„Cloning and Characterization of Mouse Ficolins -A and -B”

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Erklärung

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

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Valeria L. Runza

To AS who came to this world
at the same time as this thesis.

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Abbreviations

#	Number
Δ	heat aggregated
A_x	absorbance at a wavelength of x nm
aa	amino acid
Amp^R	ampicilin resistance cassette
AP	alkaline phosphatase or alternative pathway
APS	ammonium persulphate
biot	biotinylated
BM	bone marrow
bp	base pairs
cDNA	complementary DNA
CP	classical pathway
DEPC-H_2O	diethylpyrocarbonated water
DES	*Drosophila melanogaster* expression system
DNA	deoxyribonucleic acid
dATP = A	deoxyadenosine triphosphate
dCTP = C	deoxycytidine triphosphate
dGTP = G	deoxyguanosine triphosphate
DMEM medium	Dulbecco's Modified Eagle Medium
dNTP	deoxyribonucleotide
dTTP = T	deoxythymidine triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminiscence
EDTA	ethylenediaminetetraacetic acid
EF	embryonic fibroblasts
ELISA	enzyme linked immunosorbant assay
ES cells	embryonic stem cells
FACS	fluorescence activated cell sorter
fbg	fibrinogen
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	grams
gDNA	genomic DNA
GlcNAc	N-acetyl-D-glucosamine
GPC	gel permeation chromatography

H_2O_d	destilled water
HAT medium	hypoxanthine-aminopterin-thymidine medium
His	histidin
HPRT	hypoxanthine-guanine phosporybosyl transferase
HRP	horseradish peroxidase
HSA	human serum albumin
HT medium	hypoxanthine-thymidine medium
IDA	iminodiacetic acid
Ig	immunoglobulin
IMAC	ion-metal affinity chromatography
IMP	inosine monophosphate
i.p.	intraperitoneal
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobases
kD	kilodalton
ko	knock out
LB medium	Luria Bertani medium
LIF	leukemia inhibitory factor
LP	lectin pathway
LPS	lipopolysaccharyde
LTA	lipothaicoic acid
mAb	monoclonal antibody
MALDI-TOF	matrix associated laser absorbance ionisation-time of light
Mb	megabases
MCS	multiple cloning site
min	minutes
mRNA	messanger RNA
MurNAc	N-acetylmuramic acid
MW	molecular weight
Neo^R	neomycin resistance cassette
Ni-NTA	nickel-nitrilotriacetic acid
NTA	2-naphthoyltrifluoracetate
OD	optical density
ORF	open reading frame
PAA	polyacrylamide
PBS	phosphate buffered saline
PCs	peritoneal cells

PCR	polymerase chain reaction
PECs	peritoneal exudate cells
Pen/Strep	penicillin/streptomycin
PMA	phorbol myristate acetate
PMNs	polymorphonuclear cells
PMSF	phenylmethylsulphonylfluoride
PVDF	polyvinylidene fluoride
RBCs	red blood cells
RIPA buffer	radioimmunoprecipitation buffer
RNA	ribonucleic acid
RT	room temperature
RT-PCR	retrotranscription PCR
S2	Drosophila Schneider-2 cells
s.c.	subcutaneously
sec	seconds
SAP	shrimp alkaline phosphatase
SDS	sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SP-A	surfactant protein A
SP-D	surfactant protein D
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
TK	thymidine kinase
TOPO	tri-o-octylphosphine oxide
TRIFMA	time-resolved immunofluorometric assay
TRITC	tetramethyl rhodamine isothiocyanate
Tween	Tween 20
U	units
vol	volume(s)
WB	Western Blot
wt	wildtype
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Summary

Innate immunity uses a variety of induced effector mechanisms to fight against infections or to control them until the causing pathogens are recognized by the adaptive immunity. The complement system represents one of the major mechanisms of effective innate host defense, proceeding as a cascade of reactions that occur on the surface of pathogens or infected cells and generate active components with various effector functions such as opsonization and pathogen lysis.

There are three distinct pathways by which the complement system can be activated: the classical, the lectin, and the alternative pathway, all of which converge to generate the same set of active products but depend on different molecules for their initiation. The lectin pathway starts when the mannan binding lectin (MBL) or ficolins bind to carbohydrate moieties on bacterial surfaces. This binding promotes the activation of the MBL-associated serine proteases (MASPs) which can cleave the next complement components, namely C2 and C4.

Ficolins are a group of proteins that contain collagen- and fibrinogen-like domains with a common binding affinity to N-acetyl-D-glucosamine (GlcNAc). While in humans two serum-type ficolins (L- and H-ficolin) and one cell-associated ficolin (M-ficolin) were described, two active forms have been found in mice, termed ficolin-A and –B. Ficolin-A is a plasma protein highly expressed in liver and spleen whereas ficolin-B mRNA is synthesized in spleen and bone marrow albeit the protein has not been localized so far.

In order to investigate the biological function of ficolins, the generation of specific tools (i.e. antibodies and mouse deficient lines) was required. For this, recombinant ficolin-A and –B were expressed in Drosophila Schneider 2 (S2) cells, purified by affinity chromatography, and used for the immunization of rats and rabbits for mono- and polyclonal antibody production, respectively. Polyclonal anti-ficolin-B antibodies were made monospecific by affinity-purification on a ficolin-B-coupled sepharose column, and characterized by ELISA, western blot, and dot blot. Furthermore, a total of four anti-ficolin-B antibody-secreting monoclonal hybridoma cell lines were obtained. One ficolin-B specific monoclonal antibody, termed 15H2D3, was purified on a protein-G sepharose column and found to recognize a conformational epitope on ficolin-B since it does not bind to the reduced form of the protein. The generation of anti-ficolin-A antibodies is still in progress.

By using the generated monospecific polyclonal anti-ficolin-B antibodies, localization experiments were performed since the cellular distribution of the protein had not been addressed before. Interestingly, ficolin-B was detected by intracellular staining of peritoneal exudate macrophages. In addition, double-staining experiments indicated that ficolin-B co-localizes with the lysosome associated membrane protein 1 (Lamp-1), suggesting an intracellular pathogen-recognition role of ficolin-B.

Biochemical characterization of the recombinant material showed that both ficolin-A and –B were able to form higher oligomeric structures which are required for biological functionality and are characteristic for the collectin family of proteins. However, no association of the S2-expressed ficolins to human MASP-2 was observed and, consequently, C4 cleavage could not be detected in a C4b deposition assay. This data indicates that at least the used recombinant mouse ficolins are not able to activate the lectin pathway of the complement system.

In addition, binding studies revealed an affinity of recombinant ficolin-B to GlcNAc and soluble peptidoglycan extracts from *Staphylococcus aureus*. The active substance which is being recognized by the lectin still remains to be identified. However, mild periodate treatment suggested that a carbohydrate moiety might be involved in the recognition. Furthermore, no binding of ficolin-B to lipoteichoic acid (LTA) and lipopolysaccharide (LPS) was detected.

Finally, a strategy to abolish the ficolin genes was designed. In this regard, specific targeting vectors for each ficolin were constructed and delivered into mouse embryonic stem cells by electroporation. Clones that underwent homologous recombination were selected and used for blastocyst injection. In this way, three chimeras were generated which are currently being backcrossed in order to obtain the homozygous ficolin-deficient mice on a pure C57Bl/6 background.

I Introduction

I.1 The innate immune system

I.1.1 The first line of host defense

The immune system is often divided into innate and adaptive. While the latter one involves the provision of a long-lasting and specific protection against formerly encountered pathogens, the term *innate immunity* refers to the basic resistance to disease that a species possesses. Upon an encounter with a new pathogen the innate immune mechanisms act immediately, and are followed by early induced responses, which can be activated by infection but do not generate immunological memory. Only if an infectious organism can breach these early lines of defense an adaptive immune response will ensue.

Our bodies are constantly exposed to microorganisms present in the environment. Contact with these microorganisms may occur through external or internal epithelial surfaces: the skin and the mucosa of the respiratory, the gastrointestinal, and the reproductive tracts, respectively. The epithelial surfaces serve not only as an effective physical barrier against most pathogens but also produce chemical substances that are microbicidal, e.g. the lysozyme and phospholipase A in tears and saliva, digestive enzymes and acid pH in the stomach, bile salts, fatty acids and lysolipids found in the upper gastrointestinal tract, and the surfactant proteins A and D (SP-A and SP-D) in the lung (see section I.3.3) (Janeway, Jr. *et al.,* 2005). Furthermore, most of the microorganisms that do succeed in crossing an epithelial surface are efficiently removed by innate immune mechanisms that function in the underlying tissues, preventing a site of infection from becoming established.

Whenever a microorganism is able to cross the epithelium, pathogen spread is countered by an inflammatory response that recruits effector cells and molecules of the innate immune system from local blood vessels, while inducing clotting further downstream in order to prevent pathogens from spreading through the blood. Macrophages, located in the submucosal tissues, are the first cells to encounter the pathogens, and they are soon reinforced by the recruitment of large numbers of neutrophils to the site of infection. Macrophages and neutrophils, both phagocytes, recognize microorganisms by means of their cell-surface receptors that can discriminate between self and non-self (see section I.1.2). These receptors include the mannose receptor (only on macrophages), scavenger receptors, which bind negatively charged ligands such as lipoteichoic acids (LTA, cell-wall components of Gram-positive bacteria), and CD14, a receptor for lipopolysaccharides (LPS, cell-wall component of Gram-negative bacteria) (Feizi, 2000) (Janeway, Jr. and Medzhitov, 2002). Upon ligation of many of these receptors by pathogen binding, phagocytosis of the microbe and its death inside the cell follow. Phagocytosis is an active process in which the bound pathogen is first surrounded by the phagocyte membrane and then internalized in a

membrane-bound vesicle known as the phagosome, where the microbe is killed by acidification of the vesicle. In addition, macrophages and neutrophils also possess vacuoles called lysosomes that contain enzymes, proteins, and peptides that can mediate intracellular killing of the bacteria. The phagosome fuses with one or more lysosomes to generate a phagolysosome in which the lysosomal contents are released to destroy the pathogen (Fig. I.1) (Janeway, Jr. *et al.,* 2005).

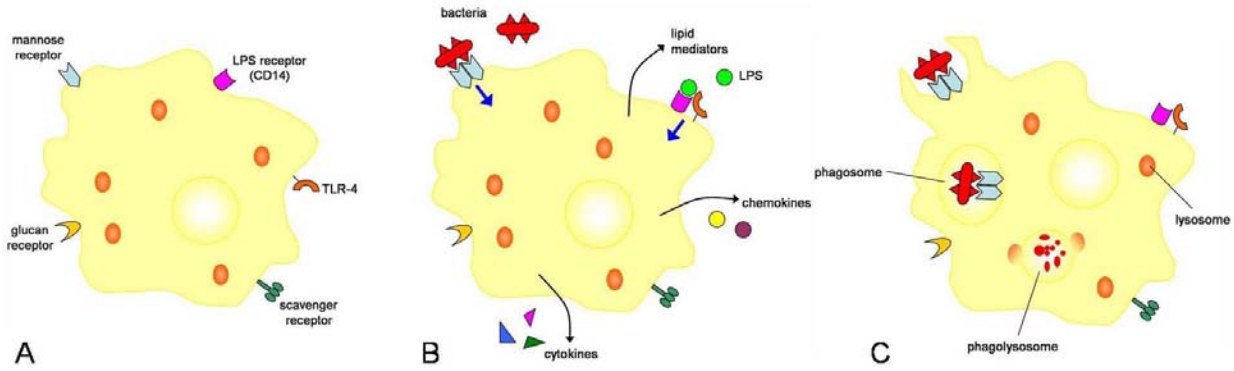

Fig. I.1: Macrophages phagocytose pathogens and initiate an inflammatory response. A) Macrophages express receptors for many bacterial components, including receptors for carbohydrates (mannose and glucan receptors), lipids (LPS receptor) and other pathogen-derived components (Toll-like receptors (TLRs) and scavenger receptors). **B)** Binding of bacterial components stimulates signaling through some receptors (blue arrows) such as TLRs, which causes the secretion of small lipid mediators, chemokines, and pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor (TNF), and **(C)** promotes the phagocytosis and the uptake of pathogens into intracellular vesicles, where they are destroyed (Janeway, Jr. *et al.,* 2005).

Upon phagocytosis, macrophages and neutrophils also produce a variety of other toxic products that help kill microorganisms. The most important of these are nitric oxide (NO), the superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2), which are directly toxic to bacteria (Janeway, Jr. *et al.,* 2005). Another important effect of the interaction between pathogens and tissue macrophages is the activation of these cells to release cytokines, chemokines (chemoattractant cytokines), and other mediators that set up a state of inflammation in the tissue and attract neutrophils and plasma proteins to the site of infection. Furthermore, receptors that signal the presence of pathogens have also the important role of inducing the expression of co-stimulatory molecules (i.e. B7.1/CD80 and B7.2/CD86) on macrophages and on dendritic cells, thus enabling these antigen-presenting cells to initiate an adaptive immune response (Janeway, Jr. and Medzhitov, 2002) (Barton and Medzhitov, 2002).

I.1.2 Innate immune receptors

Although the innate immune system lacks the specificity of the adaptive immunity that is necessary to produce immunological memory, it can distinguish self from non-self. This task is achieved through receptors on macrophages, dendritic cells, and neutrophils, which recognize features common to many pathogens. Unlike the receptors that mediate adaptive immunity, the receptors of the innate immune system promote very rapid responses upon binding to specific pathogen components without the delay of the clonal expansion of lymphocytes. They also mediate different functions: 1) some of them stimulate the ingestion of the pathogens they recognize ("phagocytic receptors"), 2) others, like the fMet-Leu-Phe receptor that binds *N*-formylated peptides produced by bacteria, are able to guide neutrophils to the site of infection ("chemotactic receptors"), and 3) some of them, including the phagocytic as well as the specialized signaling receptors, are able to induce the expression of effector molecules that contribute to the initiation and nature of any subsequent adaptive immune response, e.g. Toll-like receptors (Linehan *et al.,* 2000) (Janeway, Jr. and Medzhitov, 2002).

Independently of the effect they trigger, the innate immune receptors are able to recognize pathogens by their repeating surface patterns or nucleic acids. Bacterial DNA, for example, contains unmethylated repeats of the dinucleotide CpG. In addition, the sugar residues on the surface of bacteria are of a particular orientation as well as spacing only found on microbes (Medzhitov and Janeway, Jr., 2000b). The innate immune receptors are able to recognize pathogens by means of these regular patterns, and are therefore known as pattern-recognition receptors (PRR).

Collectins such as the mannose-binding lectin (MBL) and the already mentioned SP-A and SP-D are examples of PRRs. They are expressed in the liver as part of an acute-phase response and can opsonize bacteria (Holmskov *et al.,* 2003). The interaction of these soluble receptors with pathogens leads in turn to the binding of the receptor-pathogen complex by phagocytes, either through the interaction with the pathogen-bound receptor or through phagocyte receptors for complement, which also binds to the pathogen (see sections I.2.2 and I.2.3). The outcome is the phagocytosis and the killing of the pathogen and the induction of other cellular responses, e.g. cytokine release. A further detailed description of collectins will follow later in this chapter.

