Efficient Convergent block synthesis of a pyruvated tetrasaccharide 5-aminopentyl glycoside related to streptococcus pneumoniae type 27. Tetrahedron. 52 (8): 2925 – 2936 (12).

Seo H.S., Cartee R.T., Pritchard D.G., Nahm M.H.. 2008. A New Model of Pneumococcal Lipoteichoic Acid Structure Resolves Biochemical, Biosynthetic, and Serologic Inconsistencies of the Current Model. J.Bacteriol.190 (7): 2379-87.

Shousun C. S., Clarke S., Robbins J.B.. 1983. Protection Against Pneumococcal Infection in Mice Conferred by Phosphocholine-Binding Antibodies: Specificity of the Phosphocholine Binding and Relation to Several Types. Infect Immun. 39 (2): 993-9.

Sleytr U.B., Egelseer E.M., Ilk N., Pum D., Schuster B.. 2007. S-Layers as a basic building block in a molecular construction kit. FEBS J. 2007: 274(2):323-34.

Sleytr U.B.. 1978. Regular arrays of macromolecules on bacterial cell walls: structure, chemistry, assembly, and function. Int Rev Cytol. 53:1-62.

Stroop C.J., Xu Q., Retzlaff M., Abeygunawardana C., Bush C.A.. 2002. Structural analysis and chemical depolymerization of the capsular polysaccharide of Streptococcus pneumoniae type 1. Carbohydr Res.. 337(4):335-44.

Sugimoto R., Yae Y., Akaiwa M., Kitajima S., Shibata M., Sato H., Hirata J., Okochi K., Izuhara K., Hamasaki N.. 1998. Cloning and characterization of the Hakata antigen, a member of the ficolin/opsonin p35 lectin family. J. Biol. Chem. 273: 20721-20727.

Szu S.C., Clarke S., Robbins J.B.. 1983. Protection Against Pneumococcal Infection in Mice Conferred by Phosphocholine-Binding Antibodies: Specificity of the Phosphocholine Binding and Relation to Several Types. Infection and Immunity. 993 – 999.

Tanio M., Kohno T.. 2008. Histidine regulated activity of M-ficolin. Biochemical Journal Immediate Publication. 1640.

Teh C., Le Y., Lee S.H., Lu J.. 2000. M-Ficolin is expressed on monocytes and is a lectin binding to N-acetyl-D-glucosamine and mediates monocyte adhesion and phagocytosis of Escherichia coli. Immunology. 101: 225-232.

Thiel S., Vorup-Jensen T., Stover C.M., Schwaeble W., Laursen S.B., Poulsen K., Willis A. C., Eggleton P., Hansen S., Holmskov U., Reid K.B. M.. 1997. A second serine protease associated with mannan-binding lectin that activates complement. Letters to the Nature. 386: 506-510.

Thielens N. M.. 2011. The double life of M-ficolin: what functions when circulating in serum and tethered to leukocyte surfaces? J. Leuk. Biol., 90: 410 – 412.

Tomasz A., Westphal M., Briles B. E., Fletcher P.. 1975. On the physiological functions of teichoic acids. J. Supramol. Struct. 3:1–16.

Townsend R., Read R.C., Turner M.W., Klein N.J., Jack D.J.. 2001. Differential recognition of obligate anaerobic bacteria by human mannose-binding lectin. Clin. Exp. Immunol. 124: 223-228.

Trzcinski K., Thompson C.M., Lipsitch M.. 2003. Construction of otherwise isogenic serotype 6B, 7F, 14, and 19F capsular variants of *Streptococcus pneumoniae* strain TIGR4. Applied and Enviromental Microbiology. 69 (12): 7364–7370.

Turner, M. W. Hamvas R.M.. 2000. Mannose-binding lectin: structure, function, genetics and disease associations. Rev. Immunogenet. 2: 305-322.

Tzianabos A.O., Wang J.Y., Jean C.. 2001. Structural rationale for the modulation of abscess formation by Staphylococcus aureus capsular polysaccharides. Proc. Natl. Acad. Sci USA. 98 (16): 9365-70.

Van S. S., Kolkman M.A.B., van der Zeijst B.A.M., Zwaagstra K.A., Gaastra W., v. Putten J.P.M.. 2002. Organization and characterization of the capsule biosynthesis locus of *Streptococcus pneumoniae* serotype 9V Microbiology. 148, 1747–1755.

Vann J.M., Hamill R.J., Albrecht R.M., Mosher D.F., Proctor R.A.. 1989. Immunoelectron microscopic localization of fibronectin in adherence of Staphylococcus aureus to cultured bovine endothelial cells. J Infect Dis..160(3):538-42.

Vann W. F., Moreau M., Sutton A., Byrd R. A. Karakawa W. W.. 1988. Bacterial Host–Cell Interaction, ed. Horwitz, M. A. (Liss, New York). 64: 187–198.

Varki A. 2008. Sialic acids in human health and disease. Trends Mol. Med. 14, 351–360.

Yae Y., Inaba S, Sato S., Okochi K., Tokunaga K., Iwanaga I.. 1991. Isolation and characterization of a thermolabile beta-2 macroglycoprotein ('thermolabile substance' or 'Hakata antigen') detected by precipitating (auto) antibody in sera of patients with systemic lupus erythematosus. Biochim. Biophys. Acta 1078: 369-376.

Yang L., Zhang J., Ho B., Ding J. L.. 2011. Histidine-mediated pH-sensitive regulation of M-ficolin:GlcNAc binding activity in innate immunity examined by molecular dynamics simulations. PLoS ONE, 5.6.

Yokota Y., Arai T., Kawasaki T.. 1995. Oligomeric structures required for complement activation of serum mannan-binding proteins. J. Biochem. (Tokyo) 117: 414-419.

Acknowledgments

The writing of this dissertation has been one of the most significant academic challenges I have ever had to face. Without the support, patience and guidance of the following people, this dissertation would have not have been completed. It is to them that I owe my deepest gratitude.

- I am sincerely and heartily grateful to my mentor, Prof. Dr. Daniela Männel for the support and guidance she showed me throughout my years of the medical school and dissertation writing. Her wisdom, knowledge and commitment to the highest standards inspired and motivated me.

- Dr. Valeria Runza, who undertook to act as my supervisor despite her many other academic and professional commitments, who also helped me with planning a great deal of the experiments and with correcting the written material.

- Our lab assistant Dorothea Steffens who always stood by my side when I was working in the lab to gather the experimental results and helped me with her great advice and her incredible wisdom.

- Daniel Kennelly, my husband, without whom this effort would have been worth nothing. Your love, support and constant patience have taught me so much about sacrifice, discipline and compromise – even if there were times when you said “I told you so”.

- Fedar and Lydzia Muraveika, my parents, and Dr. Alena Muraveika, my sister, who have always supported, encouraged and believed in me, in all my endeavors

This dissertation is dedicated to Daniel and to all my family.