Among the cell-surface receptors present on phagocytes is the macrophage mannose receptor (Feizi, 2000) (Linehan *et al.,* 2000). This is a calcium dependent (C-type) lectin that binds certain sugar molecules found on the surface of many bacteria and viruses, including the human immunodeficiency virus (HIV). Although its recognition properties are very similar to those of the MBL, it can only function as a phagocytic receptor due to its cell association.

The so-called scavenger receptors are also phagocytic. They recognize anionic polymers and acetylated low-density lipoproteins, and are very heterogeneous in structure, existing in at least six different molecular forms. Moreover, some of them are involved in the removal of senescent red blood cells that have lost their sialic acids (which otherwise shield the scavenger receptors activity) (Janeway, Jr. and Medzhitov, 2002).

In addition to phagocytosis, stimulation of certain receptors by pathogen products can also lead macrophages and dendritic cells to display co-stimulatory molecules, which will eventually induce an adaptive immune response. The best known sets of receptors bearing these features are the Toll-like receptors (TLRs). There are 10 characterized TLRs expressed in mice and humans, each able to recognize a distinct set of molecular patterns that are not found in vertebrates. For instance, TLR4 on macrophages signals the presence of LPS by associating with CD14, the monocyte/macrophage receptor for LPS, while TLR2 senses peptidoglycans of Gram-positive bacteria among other bacterial constituents. In addition, TLR3 signals the presence of double stranded DNA (dsDNA), and TLR9 detects unmethylated CpG motifs (Medzhitov and Janeway, Jr., 2000a) (Janeway, Jr. and Medzhitov, 2002).

Stimulation of these receptors gives rise to an activated Toll signaling pathway which leads to the production of cytokines and chemokines upon NFκB activation. As stated before, this pathway also promotes the expression of co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86), which are expressed on the surface of both macrophages and tissue dendritic cells in response to LPS signaling through TLR4. Together with the antigenic microbial peptides presented by MHC-class-II proteins on the antigen-presenting cells (APC, dendritic cells, monocytes, macrophages or B-cells), these co-stimulatory molecules activate naïve $CD4^+$ T-cells, which in turn are needed to initiate adaptive immune responses (Fig. I.2). Furthermore, the APC must migrate to a nearby lymph node in order to encounter a $CD4^+$ T-cell. This migration is stimulated by cytokines such as the tumor necrosis factor (TNF), which is also induced by TLR4 signaling (Janeway, Jr. *et al.,* 2005). Thus, a proper adaptive immune response clearly depends on molecules induced as a consequence of the innate immune recognition and signaling.

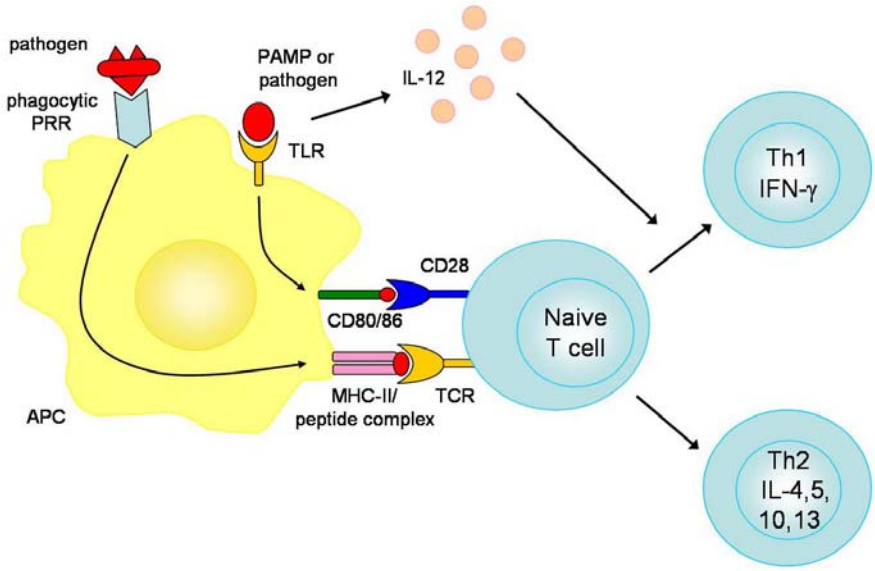

Fig. I.2: The interaction between pathogens and antigen-presenting cells is critical for the activation of naïve T-cells and starting of a proper adaptive immune response. Phagocytosed pathogens are processed inside the APC and the derived peptides are presented on the MHC-II molecule on the surface of the cell. In addition, upon binding to the pathogen, TLRs mediate signaling through the expression of co-stimulatory proteins (CD80/86). Both events contribute to the interaction of APCs with naïve T-cells via the T-cell receptor (TCR) and the CD28 molecule, respectively, which leads to T-cell activation. Many pathogens, especially intracellular bacteria and viruses, activate the APCs to produce IL-12 which causes proliferating $CD4^+$ T-cells to differentiate into Th1-cells. (Jones, 2005).

I.2 The complement system

The complement system was discovered by Jules Bordet in 1899 as a heat-labile component of plasma that “complements” the antibacterial activity of antibodies, promoting the opsonization and killing of bacteria. Today it is clear that complement can be activated in the absence of antibodies and that it probably evolved as part of the innate immune system, where it plays a very important role.

The complement cascade consists of more than twenty plasma proteins that react with one another in order to induce a series of inflammatory responses that help fight infection. Some of these proteins are zymogens that upon activation cleave their substrate, another complement zymogen, to its active form and in this way the activation of a small number of proteins at the start of the cascade is hugely amplified by each successive enzymatic reaction, resulting in the rapid generation of a large complement response (Janeway, Jr. *et al.,* 2005).

In the following sections, the activation, regulation, and biological effects of the complement system will be described.

I.2.1 Activation of the complement system

There are three distinct pathways through which the complement system can be activated: the classical, the lectin, and the alternative pathway (Janeway, Jr. *et al.,* 2005). Although they all converge to generate the same set of effector molecules, they depend on different molecules for their initiation (Fig. I.4)

The **classical pathway** (CP) starts when C1q binds to the pathogen surface. The binding of this molecule to a microorganism can occur in three different ways. First, it can bind directly to bacterial surface components such as certain proteins of the cell wall and polyanionic surface structures (i.e. LTA on Gram-positive bacteria). Second, C1q can bind to the C-reactive protein, which is a human plasma protein with specificity for phosphocholine residues in bacterial polysaccharides (i.e. the pneumococcal C polysaccharide). Third, as a linker between the innate and the adaptive immune systems, C1q binds to the Fc domains of antibody:antigen complexes (Arlaud *et al.,* 2002).
C1q is composed of six identical subunits joined together through their collagen-like tails that end in globular heads. In addition to C1q, the C1 complex consists of two other zymogens, C1r and C1s, resulting in the C1q:$C1r_2$:$C1s_2$ structure (Fig. I.3). When the globular heads of C1q bind to a pathogen surface or to an immune complex the $(C1r:C1s)_2$ complex is activated by a conformational change leading to the autocatalytic activity of C1r, which in turn cleaves C1s to generate an active serine protease (Arlaud *et al.,* 2002).

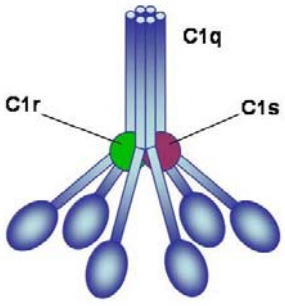

Fig. I.3: Structure of the first component of the classical pathway of complement activation. C1 is a complex of C1q, C1r, and C1s. C1q consists of six identical subunits with globular heads and long collagen-like tails which bind to two molecules of each C1r and C1s, forming the complex C1q:$C1r_2$:$C1s_2$ (Janeway, Jr. *et al*., 2005).

The **lectin pathway** (LP) uses similar molecules to C1q to trigger the complement cascade. These are the mannose-binding lectin (MBL) and ficolins, both members of the collectin family. As such, MBL and ficolins bind to carbohydrate structures typically present on microorganisms but masked on vertebrate cells. The way they activate the complement

cascade is also similar to the CP since they form a complex with protease zymogens called MBL-associated serine proteases (MASP-1, MASP-2, MASP-3 and MAp19, see section I.3.1) (Jack *et al.,* 2001). Due to a conformational change upon binding of the collectin to the bacterial surface, MASP-2 becomes active and cleaves the next complement component. The function of the other proteases in this complex still remains unknown (Matsushita *et al.,* 2000b), (Matsushita *et al.,* 2001). The structure of the molecules involved in this activation pathway will be discussed later (see section I.3).

When the complement system is activated by either the CP or the LP, the active protease (C1s or MASP-2, respectively) cleaves C4 to generate C4b, which binds covalently to the pathogen surface. Bound C4b then binds C2, making it susceptible to cleavage by the same proteases to produce the large fragment C2b, which is itself a serine protease. In this way the complex C4b2b remains attached to the pathogen where it serves as the C3 convertase for both the classical and the lectin pathways (Fig. I.4) (Janeway, Jr. *et al.,* 2005).

Finally, the **alternative pathway** (AP) can proceed on many microbial surfaces in the absence of specific antibodies or bacterial carbohydrates since it is thought to start by spontaneous hydrolysis of C3 in plasma. This cleavage, also known as “tickover”, occurs upon the hydrolysis of the thioester bond in C3 to form C3(H_2O). The altered conformation of C3(H_2O) favours the binding of the plasma protein factor B, which then allows the plasma protease factor D to cleave factor B into Ba and Bb, the latter remaining attached to C3(H_2O). The new molecule C3(H_2O)Bb is a fluid-phase C3 convertase which can cleave C3 into C3a and C3b. Although most of the C3b fragments will be inhibited by factor I (see section I.2.3), some C3b can still attach to pathogen surfaces where it can bind factor B, allowing its cleavage by factor D (Fig. I.4). This results in the formation of the AP C3 convertase, C3bBb, which is additionally stabilized by a positive regulatory factor known as properdin or factor P (Holers and Thurman, 2004). Properdin binds to many microbial surfaces and favours the stability of C3bBb (Janeway, Jr. *et al.,* 2005).

The formation of C3 convertases is the point at which the three pathways of complement activation converge, since both the C4b2b and C3bBb complexes can cleave C3 into C3a and C3b. The next step in the cascade is the generation of the C5 convertases. For the CP and the LP, this happens when multiple C3b molecules bind C4b2b to yield C4b2b$(3b)_n$. In the same way, the C3b fragment binds to C3bBb to form C3b_2Bb in the AP. Both C5 convertases are 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 C2b 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).

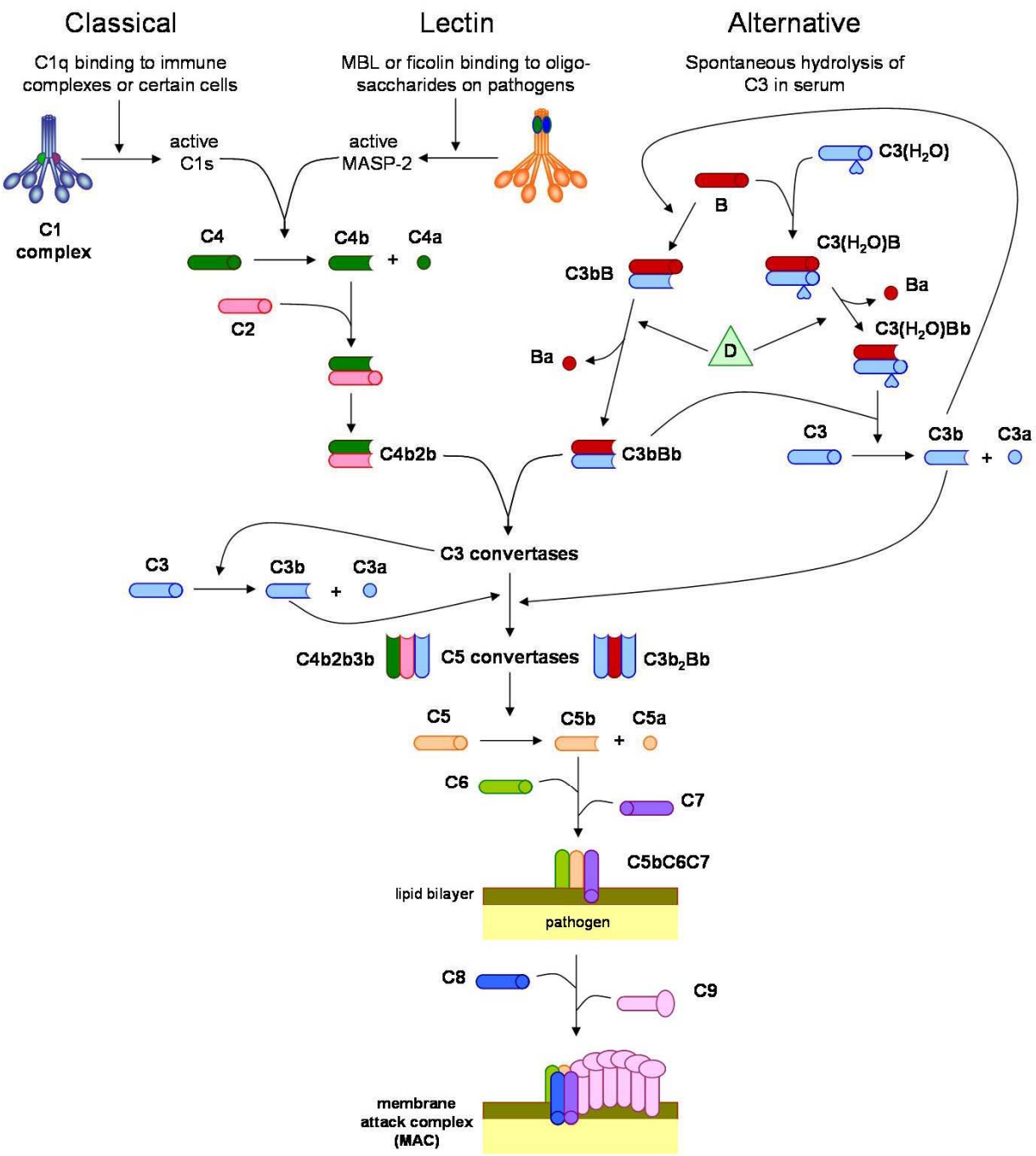

Fig. I.4: The three activation pathways of the complement cascade. In the classical pathway, the binding to an immune complex induces a conformational change in the C1 complex, leading to the activation of the serine protease C1s. In the lectin pathway, the MBL-associated serine proteases are activated when MBL or ficolins bind to sugar moieties on a microbial surface. In both cases, the active proteases cleave C4 and C2 to generate the C3 convertase C4b2b. In the alternative pathway C3 undergoes spontaneous hydrolysis that makes it susceptible for binding to factor B, which is in turn cleaved by factor D to generate the C3 convertase C3bBb. Convergence of the three activation pathways at the C3 cleavage leads to deposition of C3b and the formation of the C5 convertases. Cleavage of C5 then initiates the terminal pathway towards the formation of the membrane attack complex (MAC). The numerous regulators and inhibitors of complement activation are described in figure I.5.

The first step in the generation of the MAC is the consecutive binding of C6 and C7 to C5b. This binding promotes a conformational change in the constituent molecules, exposing a hydrophobic site on C7, which inserts into the lipid bilayer of the pathogen. The same exposure occurs to the later component C8 upon binding to the C5bC6C7 complex, enabling its insertion into the membrane. Finally, the inserted C8 induces the polymerization of 10 to 16 molecules of C9 into a pore-forming structure, the MAC (Parker and Sodetz, 2002). A scheme summarizing all these steps of the complement cascade is shown in figure I.4.

I.2.2 Biological effects of complement activation

There are different ways in which the complement system protects the body against infection. As shown in figure I.4, the last steps of the complement cascade lead to the formation of the MAC on the pathogen surface. This complex has a hydrophobic external face, allowing the association with the pathogen membrane, and a hydrophilic internal channel that enables the passage of solutes and water. The disruption of the lipid bilayer leads to the loss of homeostasis and the proton gradient across the membrane, the penetration of enzymes into the cell, e.g. lysozyme, and the eventual destruction of the pathogen (Parker and Sodetz, 2002) (Janeway, Jr. *et al.,* 2005).

However, this is not the only consequence of the activation of the complement system. In addition to the MAC formation, all along the cascade the complement generates large numbers of activated proteins that either opsonize the pathogen or act as chemoattractant molecules.

The opsonization of microorganisms is a very important effect because it promotes the uptake and destruction of pathogens by phagocytic cells. Both C3b (together with its proteolytic derivatives iC3b and C3dg) and C4b act as opsonins since phagocytes bear complement receptors (CRs) on their surface that can bind them. The first one of these receptors, CR1 (CD35), is expressed on macrophages and polymorphonuclear lymphocytes (PMNs), and can bind to C3b, C4b and iC3b (Whaley and Schwaeble, 1997). However, the interaction of CR1 with these complement products leads to phagocytosis only in the presence of other immune mediators that activate macrophages, e.g. the anaphylatoxin C5a. The other three complement receptors, named CR2 (CD21), CR3 (CD11b:CD18), and CR4 (CD11c:CD18), bind to the inactive forms of C3b that remain attached to the pathogen surface. These C3b derivatives are breakdown products that result from the fine regulation of the cascade (see section I.2.3) and are unable to form an active convertase. CR2 is found on B cells as part of a co-receptor complex that amplifies the signal received through the antigen-specific immunoglobulin receptor. In this way, a B cell that has been activated for a specific pathogen will be further stimulated if the pathogen is coated with an

inactive form of C3b, establishing a link between the innate and the adaptive immune system. Both CR3 and CR4 are found on macrophages, monocytes, PMNs and follicular dendritic cells (FDCs). Unlike the binding to CR1, the binding of iC3b to CR3 is sufficient to stimulate phagocytosis. The inactive derivative of C3b, C3dg, only binds to CR2 (Whaley and Schwaeble, 1997) (Meri and Jarva, 1998).

During complement activation, the cleavage of a molecule typically yields a small fragment named “a” and a big fragment designated “b”. Although it is the latter one binding to another molecule to keep the cascade going, the small fragments still play very important biological roles. When produced in large amounts, C3a, C4a and C5a (also referred to as anaphylatoxins) induce a generalized circulatory collapse similar to the anaphylactic shock, a systemic IgE-mediated allergic reaction. Although C5a has a higher specific activity, C3a is also able to promote smooth muscle contraction and increase vascular permeability. Furthermore, C5a and C3a act on the endothelial cells lining blood vessels to induce the expression of adhesion molecules, and on mast cells from the submucosal tissue to release mediators such as histamine and TNF that cause similar effects (Kohl, 2001).

As mentioned before, C5a, and to a smaller extent C3a and C4a, can increase the CR1 expression on macrophages to promote the destruction of pathogens. Both C3a and C5a signal through transmembrane receptors that have seven membrane-spanning domains and are coupled with intracellular guanine-nucleotide-binding proteins called G proteins. In addition, C5a can also act on neutrophils and monocytes to increase their adherence to vessel walls, their migration to the sites of infection, and their ability to ingest particles (Kohl, 2001), (Janeway, Jr. *et al.,* 2005).

In summary, the changes induced mainly by C5a and C3a recruit antibodies, complement components, and phagocytic cells to the site of infection. In this way, the increased fluid in the tissue accelerates the movement of pathogen-bearing APCs to the local lymph nodes, contributing to the initiation of the adaptive immune response.

I.2.3 Regulation of the complement system

Due to its tendency for rapid activation and ability to autoamplify the complement system must be well controlled. The relatively short half-life of the activated components naturally restricts the complement activation in a temporal and spatial manner. It is very important that every enzymatic event is confined to the same site where the cascade was triggered, so that C3b is deposited on the surface of the pathogen and not in the plasma or on host cells. When the complement system is activated via the CP or the LP, this restricted microenvironment is achieved by the covalent binding of C4b to the pathogen surface. If the binding does not occur, the exposed reactive thioester bond of C4b will be rapidly inactivated

by hydrolysis. In this way, the serine protease C2b is also confined to the pathogen surface since C2 is susceptible to cleavage only when it is bound to C4b. Therefore, the CP/LP C3 convertase C4b2b generates C3b molecules that are rapidly inactivated unless they opsonize the surface on which complement activation has taken place (Janeway, Jr. *et al.,* 2005).

In addition, to fully prevent self-damage and excessive production of active substances, a set of both fluid-phase and membrane-bound regulators is needed.

Among the fluid-phase regulators are factor I, the C1 inhibitor (C1-INH), C4 binding protein (C4bp), factor H, clusterin, and S-protein (vitronectin). Factor I is a plasma protease that inactivates C3b to iC3b in conjunction with membrane C3b-binding proteins that can act as cofactors (i.e. CR1 and MCP). The C1-INH belongs to the family of serine protease inhibitors (also known as serpins) and inhibits both the classical and the lectin pathways by blocking the C1r and C1s serine proteases of the C1 complex and MASP-2 and MASP-1, respectively (Matsushita *et al.,* 2000b) (Ambrus *et al.,* 2003). MASP-1 is also inhibited by α_2-macroglobulin (Ambrus *et al.,* 2003).

On the other hand, C4bp acts in two different ways: as a decay accelerating factor for the CP C3 convertase (C4b2b), by irreversibly displacing C2b from the complex, and as a cofactor for the cleavage of C4b by factor I leading to inactivation of C4b into C4c and C4d. Factor H acts as the C4bp analogue for the alternative pathway by inhibiting the AP C3 convertase (C3bBb). The mechanism by which Factor H can distinguish C3b bound to a host cell or a pathogen relies on its high affinity for the terminal sialic acids of host cell membranes.

Clusterin is a multifunctional plasma protein that binds to the terminal complement complex and prevents its insertion into cell membranes. The resulting complexes are therefore soluble (SC5b-9) and unable to induce complement lysis.

Finally, the S-protein also prevents the assembly of the cytolytic MAC on cell membranes and can also inhibit polymerization of C9. Targets marked by S-protein like apoptotic or complement-attacked cells may become phagocytosed by macrophages expressing the vitronectin receptor ($\alpha_v\beta3$) (Whaley and Schwaeble, 1997) (Meri and Jarva, 1998).

Membrane regulators of the complement system include four well-characterized molecules. Three of them, the C3b receptor (CR1, CD35), the decay accelerating factor (DAF, CD55), and the membrane cofactor protein (MCP, CD46) act as inhibitors of the C3/C5 convertases whereas protectin (CD59) is an inhibitor of the MAC. CR1, as mentioned before, has binding affinity for C3b and also for C4b, acting as a decay accelerating factor of the C3/C5 convertases. In addition, CR1 serves as a cofactor for factor I-mediated cleavage of C3b and C4b as well as for the cleavage of iC3b to C3c and C3dg. DAF is present on peripheral blood cells, vascular endothelial cells, placenta, and many types of epithelial cells, where it binds and dissociates both the CP and AP C3/C5 convertases acting intrinsically, i.e. on the same

cell where it is located. MCP has a similar structure to DAF and binds to C3b but, although it acts as a cofactor for factor I-mediated C3b cleavage, it has weak activity for the cleavage of C4b. Finally, the function of protectin is to inhibit the final steps of the MAC assembly on cell membranes by binding to the C5b-8 complex and limiting the C9 input and polymerization (Whaley and Schwaeble, 1997), (Meri and Jarva, 1998), (Janeway, Jr. *et al.,* 2005).

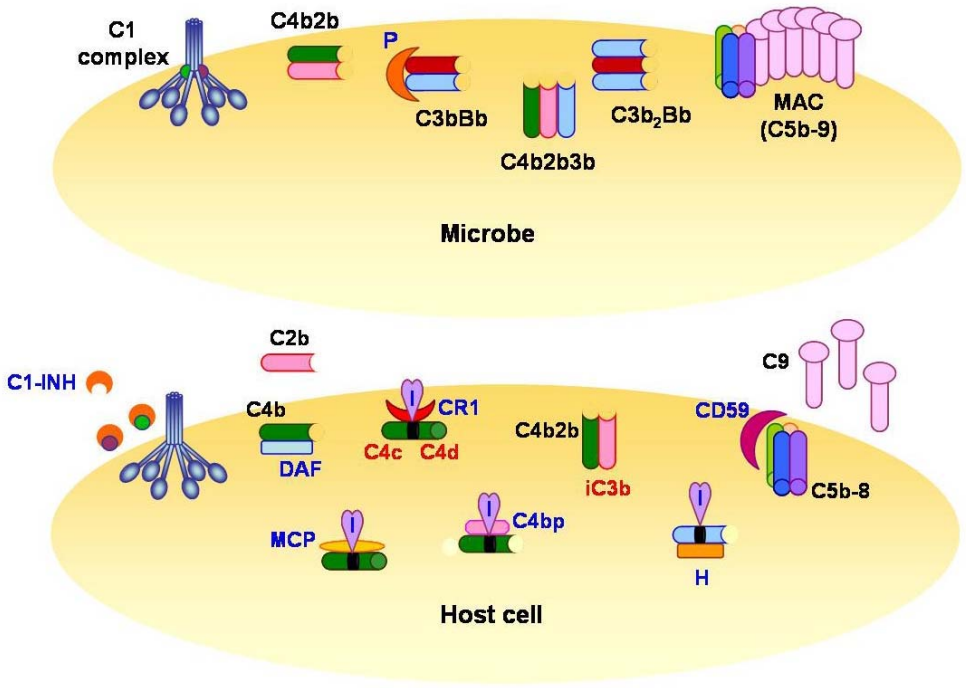

Fig. I.5: Regulation of the complement system: comparison of the complement components found on a microbial surface (upper cell) and a host cell (lower cell). Complement activation is regulated by a series of proteins (shown in blue) that serve to protect host cells from accidental damage, acting on different stages of the cascade. They can dissociate complexes (C1-INH, DAF, C4bp, factor H), catalyse the enzymatic degradation of covalently bound complement proteins (factor I) or serve as cofactors for factor I-mediated cleavage (CR1, MCP). Protectin (CD59) avoids the complete assembly of the MAC by preventing C9 input and polymerization. Clusterin and S-protein are soluble proteins that also interfere with the MAC formation (not shown in the picture). Factor P (properdin) is a positive regulator of the complement system acting on the pathogen surface where it stabilizes the AP C3 convertase, C3bBb. Breakdown products of the cascade and degradation products are shown in black and red, respectively.

I.2.4 Complement deficiency

Inherited complete or partial deficiencies of single complement components have been reported for most constituents and are associated with an increased susceptibility to infections from organisms that are normally opsonized or lysed by complement. The deficiencies can be grouped according to the most prominent symptoms.

A deficiency of proteins of the CP (C1, C2, C4) results in immune complex disease, presented frequently as systemic lupus erythematosus (SLE) (Ohashi and Erickson, 1998). Rare deficiencies of AP components (i.e. properdin and factor D) emphasize the importance of the amplification role of this pathway in host defense. Approximately 50% of the patients suffer severe, fulminant, pyogenic *Neisseria* infections, with meningococci frequently involved and a fatality rate of 75%. The susceptibility to *Neisseria* infections is also found in patients with deficiencies of the terminal components of the complement cascade (C5 to C9). Patients with deficiencies of C3, factor H, and factor I are predisposed to a broader range of bacterial infections, particularly to pyogenic bacteria (Janeway, Jr. *et al.,* 2005).

Hereditary angioedema (HAE) is an inherited autosomal dominant illness caused by the lack of C1-INH. The absence of this regulator lowers the threshold of CP activation and as a result C4 and C2 are consumed during HAE attacks. In its most severe form an untreated attack can lead to suffocation by obstruction of the upper airways (Ohashi and Erickson, 1998). Another illness involving a deficiency in complement regulators is the paroxysmal nocturnal hemoglobinuria (PNH), which is a rare hemolytic disorder where the lack of protectin (CD59) and DAF (CD55) accounts for most of the symptoms. The reason of the deficiency is a mutation in a hematopoietic stem cell leading to the lack of all GPI-anchored proteins in the daughter cells. With regard to the symptoms the deficiency of protectin seems to be more important than that of DAF (Meri and Jarva, 1998).

Concerning the LP, MBL deficiency has been investigated in many clinical studies. MBL binds to a wide range of pathogenic bacteria, e.g. *Staphylococcus*, *Neisseria*, *Salmonella* and *Escherichia coli* (Jack *et al.,* 2001) (Jack and Turner, 2003), and MBL-deficiency is associated with an increased risk of infections in children (Super *et al.,* 1989) (Summerfield *et al.,* 1997) (Cedzynski *et al.,* 2004) and in adults (Summerfield *et al.,* 1995).

However, the fact that most MBL-deficient individuals are healthy (Dahl *et al.,* 2004), has given rise to the theory that a second immune defect needs to be present for a higher susceptibility to infection. Patients deficient in MBL have an increased risk of severe infections after operations (Siassi *et al.,* 2003), or stem cell transplantation (Mullighan *et al.,* 2002). Patients admitted to intensive care units suffering from systemic inflammatory response syndrome are more likely to develop severe sepsis or septic shock when they have low MBL serum levels (Garred *et al.,* 2003).

Furthermore, MBL plays a role in viral infections. MBL-deficient individuals have an increased risk of HIV infection and an increased susceptibility to coinfections (Garred *et al.,* 1997).
MBL is also involved in parasitic infections. It is known to bind to *Trichinella spiralis* (Gruden-Movsesijan *et al.,* 2003) and to recognize parasite-derived proteins on the surface of cells infected with *Plasmodium falciparum* (Klabunde *et al.,* 2002).
MBL has also been associated with autoimmune diseases like SLE or rheumatoid arthritis (RA). MBL-deficiency is a risk factor for acquiring SLE and increases the risk of complicating infections (Garred *et al.,* 1999). In RA the frequency of variant alleles is higher in patients with early onset of disease (Garred *et al.,* 2000) and fast progression of radiographic joint destruction (Graudal *et al.,* 2000) (Saevarsdottir *et al.,* 2001).
To date no ficolin deficiencies have been reported. However, two independent studies on L-ficolin polymorphisms have been recently reported (Hummelshoj *et al.,* 2005) (Herpers *et al.,* 2005), and it seems that these genetic variations are associated with both the serum levels and the N-acetyl-D-glucosamine (GlcNAc) binding activity of this lectin. In addition, it was also reported that children with recurrent infections have low L-ficolin concentrations (Atkinson *et al.,* 2004).

I.3 Pattern recognition molecules

As mentioned before (see section I.1.2), macrophages and dendritic cells do not possess the antigen receptors that are expressed by lymphocytes. Instead, these phagocytes employ a limited number of germline-encoded receptors or soluble molecules for pathogen recognition. These molecules are called pattern recognition receptors (PRRs) and appear to have evolved to distinguish microbial pathogens from self-antigens through the recognition of molecular arrays, called pathogen-associated molecular patterns (PAMPs). PAMPs are essential for the survival of certain microbial groups, and are, therefore, highly conserved among the different classes of pathogens (Medzhitov and Janeway, Jr., 2000b). Lectins form a class of PRRs that bind specifically to the unique carbohydrate moieties on microbes (Lu *et al.,* 2002).
One of the outstanding advances in recent research on the complement system was the discovery of the lectin pathway which is activated by the mannose-binding lectin (MBL) in association with MBL-associated serine proteases (MASPs) upon binding to mannose or GlcNAc residues on pathogen surfaces. Ficolins are lectins containing both collagen- and fibrinogen-like domains (Lu and Le, 1998) and, recently, human serum ficolins have also been shown to be complexed with MASPs. Together with the characterization of human,

murine and porcine ficolins that revealed a common binding specificity for GlcNAc (Matsushita *et al.,* 1996), this fact suggests that serum ficolins are also lectin pathway-activating lectins like MBL (Matsushita *et al.,* 2000a).

I.3.1 MBL and MASPs

The mannose-binding lectin is the best studied member of the collectin family. 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 and Hamvas, 2000). MBL forms homotrimers composed of a collagenous triple helix subunit and several of these homotrimers assemble to form higher order oligomers (Fig. I.6.A). 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 *et al.,* 1995).

While only one MBL gene is expressed in humans, two MBL genes are expressed in rodents, namely MBL-A and MBL-C. Both genes encode a polypeptide with a molecular weight of 28 kDa (Ihara *et al.,* 1991) and both are present in serum (Hansen *et al.,* 2000). Mouse MBL-C, however, seems to form higher oligomeric structures, with a molecular weight of 950 kDa (compared to 850 kDa for MBL-A), and also has a higher serum concentration (Liu *et al.,* 2001). Mouse MBL-A and MBL-C show a 60% sequence identity at the DNA level, whereas human MBL is more closely related to MBL-C (Holmskov *et al.,* 1994). The liver is the main organ of MBL biosynthesis, but its expression was also found in other tissues in the mouse (Uemura *et al.,* 2002) (Wagner *et al.,* 2003).

MBL binds to monosaccharides such as N-acetyl-D-glucosamine, mannose, N-acetyl-D-mannosamine, L-fucose and glucose (Hansen *et al.,* 2000) in a Ca^{++}-dependent manner. 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 *et al.,* 2001) (Townsend *et al.,* 2001) (Jack and Turner, 2003), but not on mammalian cells, because of the prevalent termination of self-glycoproteins with sialic acid or galactose (Ezekowitz, 1998) (Wallis, 2002). Some bacteria protect themselves from MBL-mediated complement attack by sialylating their surface structures (Jack *et al.,* 2001).

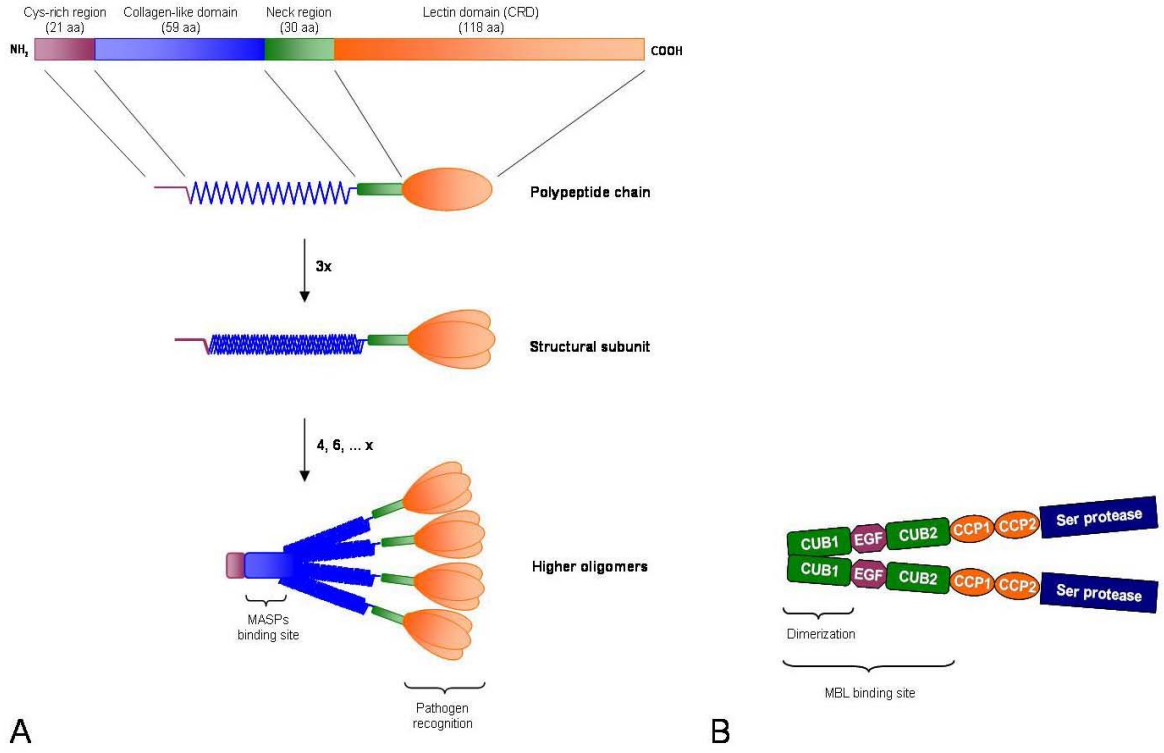

Fig. I.6: Structural organization of MBL and MASP-2. A) An MBL tetramer is shown. Lower and higher oligomers have similar organizations. **B)** Schematic representation of MASP-2 dimer. **CRD:** carbohydrate recognition domain; **CUB:** C1r/C1s/Uegf/bone morphogenic protein 1; **CCP:** complement control protein domain; **EGF:** epidermal growth factor domain. (Petersen *et al.,* 2001).

Initially it was believed that MBL associates with the $C1r_2$-$C1s_2$ complex to activate the lectin pathway (Lu *et al.,* 1990), but later it was demonstrated that a novel C1s-like protease is attached to MBL, which cleaves C4 and C2 (Matsushita and Fujita, 1992). To date three serine proteases, named MBL-associated serine proteases (MASP-1, -2 and -3) and an enzymatically inactive product of the MASP-2 gene (MAp19) have been identified in complex with MBL. The organization is similar for all four proteins and consists of a CUB domain (also called C1r/C1s-like domain), followed by an epidermal growth factor (EGF) domain, a second CUB domain, two complement control protein (CCP) domains, and a serine protease domain (Fig. I.6.B). MAp19 lacks not only the serine protease region but also the second CUB domain and both CCP domains, thus explaining its low affinity for MBL binding and the inability to activate complement (Wong *et al.,* 1999).

MASPs form homodimers through interactions of the N-terminal CUB domain. These homodimers bind through the N-terminal CUB1-EGF-CUB2 domains (Chen and Wallis, 2001) to the N-terminal portion of the collagenous domain of MBL (Wallis, 2002) in a Ca^{++}-dependent manner.

As mentioned before, in circulating complexes MASP-2 is inactive as a zymogen. However, upon binding of MBL to its microbial target, a conformational change exposes an accessory C4 binding site on MASP-2 (Chen and Wallis, 2004). Subsequently, MASP-2 cleaves C4 and C2, thereby activating the lectin pathway (Thiel *et al.,* 1997) (Vorup-Jensen *et al.,* 2000).
The exact role of MASP-1 is not clear at the moment. Initially it has been reported to directly cleave C3 (Matsushita *et al.,* 2000b), although other studies have reported contrary results (Wong *et al.,* 1999) (Ambrus *et al.,* 2003). However, it has been shown that MASP-1 seems to cleave C2 (Ambrus *et al.,* 2003), and its role as an enhancer of complement activation has been suggested (Chen and Wallis, 2004).
MASP-3 is derived from the same structural gene as MASP-1 by alternative splicing (Stover *et al.,* 2003). Its natural substrate has not been identified yet, but it has been proposed that it might act as an antagonist of MASP-2 (Zundel *et al.,* 2004).
MAp19 is an alternative splice product of the MASP-2 gene (Stover *et al.,* 1999a) (Stover *et al.,* 1999b). It is found in association with MBL or in complex with MASP-1 in serum, but its physiological role has not been determined yet (Thiel *et al.,* 2000).

I.3.2 Ficolins

Ficolins were first documented as transforming growth factor-β1-binding proteins on pig uterus membranes by Ichijo and co-workers in 1991 (Ichijo *et al.,* 1991). Upon characterization and sequencing, they described the novel proteins as molecules containing both collagen- and fibrinogen-like (fbg-like) domains and, thus, named them ficolins (Ichijo *et al.,* 1993). Since the first description of porcine ficolins, proteins having such structural features have been identified at the cDNA and/or protein levels in human (Matsushita *et al.,* 1996) (Lu *et al.,* 1996) (Sugimoto *et al.,* 1998), rodents (Fujimori *et al.,* 1998) (Ohashi and Erickson, 1998), *Xenopus* (Kakinuma *et al.,* 2003) and invertebrates (Kenjo *et al.,* 2001), showing different tissue distributions, thus suggesting different local functions. Moreover, it has been shown that ficolins present in human, mouse, and pig serum/plasma are lectins with a common binding specificity for GlcNAc (Matsushita *et al.,* 1996).
Like collectins, ficolins are built of structural subunits each composed of three identical polypeptide chains. In each chain, a short N-terminal region with one or two cysteine residues is followed by a collagen-like domain, a short link region, and a subsequent fbg-like domain. Although ficolins do not have a coiled-coil structure acting as the neck region like MBL (Holmskov *et al.,* 2003), they form active oligomers when normally four subunits join together at the N-terminal regions with the polypeptide chains radiating out in a sertiform structure. Higher or smaller oligomers appear to be less common for ficolins than for MBL (Holmskov *et al.,* 2003).

The carbohydrate-binding activity of ficolins is assigned to the fibrinogen-like domain which, in contrast to the CRD of MBL, has a Ca^{++}-independent lectin activity (Le *et al.,* 1997). The fbg-like domain consists of 220-250 residues and is characterized by the presence of 24 invariant, mostly hydrophobic, amino acids including 4 cysteines and 40 highly conserved residues. According to the crystal structure resolved for the fibrinogen CRD of tachylectin 5A (a nonself-recognising lectin from the hemolymph plasma of *Tachypleus tridentatus*), the contact between the protein and the carbohydrate is mediated by four aromatic side chains that form a funnel in which the methyl group of GlcNAc fits in the center (Kairies *et al.,* 2001). Figure I.7 shows a schematic representation of the best known collectins and ficolins (i.e. MBL, surfactant proteins A and D, and human ficolins) in their trimeric subunits. In the following sections pig, human and mouse ficolins will be described in detail.

I.3.2.1 Pig ficolins

Pigs have two distinct but closely related ficolin genes, α and β (Ichijo *et al.,* 1993). Ficolin-α is expressed in liver, bone marrow, spleen, lung (Ohashi and Erickson, 1998) and very weakly in the uterus (Ichijo *et al.,* 1993), whereas ficolin-β is expressed in bone marrow and neutrophils (Brooks *et al.,* 2003b). Ficolin-α and –β share 81-84% identity at the amino acid level.
A study using recombinant ficolin-α with mutations at the N-terminal cysteine residues (Cys^4, Cys^{24} and Cys^4/Cys^{24}) revealed that these amino acids are responsible for the intermolecular disulphide bonds that keep the subunits together, which is consistent with previous findings on collectin multimerization (Ohashi and Erickson, 2004).

Ficolin-α is the major plasma ficolin and consists of N-glycosylated subunits of 35 kDa (Ohashi and Erickson, 1998). It was shown to bind *Actinobacillus pleuropneumoniae* serotype 5B (APP5), which is the pathogen causing economically-significant pneumonic and septicemic diseases in young pigs, in a GlcNAc-dependent manner (Brooks *et al.,* 2003a). In addition, Nahid and co-workers recently described that native ficolin-α, purified from porcine serum, is able to bind to LPS from Gram-negative bacteria of both the rough- and smooth-types, such as *Escherichia coli, Salmonella typhimurium, S. enteriditis, S. abortus equi, Shigella flexeneri, Pseudomonas aeruginosa,* and *Serratia marcescens*. Furthermore, it was also shown that ficolin-α reacts with LTA from Gram-positive bacteria, such as *Streptococcus sanguis, S. pyogenes, Bacillus subtilis, and Staphylococcus aureus* (Nahid and Sugii, 2006).

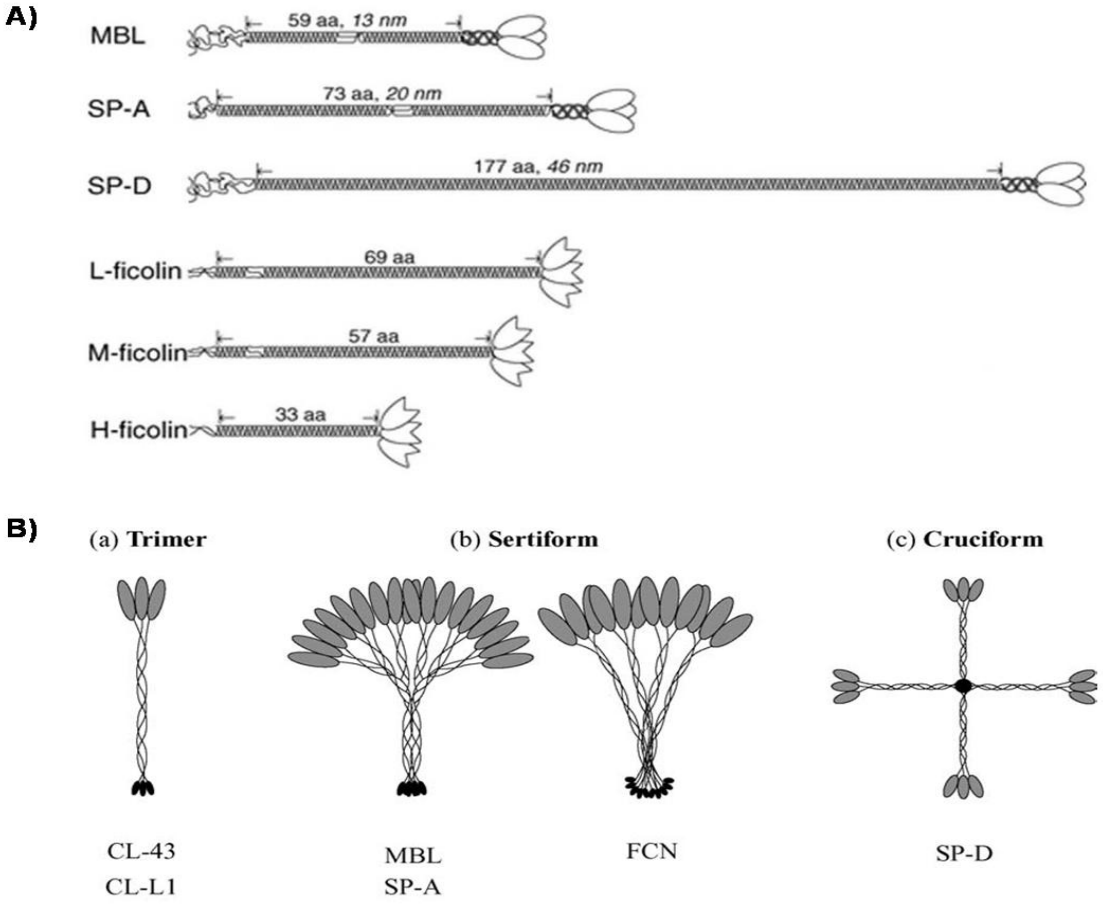

Fig. 1.7: The structures of collagenous lectins in animals. A) Trimeric subunit structures of human collectins and ficolins. The molecules are drawn approximately to scale. The number of amino acids spanning the collagen like domains, including interruptions, is indicated. Fbg-like domains are represented 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).

Ficolin-β has an apparent molecular weight of 39 kDa and was found to be synthesized, stored, and secreted by porcine neutrophils but not by peripheral blood monocytes or platelets (Brooks *et al.,* 2003b). Ficolin-β is present in both cytoplasmatic and membrane fractions of neutrophil preparations but its subcellular distribution has not been shown. It seems that ficolin-β might function locally as a secreted collagenous defense lectin at sites of inflammation where neutrophils are activated. The secreted contents of PMA-activated neutrophils are bactericidal and ficolin-β may participate with other antibacterial neutrophil products in tissue antisepsis (Brooks *et al.,* 2003b). However, it is still unknown if pig ficolins can activate the complement system.

I.3.2.2 Human ficolins

In humans three ficolins have been characterized: L-ficolin (also known as ficolin-2, ficolin/P35, EBP-37 or hucolin), H-ficolin (also known as the Hakata antigen or thermolabile β-2 macroglycoprotein), and M-ficolin (also known as ficolin-1 or ficolin/P35-related protein). While M- and L-ficolin share a 79% identity at the amino acid level, the primary sequences of H- and M-ficolin are only 45% identical. In addition to a high sequence similarity, L- and M-ficolin also share a similar exon-intron structure, and it was suggested that the gene encoding L-ficolin originated after gene-duplication of the M-ficolin gene (Endo *et al.,* 2004)

L-ficolin is a multimeric plasma protein consisting of 35 kDa subunits with the typical ficolin organization (see section I.3.2), and it has been proposed to form a tetrameric structure of four triple helices composed of twelve subunits (Ohashi and Erickson, 1998). The gene encoding L-ficolin (*FCN2*) consists of eight exons and seven introns and has been assigned to chromosome 9.

The primary site of synthesis for L-ficolin is the liver and its concentration in sera from 181 blood donors was found to range from 1.1 to 12.8 (median 3.7) μg/ml (Kilpatrick *et al.,* 1999). Like MBL, L-ficolin shows lectin activity for GlcNAc. However, L-ficolin binds to the GlcNAc residue next to galactose at the nonreducing terminal of the complex-type oligosaccharide and it does not bind to mannose (Matsushita *et al.,* 1996).

L-ficolin binds to *Salmonella typhimurium* TV119 (Ra chemotype strain with exposed GlcNAc) and enhances phagocytosis by polymorphonuclear neutrophils and monocytes. In addition to this opsonic activity, it can also activate complement through a Ca^{++}-dependent association with MASPs (Matsushita *et al.,* 2000a). In addition, it has been demonstrated that L-ficolin can bind to *Escherichia coli* and be eluted with a mixture of monosaccharides (Lu and Le, 1998).

Recently, it was demonstrated that L-ficolin/MASP complexes from sera specifically bind to LTA from *S. aureus* and initiate the C4 turnover (Lynch *et al.,* 2004). This was also shown to be true for other clinically relevant bacteria such as *S. pyogenes* and *S. agalactiae* (Lynch *et al.,* 2004).

In another work on bacterial recognition by ficolins, it was reported that L-ficolin binds to some capsulated *S. aureus* and *S. pneumoniae* serotypes but not to noncapsulated strains, which differed from MBL and H-ficolin binding properties (Krarup *et al.,* 2005). These results indicated that the binding of each lectin is directed toward specific and different PAMPs.

Interestingly, Krarup and co-workers showed that L-ficolin binds to *Streptococcus pneumoniae* 11F and that this interaction could 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.
In addition, Gaboriaud and co-workers resolved the structure of a trimer of the recombinant L-ficolin fbg-like domain by X-ray crystallography, and revealed that the ligand binding site of L-ficolin is situated at a position different from that seen in the tachylectin (see section I.3.2), at the opposite side of the globular domain (Krarup *et al.,* 2004). This unexpected structure called for caution when trying to deduce functionalities from orthologous structures.
Finally, it was also proven that, like MBL and H-ficolin, L-ficolin participates in the clearance of apoptotic cells through complement activation (Kuraya *et al.,* 2005).

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 synthesized by hepatocytes and bile duct epithelial cells, as well as 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 in the same report it was shown that H-ficolin displays 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). Sequence analysis of clones derived from a human uterus cDNA library revealed that 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 fbg-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). Even though its primary structure lacks an apparent transmembrane domain, M-ficolin was found on the surface of PBMs (Teh *et al.,* 2000). In the same report, recombinant M-ficolin isolated from transfected COS-7 cells migrated as 40 kDa bands on SDS-PAGE, and as expected 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-fbg 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, as the (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. 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 bacteria recognition might be different among ficolins.

I.3.2.3 Mouse 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). The protein encoded by this gene located on chromosome 2 is 60%, 59.3%, 59.1%, and 59% identical to those of porcine ficolin-α, –β, human M-ficolin, and L-ficolin, respectively (Fig. I.8) (Ohashi and Erickson, 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 fbg domains (12-mers) (Ohashi and Erickson, 1998).

In a recent report, 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 Kupfer cells in liver, suggests that ficolin-A mRNA is expressed by macrophage (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). More recently, ficolin-B mRNA levels were described as progressively increasing from E.13.5 to E18.5 with a subsequent rapid postnatal decline to undetectable levels in liver prior to the age of 4 weeks. However ficolin-B mRNA was detected in 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 (CD3e$^+$), or the B-cell (B220$^+$) lineages (Liu *et al.,* 2005a). To date, however, all the reports on ficolin-B were performed at DNA or RNA level, whereas ficolin-B protein has never been detected.

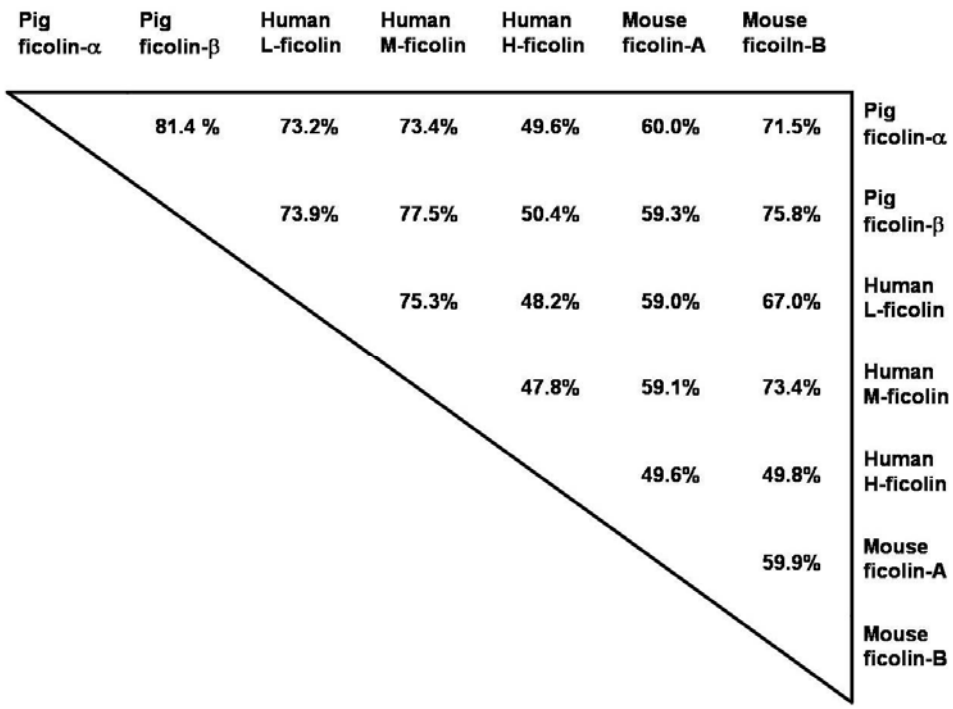

Fig. I.8: Comparison of the sequences of pig, human, and mouse ficolins at the amino acid level. (Ohashi and Erickson, 1998).

The fact that mice possess two and humans three forms of ficolins, led Endo and co-workers to perform phylogenetic studies on the structures and organization of these ficolin genes. Evidence had suggested that L-ficolin was closely related to ficolin-A (serum-type), and M-ficolin to ficolin-B (non serum-type). However, the phylogenectic tree based on amino acid sequences indicated that L-ficolin diverged from the B/M-ficolin lineage, suggesting that mouse ficolin-A is not the orthologue of human L-ficolin, although both are mainly expressed in liver and have similar sugar specificities. These results imply that comparable selective pressures acted independently on both the murine and primate lineages to produce these hepatic serum-type ficolins from a non serum-type lineage (Endo *et al.,* 2004). In addition, the tree suggested that H-ficolin had an ancient origin back to an evolutionary stage before the divergence of the *Xenopus* lineage, although no H-ficolin had been identified in mice. By computer analysis of the mouse genome data base, Endo and others characterized the genomic region homologous to the human H-ficolin gene and identified the mouse H-ficolin gene as a pseudogene on chromosome 4 (Endo *et al.,* 2004). In contrast to the human gene, the first exon of the mouse ficolin-H pseudogene has stop codons in all three frames, which were generated by base changes and micro-deletions, and no start codon could be identified, despite its 67% nucleotide sequence identity to the human sequence. This fact, together with some other stop codons distributed along the gene, suggests that it is unlikely that the H-ficolin gene in mice functions to produce a normal protein (Endo *et al.,* 2004). The same seems to be true for rat H-ficolin (Endo *et al.,* 2004).

I.3.3 Other collectins

Collectins are calcium-dependent carbohydrate-recognising proteins that are composed of collagenous structures and C-type CRDs (Holmskov *et al.,* 1994). All vertebrates employ collectins in their innate immune defense, and to date the collectin family has five well-characterized members: MBL, lung surfactant protein A (SP-A) and D (SP-D), bovine conglutinin and collectin-43 (CL-43). Recently, another novel collectin, called collectin liver 1 (CL-L1) has been found in most tissues except skeletal muscle (Lu *et al.,* 2002).

Both SP-A and SP-D are mostly synthesized in the lung by type II alveolar cells and are secreted into the alveolar space. SP-A was initially identified in the lung surfactant as a relatively hydrophilic protein component, which also appears in the amniotic fluid. However, SP-A or SP-A-like proteins have also been detected in extrapulmonary tissues such as prostate and thymus. Bovine conglutinin, which is synthesized in the liver, was the first member of the collectin family to be characterized and probably the first animal protein that was demonstrated to have lectin activity. CL-43 is also found exclusively in the *bovidae* as a serum protein synthesized in the liver (Lu *et al.,* 2002).

	Tissues of origin	Tissues of presentation	Sugar specificity	Pathogen interaction
Collectins				
MBL (human)	Liver (hepatocytes) and other tissues	Serum	GlcNAc>mannose/fucose> ManNAc>>maltose>glucose	*S. aureus, E. coli, H. influenzae, S. pneumoniae, S. cerevisiae, A. fumigatus, C. albicans*, HIV, IAV, RSV.
MBL-A (mouse)	Liver, kidney, lung, testis, spleen, brain	Serum, kidney, spleen	ManNAc, GlcNAc, mannose, glucose	*A. suis, A. pleuropneumonieae, H. parasuis.*
MBL-C (mouse)	Liver, kidney, small intestine, thymus, spleen, brain	Serum, small intestine	ManNAc, GlcNAc, mannose, fucose, glucose, maltose, galactose, lactose, GalNAc	*Neisseria* spp*., S. typhimurium, Y. enterocolitica, P. aeruginosa, E. coli, H. pylori, M. bovis, C. albicans,* RSV, HIV, IAV
SP-A	Lung epithelium, prostate, thymus, intestinal mucosa and other tissues	Bronchus, alveolus, mucosal surfaces, semen	ManNAc>fucose/maltose>glucose>mannose/galactose	*S. aureus, E. coli, S. pneumoniae*, *S. cerevisiae, A. fumigatus, P. carinii,* IAV, RSV, HSV-1
SP-D	Lung and gastrointestinal epithelium and other tissues	Bronchus	maltose>fucose>mannose> glucose>>glucosamine	*M. tuberculossis, P. aeruginosa, E. coli, S. cerevisiae, A. fumigatus, C. albicans, P. carinii, IAV, RSV*
Conglutinin	Bovine liver	Serum	GlcNAc>>mannosamine>fucose/ mannose>glucose>ManNAc/ glucosamine>maltose	acapsular *C. neoformans,* IAV, rotavirus
CL43	Bovine liver	Serum	mannose/ManNAc>fucose> GlcNAc>glucose/maltose> galactose>lactose>>GalNAc	acapsular *C. neoformans*, rotavirus
CL-L1	All tissues except skeletal muscle	n.d.	Mannose	n.d.
Ficolins				
L-ficolin	Liver	Serum	GlcNAc/ManNAc>>GalNAc/ CysNAc/GlyNAc, acetylcholine, elastin, corticosteroids, 1,3-b-D-glucan, LTA from *S. aureus, S. pyogenes, S. agalactiae, B. subtillis*	*S. typhimurium (Ra), E. coli, S. pneumoniae*
H-ficolin	Liver (hepatocytes and bile epithetlium), type II alveolar cells	Serum, bronchus, alveolus, bile	GlcNAc, GalNAc, fucose, glucose, PSA	*A. viridans*
M-ficolin	Monocytes	Monocyte surface	GlcNAc-BSA, GalNAc-BSA, SiaLacNAc-BSA	*S. aureus*
Ficolin-A	Liver and spleen	Serum	GlcNAc	n.d.
Ficolin-B	Bone marrow and spleen	n.d.	n.d.	n.d.

Table I.1: Expression, sugar specificity and target pathogens of collectins and ficolins. ManGlcNAc: N-acetyl-D-glucosamine; GalNAc: N-acetyl-D-galactosamine; ManNAc: N-acetyl-D-mannosamine; HIV: human immunodeficiency virus; IAV: influenza A virus; RSV: respiratory syncytial virus; HSV-1: herpes simplex virus type 1; n.d.: not determined. (Lu *et al.,* 2002) and (Lillie *et al.,* 2005).

The primary and tertiary structures of collectins are defined by the unique domain organization of the polypeptide sequences, which was described before for MBL (see section I.3.1 and Fig. I.6).

Regarding their binding specificity, collectins have shown to react with a range of sugar residues including mannose, fucose, glucose, maltose, GlcNAc and ManNAc, although individual collectins display different preferences to certain sugar residues over the others (Hakansson and Reid, 2000). In addition, LPS is the major glycolipid component of the outer membrane of Gram-negative bacteria which is a common microbial structure recognized by collectins. Table I.1 summarizes the main features and target pathogens for collectins and ficolins.

Unlike MBL and ficolins, SP-A, SP-D, conglutinin and CL-43 do not activate complement but function as opsonins acting through phagocytic receptors on immune cells. For example, the surfactant protein receptors SPR-210 and gp340 exhibit affinity for SP-A and –D, while both CD91 and CR1 bind to MBL. SP-A and SP-D also modulate the production of reactive oxygen species by alveolar macrophages and monocytes, and are also known to enhance chemotactic, phagocytic and superoxidative burst properties of neutrophils (Lu *et al.,* 2002).

II Materials and Methods

II.1 Materials

II.1.1 Chemicals, solutions and media

Agarose, electrophoresis grade	Invitrogen
Ampicillin, >98%	Sigma-Aldrich
APS, ammonium persulphate	Biorad
BSA, bovine serum albumin, fraction V	Biomol
Chelating Sepharose Fast Flow	GE Healthcare
CNBr-activated Sepharose 4B	GE Healthcare
Coomassie Brilliant Blue R250	Fluka
[α-^{32}P]dCTP (3000 Ci/mmol)	GE Healthcare
Deoxynucleotides, PCR grade	Roche Applied Science
DMEM medium	Invitrogen
DMSO, dimethyl sulfoxide	Sigma-Aldrich
DNA-Molecular weight standards X and XIV	Roche Applied Science
Ethidium bromide	Sigma-Aldrich
FCS, fetal calf serum	PAN Biotech
Gentamycin	PAA Laboratories
Glutamine	Biochrom AG
Glycerol, ultrapure	Invitrogen
H_2O (deionized)	Milli Q UF Plus system
HAT medium	Boehringer Mannheim
Hi-Di Formamide, Genetic Analysis Grade	Applied Biosystems
HT medium	Boehringer Mannheim
Hygromycin-B	Invitrogen
IPTG, isopropyl-β-D-thiogalactoside	Biomol
Kanamycin	Invitrogen
ß-mercaptoethanol for cell culture	Invitrogen
ß-mercaptoethanol for molecular biology	Sigma
Methanol, technical grade	Merck

Ni-NTA (Nitrilotriacetic acid) Agarose	QIAGEN
Nowa Solution A+B (ECL)	MoBiTec
Nuclease free water	Promega
Polyacrylamide, Rotiphorese® Gel30 (37,5:1)	Carl Roth
Penicillin/Streptomycin	Gibco
Phenol/chloroform, for DNA purification	Carl Roth
Pronase E	Sigma-Aldrich
Protein G Sepharose 4 Fast Flow	GE Healthcare
Proteinase K	Sigma-Aldrich
RNAse A, DNAse free	Roche Applied Science
RPMI-1640	Sigma-Aldrich Chemie
SDS-PAGE Molecular weight standard, broad range	Biorad
Shrimp alkaline phosphatase, SAP	Roche Applied Science
T4 DNA ligase	New England Biolabs
Taq Polymerase	Roche Applied Science
TEMED, Tetramethylethylendiamin	Biorad
Triton X-100	GE Healthcare
Trypsin	Invitrogen
Tryptone	Difco
TSR (Template suppression reagent)	Applied Biosystems
Tween 20	Fluka
X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside)	Biomol
Yeast extract	Difco
all other chemicals and solutions of analytical grade	Sigma-Aldrich or Merck

Soluble peptidoglycan extracts (prepared according to Rosenthal and Dziarski, 1994), lipoteichoic acid (LTA) from *S. aureus* and lipopolysaccharide (LPS) from *Y. enterocolitica* were kindly provided by Prof. Dr. Ulrich Zähringer, Borstel; LPS from *Salmonella abortus equi* was kindly provided by M. Freudenberg, Freiburg, Germany.

II.1.2 Kits

ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit	Applied Biosystems
Dc Protein Assay	Biorad
Gel Extraction Kit QIAEX II	QIAGEN
HiFi PCR Master Kit	Roche
pBluescript®II SK(-) Phagemid Kit	Stratagene
Plasmid Purification Maxi Kit	QIAGEN
QIAshredder Kit	QIAGEN
Prime-It® II Random Primer Labelling Kit	Stratagene
Reverse Transcription Kit	Promega
RNeasy Mini Kit	QIAGEN
RNase-free DNase Set	QIAGEN
TOPO TA Cloning® Kit	Invitrogen
Wizard® *Plus* Miniprep DNA Purification System	Promega

II.1.3 Nucleic acids

II.1.3.1 Oligonucleotides

All primers used for cloning, sequencing or screening were purchased from Metabion at a concentration of 100 pmol/μl, and stored at -20°C in aliquots of 10 pmol/μl until use.
The primers used in this work are listed below:

	Oligo name	5' → 3' Sequence
1	ficolinA-EcoRI-ATG-forw	CCC GAA TTC CCA GCC ATG CAG TGG CCT ACG CTG TGG
2	ficolinA-XhoI-Stop-rev	CCC CTC GAG TTA AGA TGC TCG GAT TTT CAT CTC G
3	ficolinB-EcoRI-ATG-forw	CCC GAA TTC CCA GCC ATG GCC CTG GGA TCT GCT GCAC
4	ficolinB-XhoI-Stop-rev	CCC CTC GAG CTA GAT GAG CCG CAC CTT CAT C
5	Mouse ficolinA 5'MTA BglII	GGG AGA TCT CTG GGT CAG GAG AGA GGT GCC
6	Mouse ficolinA 3'MTA XhoI	GGG CTC GAG AGA TGC TCG GAT TTT CAT CTC

7	Mouse ficolinB 5'MTA BamHI	GGG GGA TCC TGC CCA GAA CTG AAG GTC CTA
8	Mouse ficolinA 3'MTA XhoI	GGG CTC GAG GAT GAG CCG CAC CTT CAT CTC
9	5'SpeI-1stfrag-fcnA	CCA CTA GTG GGA AGC CAT TGC TGT TAG AGA G
10	3'XhoI-1stfrag-fcnA	CCC CTC GAG GGC TCT GGA CGG AGG CTG AGG
11	5'XhoI –2ndfrag-fcnA	CCC CTC GAG ACA GGT CTT GAG AGT GGT GCC
12	3'HindIII –2ndfrag-fcnA	CCC CAC CAA TAG CTT TAA GCT TTC TG
13	5'HindIII -3rdfrag-fcnA	AGA AAG CTT AAA GCT ATT GGT GGG G
14	3'SpeI -3rdfrag-fcnA	CCC ACT AGT TCT AGA GCA ACA CAG ATA CTT C
15	5'beginProbeA	CCC AGT CAA TTC AGC AGG TGA GG
16	3'beginProbeA	GGC AGG GCT TCA CTG TGT AGC CTG
17	5' Neo-XhoI	CTC GAG GGA CCT AAT AAC TTC GTA TAG C
18	3' Neo-SalI	GTC GAC CTT AAG TGA TCA CTA ATT AAG GCT AG
19	5'SpeI-fragment A-fcnB	CCC ACT AGT CCT ACA ATC GTC CTA TCA CAA GGGG
20	3'XhoI-fragment A-fcnB	CCC CTC GAG AAC CCC CTC TAT AGC TTT G
21	5'XhoI-fragment B-fcnB	CCC CTC GAG AAG GAG GGG ATA TGG AAG
22	3'XbaI-fragment B-fcnB	GGT TCT AGA ATG TGA GCC TCA GGG
23	5'XbaI-fragment C-fcnB	TCT AGA ACC TGT AAG TGG TCT GTC TGA GTG G
24	3'BamHI-fragment C-fcnB	CCC GGA TCC CTT CTA CTC CCA GAG TAG
25	5'ES-PCR ficolin A	GTG CTC AGC TTG CTG CAG TTG
26	3'Neo Out	GAT CGG AAT TAA TCA CTA GTG AAT TC

II.1.3.2 Plasmids

The following vectors were used:

pCR®2.1-TOPO: (3,9 kb) (Invitrogen) supplied as a linearized vector with single 3'-thymidine overhangs for direct cloning of *Taq*-amplified PCR products. The vector also bears the topoisomerase I from *Vaccinia* virus covalently bound to the 3'-phosphate of the 3'-thymidine, taking advantage of its ligating activity (Shuman, 1994). Such a TOPO-activated vector is ready to accept PCR products with compatible ends as inserts.

pMT/BiP/V5-His A: (3,6 kb) (Invitrogen) is designed for use with the Drosophila Expression System (DES®, Invitrogen). The vector includes the *Drosophila* metallothionein (MT)

promoter for high-level, copper-inducible expression of the gene of interest in S2 cells (Maroni *et al.,* 1986), (Bunch *et al.,* 1988), (Angelichio *et al.,* 1991), (Olsen *et al.,* 1992). In addition, the *Drosophila* BiP secretion signal that encodes an immunoglobulin-binding chaperone leads to the channeling of BiP into the secretory pathway of S2 cells and directs the recombinant protein into the culture medium (Kirkpatrick *et al.,* 1995). In addition, the vector bears the sequence for a C-terminal peptide containing the V5 epitope (GKPIPNPLLGLDST) and a polyhistidine (6xHis) tag for detection and purification of the expressed recombinant protein.

pBluescript II KS (+): (3kb) (Stratagene) allows several cloning and sequencing procedures as well as RNA synthesis. It also contains the β-galactosidase coding sequence for blue/white selection of transformants (see section II.2.1.2.1).

pSL301: (3,2 kb) (Invitrogen) offers a superlinker multiple cloning site which makes it convenient when multiple fragments are to be cloned together.

II.1.4 Antibodies

Description	Application	Working dilution	Source
Primary antibodies			
Mouse anti-V5	WB	1:5000	Invitrogen
Mouse anti-His	WB / TRIFMA	1:1000 - 1μg/ml	QIAGEN / BD
Rabbit anti-ficolin B (polyclonal)	ELISA / WB / IP	1:2000	This work
Rat anti-ficolin B (monoclonal)	ELISA	1:500	This work
Mouse anti-hMASP-2 (8B5)	TRIFMA	3 μg/ml	Dept. of Medical Microbiology, Univ. of Aarhus, Denmark
Primary labeled antibodies			
Goat anti-mouse Lamp-1 (CD107a)-FITC	IHC	1:100	BD
Mouse anti-hMASP-2 (6G12)-biot	TRIFMA	1:1500	Dept. of Medical Microbiology, Univ. of Aarhus, Denmark
Mouse anti-C4 Hyb 161-1-biot	TRIFMA	0,25 μg/ml	Immunolex
Mouse anti-C4 Hyb 161-2-biot	TRIFMA	0,25 μg/ml	Immunolex
Secondary labeled antibodies			
Goat anti-rabbit-IgG-HRP	WB	1:1000	Sigma

Goat anti-rat-IgG-HRP	WB	1:1000	Sigma
Goat anti-rabbit-IgG-AP	ELISA	1:1000	Sigma
Goat anti-rat-IgG-AP	ELISA	1:1000	Sigma
Goat anti-rabbit-IgG-FITC	IHC	1:100	Sigma
Goat anti-rabbit-IgG-TRITC	IHC	1:200	Sigma
Blocking antibodies			
Rat anti-mouse FcγRII/III 2G4.2	IHC	2 μg/section	Inst. of Immunology, Univ. of Regensburg
Heat aggregated human-IgG	TRIFMA	100 μg/ml	Dept. of Medical Microbiology, Univ. of Aarhus, Denmark

II.1.5 Restriction enzymes

Restriction endonucleases for cloning and analytical screening of plasmids were purchased from New England Biolabs (NEB). They were used at the optimal temperature and in the right buffer conditions as recommended by the manufacturer.

II.1.6 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.

Embryonic fibroblasts (EF): for long-term culture and maintenance, pluripotent embryonic stem cells must be grown either on monolayers of mitotically inactivated fibroblast cells or on gelatinized tissue culture dishes. In this work, EF feeder cells were were prepared as stated in section II.2.3.2.1.

E14.K embryonic stem (ES) cell line: ES were kindly provided by Prof. Dr. Klaus Pfeffer, Universität Düsseldorf, Germany. The ES medium (see Appendix A) used to culture the stem cells contained the myeloid regulatory leukaemia inhibitory factor (LIF) which prevents ES cell differentiation and promotes undifferentiated cell proliferation. Recombinant LIF was

obtained from the conditioned medium of CHO transfected cells and titrated to determine the optimal concentration of LIF to keep the ES cells in an undifferentiated stage.

SP2/O-Ag14 myeloma cells: (ATCC No. CRL 1581) formed by fusing BALB/c spleen cells (from a mouse immunized with sheep RBCs) with P3X63Ag8 myeloma cells (Shulman *et al.,* 1978). The SP2/O-Ag14 cells do not secrete immunoglobulins, are resistant to 8-azaguanine at 20 mg/ml and are aminopterin-sensitive (Shulman *et al.,* 1978). The cells can be used as fusion partners of murine B cells for the production of hybridomas (Ozato *et al.,* 1981). They were cultured in suspension in RPMI medium at 37°C with 5% CO_2 and split every approximately 3 days in a 1:10 ratio.

II.1.7 Competent *E. coli* strains

OneShot®TOP10F': (Invitrogen) overexpresses the Lac repressor (*lac*I^q gene). For blue/white screening, it is necessary to add IPTG to the plates to obtain expression from the *lac* promoter (see section II.2.1.2.1).
Genotype: F´*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80lacZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

INV110: (Invitrogen) bears *dam* and *dcm* deficiencies to allow production of DNA that is unmethylated at the G^{m}ATC (Dam) and C^{m}C(A/T)GG (Dcm) sites, thus allowing digestion with *dam*- and *dcm*-sensitive restriction enzymes. The Δ(*mcrC-mrr*) allele eliminates two restriction systems to allow more efficient transformation of DNA from highly methylated sources (i.e. eukaryotic DNA). The *end*A1 mutation permits the isolation of higher quality plasmid DNA, and the Tn*10* mutation the selection using tetracycline. This strain also contains the *lac*I^q allele.
Genotype: F´ {*tra*Δ36 *pro*AB *lac*I^q *lac*ZΔM15} *rps*L (Str^R) *thr leu end*A *thi*-1 *lacY galK gal T ara ton*A *tsx dam dcm sup*E44 Δ(*lac-proAB*) Δ(*mcrC-mrr*)*102*::Tn*10* (Tet^R).

XL-1 blue: (self-made competent; see section II.2.1.1) allows blue/white color screening for recombinant plasmids and is an excellent host strain for routine cloning applications using plasmid or lambda vectors. XL-1 cells are tetracycline resistant, endonuclease (*endA*) and recombination (*recA*) deficient, for an improved insert stability. The strain also bears the *hsdR* mutation that prevents the cleavage of cloned DNA by the *Eco*K endonuclease system.
Genotype: *rec*A1 *end*A1 *gyrA96 thi-1 hsdR17 supE44 relA1 lac* {F´ *proAB lac*Iq*ZΔM15* Tn*10* (Tetr)}

II.1.8 Animals

Male C57BL/6 mice 9 to 12 weeks of age as well as female rabbits (Chinchilla Bastard) and Wistar rats were purchased from Charles River. Animals were kept under conventional conditions with food and water *ad libitum* in the animal facilities of the University of Regensburg and handled in accordance with institutional guidelines and the German Federal Regulations of Animal Experimentation.

II.1.9 Software and databases

Ficolin sequences (at both genomic and protein level) were obtained from PubMed publications and databases. Sequence analysis of plasmid and genomic DNA was performed using the Clone Manager 4.01 (1995, Scientific and Educational Software), the Generunner (Hastings Software Inc.), and the Web Cutter 2.0 (1997, Max Heiman) programmes. Calibration curves were plotted with Microsoft Excel 2002 for Windows XP.

II.2. Methods

II.2.1 Standard DNA-cloning techniques

II.2.1.1 Preparation of competent bacteria

The *E. coli* strain "XL-1 blue" was made competent by the following protocol according to Hanahan (1983).
Colonies were picked from a SOB-agar plate (containing 20 mM $MgSO_4$) and used to inoculate 100 ml of SOB_{MgSO4} medium (see Appendix A). The culture was incubated at 37°C with vigorous shaking until it reached an OD_{600} of 0.3 (<10^8 CFU/ml), dispensed into ice-cold polypropylene tubes (Falcon), and kept on ice for 10 min. After centrifugation (10 min, 3200x*g*, 4°C), the supernatant was decanted for 1 minute and the cells resuspended in 20 ml ice-cold TFB (see Appendix A) by gentle vortexing. Following a 10-minute incubation on ice and further centrifugation, the cells were resuspended in 4 ml ice-cold FSB (see Appendix A). Finally, 280 µl DMSO were added in two steps (swirling in-between), cells dispensed in 200 µl aliquots, and frozen immediately at -80°C.

II.2.1.2 Transformation of bacteria

Incorporation of DNA into competent *E. coli* cells was performed by chemical transformation. For this, 2 µl of the TOPO cloning reaction (see section II.2.1.6.1) or up to 1 µg of ligated DNA (see section II.2.1.6.2) was added to a vial of competent bacteria, which had been previously thawed on ice, and mixed gently. After a 15-minute incubation on ice, cells were heat-shocked for 30 seconds at 42°C without shaking, and recovered by adding 3 volumes of SOC medium (see Appendix A). Tubes were gently shaken at 37°C for 1 hour and then plated on pre-warmed LB (Luria Bertani)-agar plates containing the proper selection antibiotics (see section II.2.1.3.1).

II.2.1.2.1 Blue/white screening of transformants

Many plasmid vectors carry a short segment of *E. coli* DNA containing the regulatory sequences and the coding information for the first 146 aa of the ß-galactosidase gene. Embedded in the coding region is a polycloning site that maintains the reading frame and results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of the ß-galactosidase. Vectors of this type are used in host cells that express the carboxy-terminal portion of the ß-galactosidase. Although neither the host-encoded nor the plasmid-encoded fragments of the ß-galactosidase are themselves active, they can

associate to form an enzymatically active protein. This phenomenon is called 'α-complementation'. The *lac*$^+$ bacteria that undergo α-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) which is converted by the ß-galactosidase into an insoluble dense blue compound (Horwitz *et al*, 1964) (Davies and Jacob, 1968). However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in the production of an amino-terminal fragment of the ß-galactosidase that is no longer capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white colonies. If the bacterial strain bears the lacI^q gene, it is necessary to include IPTG on the agar plates (in addition to X-gal), which is a nonfermentable lactose analogue that inactivates the *lacZ* repressor.

For blue/white screening bacterial cells were plated on LB-agar plates containing X-gal (40 µl of a 20 mg/ml stock solution) and IPTG (4 µl of a 200 mg/ml stock solution), and 100 µg/ml ampicillin. After overnight incubation at 37°C, white colonies were picked for plasmid DNA isolation and restriction analysis (see sections II.2.1.4.1 and II.2.1.7).

II.2.1.3 Bacterial cultures

II.2.1.3.1 Agar-plates

After transformation bacteria (120 µl) were plated on LB_{amp}-agar dishes (additionally containing IPTG/X-Gal for blue/white selection) with a sterile Drigalski spatula and incubated overnight at 37°C. In order to make competent bacteria (see section II.2.1.1) XL-1 blue cells were plated from a glycerol stock with a sterile inoculating loop.

II.2.1.3.2 Liquid cultures

In order to amplify and isolate plasmid DNA from transformants, tubes containing sterile LB_{amp}-medium were inoculated with one single colony from the agar-plates and incubated aerobically at 37°C overnight (~16 hours) in an orbital shaker at 220 rpm.

II.2.1.3.3 Glycerol stocks

For long-term storage of positive transformants, bacterial cultures were mixed 1:1 with 60% sterile glycerol in cryotubes (CryoTube™ vials, Nunc) and frozen at -80°C.

II.2.1.4 Isolation and purification of plasmid DNA

II.2.1.4.1 Mini-scale isolation of plasmid DNA („Minipreps“)

Colonies were picked and cultured in LB medium (see Appendix A) containing 100 μg/ml ampicillin at 37°C overnight under vigorous shaking.
Mini-scale isolation of plasmid DNA was performed using the Wizard Plus SV Minipreps DNA Purification System (Promega). This kit combines two techniques: alkaline lysis and silica resin-based DNA purification. Cells are partially lysed using an alkaline solution of the detergent SDS. This allows small plasmid DNA molecules to escape from the cell, while genomic DNA remains within the cells. When a concentrated potassium acetate solution is added to the cell lysate, cell debris (containing high molecular weight genomic DNA) is precipitated, while plasmids and soluble proteins remain in solution. If cell membranes are dissolved completely, sheared genomic DNA may be released, contaminating the plasmid preparation. To avoid this, the lysis step is carried out for a limited time – just enough for the solution to clear. The adhesion of DNA to a silica matrix is based on the observation that nucleic acids adhere to silica in high-salt conditions, but not in low-salt conditions. DNA will bind to silica in the lysis solution, and will be eluted from the matrix by TE buffer (see Appendix A).
The experimental procedure was carried out according to the manufacturer's instructions. Briefly, 3 ml of overnight bacterial culture were centrifuged for 5 min and the pellet resuspended in 250 μl of Resuspension Solution. After lysis with one volume of Cell Lysis Solution the reaction was stopped by adding 350 μl of Neutralization Solution and pelleted at top speed for 10 min at RT. The clear lysate was then decanted into a Spin Column containing the silica-resin, and washed extensively before the DNA was eluted with 100 μl of TE buffer. Plasmid identity was checked by restriction digestion and the DNA stored at –20°C.

II.2.1.4.2 Scaled-up preparation of plasmid DNA („Maxipreps“)

In order to obtain high yields of purified plasmid DNA for further transfections (see section II.2.3.1.1), positive clones were chosen and plasmids were isolated using the Plasmid Maxi Kit (QIAGEN). This protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. While RNA, proteins, dyes and low-molecular-weight impurities are removed by a medium-salt wash, plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.
Briefly, 200 ml LB medium containing 100 μg/ml ampicillin was inoculated with the starter culture in a 1/1000 ratio, and incubated overnight at 37°C under vigorous shaking. After

spinning down at 6500x*g* for 15 minutes, cells were resuspended extensively in Buffer P1 containing RNase A, sequentially lysed in Buffer P2 for 5 min and neutralized in chilled Buffer P3. The lysate was then centrifuged (20,000x*g,* 30 min) to pellet the cell debris and the clear supernatant was decanted into a QIAGEN-tip 500 containing the anion-exchange resin. The resin was washed with medium-salt- and DNA eluted with high-salt-containing buffers. DNA precipitation was performed by adding 0,7 volumes of isopropanol and further centrifugation (14,500x*g* for 30 min). Before resuspending the DNA in water, the pellet was washed with 70% ethanol. All centrifugation steps were carried out at 4°C. Plasmid identity was checked by restriction digestion and the DNA stored at –20°C.

II.2.1.5 Quantification of DNA

In order to estimate the concentration of isolated purified DNA, absorbance of diluted samples was measured at 260 nm in a GeneQuantII photometer (Pharmacia Biotech). DNA concentration was calculated according to the Lambert-Beer's law where an optical density of 1 (OD_{260}=1) corresponds to 50 μg of dsDNA/ml.

II.2.1.6 Ligation of DNA fragments

II.2.1.6.1 Cloning into the pCR2.1 vector

Since the *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine to the 3'-end of PCR products, amplified DNA fragments were cloned into the pCR2.1 TOPO vector. Ligation of freshly purified PCR products to this vector was performed according to the manufacturer's instructions. Briefly, 2.5-6 ng of PCR product was mixed with 1 μl of Salt Solution and water to a final volume of 5 μl before adding 10 ng of pCR2.1 TOPO vector. This reaction mixture was incubated for 5 minutes at RT and used to transform TOP10F' cells (see section II.2.1.2).

II.2.1.6.2 Standard ligation

DNA fragments and vectors which were digested by restriction enzymes (see section II.2.1.7) were analyzed by agarose gel electrophoresis, purified (see section II.2.1.9.2), and ligated together in the ratio given by (Sambrook *et al.,* 1989):

$$\text{g insert DNA} = \frac{10 \times (\text{g vector DNA}) \times (\text{\# of bp insert DNA})}{(\text{\# of bp vector DNA})}$$

The ligation reactions were carried out in an optimal buffer for the T4-DNA Ligase in 20 μl final volume, and incubated at 16°C overnight, before proceeding to transform XL-1 or INV110 cells (see section II.2.1.2)

II.2.1.7 Restriction digestion of plasmid DNA

Unless otherwise specified, isolated and purified plasmids were digested (~1 μg) in a 20 μl-reaction containing 10-20 U of the corresponding restriction endonuclease in a 1x optimal buffer according to the manufacturer's instructions. In addition, BSA (100 μg/ml) was added when the enzyme required it. The temperature and duration of the restriction reactions depended on the enzyme as specified by the provider. Digested fragments were checked by agarose gel electrophoresis, and eventually extracted for further cloning (see section II.2.1.9.2). For double digestions the total enzyme volume was kept at 5% of the total volume to avoid interference of glycerol in the reaction.

II.2.1.8 Dephosphorylation of a linearized plasmid by SAP

In order to avoid re-ligation of a linearized plasmid after a single digestion, alkaline dephosphorylation using shrimp alkaline phosphatase (SAP) (Roche) was performed according to the manufacturer's instructions. Briefly, 3 units of enzyme were required to dephosphorylate 50 ng of plasmid DNA at 37°C during 1 hour. DNA was then purified by phenol/chloroform extraction and alcoholic precipitation (see section II.2.2.2.1 with slight modifications) and ligated with the insert as described before (see section II.2.1.6.2).

II.2.1.9 Agarose gel electrophoresis

II.2.1.9.1 Gel preparation

Unless otherwise specified, agarose gels were prepared by dissolving 1% agarose in 1xTAE buffer (see Appendix A). Samples were diluted in 6x DNA loading buffer (see Appendix A) and separated according to their size at 120 Volts. DNA bands were visualized by ethidium bromide staining under a 366 nm UV light (Bachofer GmbH).

II.2.1.9.2 DNA purification from agarose gels

DNA was purified from agarose gels by using the Gel Extraction Kit (QIAGEN). Briefly, the bands were excised from the gel with a clean scalpel and melted in Binding Buffer at 50°C, before adsorption of nucleic acids to the silical-gel particles took place in the presence of

high salt concentration. After purification, a small aliquot of DNA was always checked on a 1% agarose gel.

II.2.1.10 Sequencing

The integrity of plasmid constructs was checked by sequencing before proceeding to further applications. Sequencing was performed with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). This kit combines the dideoxynucleotide sequencing method (Sanger and Coulson, 1978) and PCR.
The concentration of plasmid DNA was calculated as stated in section II.2.1.5, and one reaction tube contained 700 ng of DNA, 6.4 pmol of the sequencing primer, and 8 µl of premix from the kit, in a final volume of 20 µl. Depending on the G/C content of the primer one of the following sequencing-programmes was run during 35 cycles:

"normal":	15sec	96°C	"low":	15sec	96°C	"high":	15sec	96°C
(10-15 G+C)	15sec	55°C	(< 10 G+C)	15sec	50°C	(> 15 G+C)	15sec	60°C
	4min	60°C		4min	60°C		4min	62°C

The sequencing reactions were purified by the addition of 1/10 volume of 3 M sodium acetate pH 4.6 and 2 volumes of 100% ethanol. Samples were precipitated at room temperature for 20 minutes in the dark, centrifuged for 30 minutes at 16000x*g*, washed with 500 µl 70% ethanol, and dissolved in 25 µl template suppression reagent (TSR) for the ABI310 Genetic Analyzer or 12 µl Hi-Di Formamide for the ABI3100-Avant Genetic Analyzer. The obtained sequence data were compared with the published sequence in the database (PubMed). Clones without any mutations were used for further work.

II.2.2 DNA and RNA-based techniques

II.2.2.1 The polymerase chain reaction

The polymerase chain reaction (PCR) is a powerful technique used to enzymatically synthesize a defined nucleotide sequence *in vitro* (Mullis, 1990). In this work, the PCR reactions were carried out in a programmable thermocycler (GeneAmp PCR System 9700, PE Applied Biosystems). In order to avoid DNA contamination, the different components of the reaction were pipetted in a nucleic acid-free room with filtered tips.

II.2.2.1.1 Standard PCR

Unless otherwise stated, standard PCR reactions were carried out with the *Taq* DNA polymerase (isolated from the eubacterium *Thermus aquaticus*) which possesses a 5'-3' exonuclease activity and a processivity of approximately 50 base pairs per second.
Whenever possible, primers were designed to be 24-36 bp long (including 5'-incorporated restriction sites), possess 40-60% GC content and similar melting temperatures (T_m) that allow annealing temperatures of 55-65°C for optimal specificity. Annealing temperatures were calculated as follows: T_a = (G+C)x4 + (A+T)x2. As template, purified plasmid DNA (~1 ng) or mouse genomic DNA (500 ng) was used. The standard PCR reactions were carried out in 50 µl final volume under the following conditions:

	Final concentration
Template DNA	x ng
Reaction buffer	1x, incl. 1.5 mM Mg^{2+}
5' primer	1.0 µM
3' primer	1.0 µM
dNTPs	0.2 mM each
Taq polymerase	0.1 U/µl

Initial denaturation:	94°C	5 min.	
Denaturation:	94°C	1 min.	35 cycles
Annealing:	T_a	1 min.	
Elongation:	72°C	~1 min/1000 bp	
Final elongation:	72°C	10 min	
Cooling down:	4°C	∞	

II.2.2.1.2 Touchdown PCR

In order to genotype the genetically modified mouse lines by PCR, a "touchdown" PCR was established. This particular PCR reaction is the method of choice when the template DNA is extremely complex and long. In touchdown PCR, the annealing temperature of the first amplification cycle is set ~3°C above the calculated melting temperature of a perfect hybrid formed between the most GC-rich oligonucleotide primer in the reaction and its target DNA. The annealing temperature is then reduced by 0.8°C for each subsequent cycle. At some point, a temperature permissive for specific priming will be reached and amplification of the target sequence will begin. The onset of non-specific amplification will be delayed for several

additional cycles until the annealing temperature has been lowered to the point where non-specific priming can occur. However, by this time, the specific amplification product will have hegemony over the reaction and will effectively suppress the accumulation of non-specific amplification products.

To establish the best parameters for this kind of reaction in our lab, different concentrations of enzyme as well as magnesium ions were tested for each particular set of primers and/or template DNA, ranging from 1.75–5 units and 1.5–5 mM, respectively.

In addition, the touchdown PCR was carried out with the "HiFi" (high fidelity) PCR master kit (Roche) which is a blend of two DNA polymerases able to overcome the limitations of single polymerase PCR systems. It is a useful system when amplifying critical sequences (e.g. repeat-rich sequences), requiring less buffer optimization than single systems.

The 50 µl-volume touchdown PCR reaction contained:

	Final concentration
Template DNA	500 ng
Reaction buffer	1x (without Mg^{2+})
5' primer	1.0 µM
3' primer	1.0 µM
dNTPs	0.2 mM each
HiFi polymerase	1.75 - 5 units
$MgCl_2$	1.5 - 5 mM

The cycling programme was:

Initial denaturation:	95°C	2 min	
Denaturation:	95°C	30 sec	15 cycles
Annealing:	72°C [-0.8°C/cycle]	30 sec	
Elongation:	72°C	40 sec	
Denaturation:	95°C	30 sec	30 cycles
Annealing:	60°C	30 sec	
Elongation:	72°C	40 sec	
Final elongation:	72°C	5 min	
Cooling down:	4°C	∞	

II.2.2.1.3 RT-PCR

In order to amplify a gene of interest without introns and clone an expression construct, or to detect the presence of a specific mRNA transcript in a particular tissue, RNA must be first reverse transcribed into cDNA ("complementary DNA") since it cannot serve itself as a template for PCR. This can be achieved by using a reverse transcriptase such as the avian myeloblastosis virus (AMV) or the Moloney murine leukaemia virus (M-MuLV) transcriptases, and primers which bind either to the endogenous poly(A)+ tail at the 3' end of mammalian RNA (Oligo(dT) primers) or to mRNA at a variety of complementary sites (random primers).
RT-PCR is the combination of a first reverse transcription (RT) step coupled to a standard PCR reaction, described for the first time by Powell *et al*. in 1987.
In this work, the RT reaction was carried out with the Reverse Transcription Kit in a 20 µl final volume under the following conditions:

	Final concentration
$MgCl_2$	5 mM
dNTPs	1 mM each
Oligo $(dT)_{15}$ primer	0.5 µg
RT Buffer	1x
AMV reverse transcriptase	15 U/µl
RNasin	1 U/µl
RNA template	1 µg

The reaction was incubated at 42°C for 15 minutes (first strand cDNA synthesis) followed by 5 minutes denaturation at 95°C and cooling down to 4°C for 5 minutes.
The first strand of cDNA synthesized during this step was used as the template for the coupled standard PCR reaction that followed (see section II.2.2.1.1.).

II.2.2.2 Genotyping of targeted cells and genetically-modified mouse lines

II.2.2.2.1 Isolation of genomic DNA

Genomic DNA was isolated from both embryonic stem (ES) cells and mouse tails by phenol/chloroform extraction after lysis of the cells/tissue followed by alcoholic precipitation. For cell lysis, a monolayer of confluent ES cells was washed with PBS, covered in lysis buffer, and incubated overnight at 37°C. For mouse tail lysis, 0.6 cm-long tails were digested in lysis buffer at 56°C overnight in the presence of proteinase K (0.2 mg/ml) and pronase E (0.5 mg/ml, pre-activated at 37°C for 1 hour) and further cleared by centrifugation. In both

cases, genomic DNA was isolated from the lysate by two subsequent (2x) phenol extractions, followed by 2x phenol/chloroform/isoamyl alcohol (in a 25:24:1 ratio) and 2x chloroform extractions (1 volume each, with 5-minute centrifugation steps in-between at 9000x*g*). Genomic DNA was precipitated by the addition of 2 volumes of 100% ethanol and 1/10 volumes of sodium acetate 3 M pH 5.2 at -20°C for 30 minutes and pelleted at 9000x*g* for 5 minutes. After a 70% ethanol wash, genomic DNA was resuspended in nuclease-free water and stored at 4°C. DNA concentration and quality was assessed by absorbance at 260 nm and the 260/280 nm ratio, respectively.

II.2.2.2.2 Southern Blotting

In order to genotype the targeted ES cells and the chimeric mice, genomic DNA was extracted from the cells/tails and subjected to Southern Blot. For this, 10 μg of genomic DNA was digested with *BamH*I (30 U) in 1x reaction buffer containing 1 mg/ml BSA in a final volume of 35 μl, at 37°C overnight. Digested samples were analyzed on a 0.8%-agarose/0.5xTAE gel which was sequentially soaked in 0.2 N HCl, denaturing buffer, and neutralizing buffer (see Appendix A) before proceeding to blotting. Samples were transferred to a nylon membrane (Osmonics Inc, 0.22 μm) by capillarity in a 20x SSC containing chamber overnight and crosslinked at $1.2x10^5$ μJoules (UV Stratalinker 2400, Strategene). The membrane was prehybridized with pre-warmed UltraHyb buffer (Ambion) for at least 1 hour at 45°C under rotation before hybridization with the radioactive probe took place. Hybridization was performed in the same solution at 45°C overnight. Before proceeding to detect the probe, the membrane was sequentially washed with 2x SSC 0.1% SDS at 45°C, 0.1x SSC 0.1% SDS at 45°C, and 0.1x SSC 0.1% SDS at 65°C, each for 20 min, and exposed to a radiographic film (Kodak Biomax MS) at -80°C.

II.2.2.2.2.1 Generation of the specific probe

To detect homologous recombination events by Southern Blot, a specific DNA probe spanning over the 5' region of the ficolin-A gene was designed.
The probe called "Begin-A" (470 bp) was amplified from genomic ES cells DNA using the primers "5'beginProbeA" and "3'beginProbeA" by standard PCR (see section II.2.2.1.1).
The DNA probe was radioactively labeled with the Random Primer Labelling Kit (Stratagene) according to the manufacturer's instructions. The procedure relies on the ability of random hexanucleotides to anneal to multiple sites along the length of a DNA template. The primer-template complexes formed represent a substrate for the Klenow fragment of the DNA polymerase I. The enzyme synthesizes new DNA by incorporating nucleotide

monophosphates at the free 3'-OH group provided by the primer. The newly synthesized DNA is made radioactive by substituting a nonradioactive nucleotide for a radiolabeled one in the reaction mixture. Furthermore, to obtain fast results, this kit provides a 3' exonuclease-deficient mutant of Klenow [Exo(-) Klenow] and random nonamer primers (9-mers).

Briefly, 25 ng of probe were heated at 95°C for 5 min in the presence of random primers (0.27 OD Units) in 34 µl final volume. The solution was allowed to cool down and then 5 units of Exo(-) klenow, 1x dCTP buffer and 5 µl [α-^{32}P]dCTP (3000 Ci/mmol) were added. After a 1-hour incubation at 37°C, the probe was filtered using the MicroSpin Columns (GE Healthcare) in order to remove unincorporated radioactive nucleotides, and further denatured at 95°C for 5 min before proceeding to the hybridization.

II.2.2.3 RNA isolation from mouse organs

To investigate the expression pattern of ficolin B in mouse tissue, total RNA was isolated from different organs using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Briefly, after excision samples (up to 30 mg, depending on the organ) were immediately placed in RNA*later* solution which permeates the tissue to stabilize and protect cellular RNA *in situ*. Stabilized tissue samples were then disrupted in buffer containing highly denaturing guanidine isothiocyanate (to inactivate RNases) by a rotor-stator homogenizer (Ultra-Turrax T25, Ika® Labortechnik), which causes disruption by a combination of turbulence and mechanical shearing. Disrupted tissue was then homogenized to reduce viscosity of the cell lysate by shearing the high-molecular-weight genomic DNA and cellular components to create a homogeneous lysate. Homogenization was performed using the QIAshredder Kit (QIAGEN). Ethanol was then added to provide appropriate binding conditions and the lysate applied to a column where the total RNA binds to the membrane and contaminants are washed away. Eventually, on-column DNase digestion was carried out with the RNase-Free DNase Set (QIAGEN) and high-quality RNA was finally eluted with DEPC-water.

II.2.3 Cell culture techniques

II.2.3.1 Drosophila Schneider-2 (S2) cells

II.2.3.1.1 Transfection of S2 cells

S2 cells were stably transfected with the "pMT-fcnA" or "pMT-fcnB" constructs in order to express the ficolin-A and -B proteins, respectively.

Stable transfection is optimal for long-term storage, high protein expression, and large-scale production of the desired protein. In addition, the Drosophila Expression System (DES®, Invitrogen) offers a convenient non-lytic system that uses simple plasmids for stable expression of heterologous proteins in S2 cells. Protein expression using the DES occurs in healthy, logarithmically growing cells, so high yields of high-quality protein can be produced (Johansen *et al.,* 1989), (Deml *et al.,* 1999). *Drosophila melanogaster* transfected cell lines generally contain multicopy inserts of the foreign gene that form arrays of more than 500-1000 copies in a head to tail fashion.

In order to generate a stable recombinant cell line it is necessary to co-transfect the plasmid containing the sequence of interest and a resistance-bearing plasmid like the pCoHygro vector that constitutively expresses the hygromycin resistance gene (HPH) (Gritz and Davies, 1983) for selection of transfectants with the antibiotic hygromycin B. When added to cultured S2 cells, hygromycin B acts as an aminocyclitol to inhibit protein synthesis by disrupting translocation and promoting mistranslation.

In this work, transfection was performed with the SuperFect® Transfection Reagent (QIAGEN) which is an activated molecule with a defined spherical architecture and branches radiating from a central core and terminating at charged amino groups. Due to these features, it assembles DNA into compact structures, thus optimizing its entry into the cell. SuperFect-DNA complexes possess a net positive charge, which allows them to bind to negatively charged receptors (e.g. sialylated glycoproteins) on the surface of eukaryotic cells. Once inside, the reagent buffers the lysosome after it has fused with the endosomes, leading to pH inhibition of lysosomal nucleases and stability of SuperFect-DNA complexes.

The day before transfection cells were seeded in a 25 cm^2 flask (~$3x10^6$ cells) to get a 50-70% confluence on the following day. The pMT-fcnA/B vector (10 μg) and the pCoHygro vector (1 μg) were diluted in 500 μl of serum-free medium and then 50 μl of SuperFect reagent was added and mixed by pipetting. While samples were allowed to complex for 5-10 minutes at RT, cells were gently washed with PBS. 2 ml of medium (supplemented with 10% FCS) was added to the SuperFect-DNA mixture and the total volume was immediately transferred onto the cells. After 2-hour incubation at RT on a rocking platform, the medium was aspirated and 5 ml of fresh medium were added. Cells were cultivated for 3 days under normal conditions and then the selection reagent was incorporated to the normal growing medium (300 μg hygromycin B per ml of medium) which was replaced every 4-6 days. Selection took place over 4 weeks and was monitored by comparison to the control flask containing cells transfected in the absence of the pCoHygro vector. Cells transfected with the empty pMT/BiP/V5-His A vector served as a negative control.

II.2.3.1.2 Induction of protein expression

Stable transfectants were induced to express the protein by the addition of 500 μM $CuSO_4$ (final concentration) to the culture medium. To check for protein expression, supernatant aliquots were taken 2-3 days after induction and analysed by Western Blot (see section II.2.5.1). For high yield production of the recombinant protein supernatant was collected 10 days after induction and subjected to metal-affinity chromatography (see section II.2.4.1.1).

II.2.3.1.3 Freezing S2 cells

Cells were removed at 90-100% confluence in a 75 cm^2 flask, counted in a hemacyto-meter to determine viability, and pelleted at 1000x*g* for 5 minutes at 4°C. After one wash with sterile PBS, cells were resuspended in freezing medium (see Appendix A) at a density of $1x10^7$ cells/ml, frozen at -20°C, and transferred to the liquid nitrogen tank.

II.2.3.2 Embryonic fibroblasts (EF) and stem (ES) cells

II.2.3.2.1 Preparation of EF cells

Pregnant (E14.5) CD1 mice were sacrificed by cervical dislocation and rinsed with 70% ethanol before being placed in the laminar flow. Using sterile surgical instruments the abdominal skin and the peritoneal wall were cut open and the embryos were localized. The uterus was excised and placed into sterile PBS for washing. In order to free the embryos, the uterine wall was disrupted and the embryos transferred to a new dish containing PBS. Fetal heads and livers were removed and the rest of the embryo cut into small pieces which were then gently pushed through a metal sieve in order to get a cell suspension. Cells were recovered by centrifugation (250x*g,* 5 min), cultured on 15 cm dishes until they were confluent, and frozen down (4 vials/15 cm dish) (see section II.2.3.2.5).
EF cells were routinely cultured at 37°C with 8% CO_2 supply and split regularly according to their growing speed (normally every 2 days). For this, cells were washed with PBS and incubated with 1x trypsin/EDTA for ~2 min at 37°C. Trypsin digestion was stopped by the addition of fresh EF medium (see Appendix A) and single cells were obtained by gently pipetting up and down. Cells were split at a 1:3 ratio and cultured up to 4 passages.

II.2.3.2.2 Mitomycin-C treatment of EF cells

The day before an ES cell vial was to be thawed or ES cells in culture to be split, the required amount of EF plates were subjected to mitomycin-C treatment to inhibit growth (mitomycin works by inserting itself into the DNA strands and binding them together. This stops the cell

from synthesizing genetic material and proteins, thus impairing the cell growth). Confluent EF plates were washed with PBS and mitomycin-containing EF medium (10 μg/ml) was added. After 2.5 hours incubation at 37°C, cells were washed three times with PBS and cultured in fresh medium. Mitomycin-treated EF cells were used up to three days after treatment.

II.2.3.2.3 ES cell culture

ES cells were routinely cultured at 37°C with 8% CO_2 supply and medium was changed every day. The density was kept under $1x10^7$ cells/10 cm plate to prevent an increased frequency of differentiation (cells were trypsinized as explained before).

II.2.3.2.4 Electroporation of ES cells and selection of clones

DNA can be transfected into ES cells by application of a high voltage electrical pulse to a suspension of cells and DNA. After application of this pulse, the DNA passes through pores in the cell membrane. This results, however, in the death of about 50% of the cells when using conditions that give optimal transfection efficiency (i.e. voltage, ion concentration, DNA concentration and cell number).
Since the vector used to target the ficolin gene contains the neomycin resistance cassette, clones were selected in the presence of G420. This aminoglycoside antibiotic, similar in structure to neomycin and kanamycin, blocks protein synthesis through interference with ribosomal functions. The bacterial enzyme aminoglycoside phosphotransferase, carried on the transposon sequence Tn5, converts G420 to a non-toxic form.
Two days after the last passing ES cells were trypsinized, counted, adjusted to $6.25x10^6$ cells/ml, and mixed with the linearized sterile plasmid. The resulting suspension was dispensed in 10 cuvettes (BioRad Gene Pulser® Cuvettes 0.4 cm electrode), and subjected to electroporation (day 0) under the following parameters:

Electroporation device: BioRad Gene Pulser
Capacity extender: 250 μF
Volts: 0.34 kV
Time constant: was always between 3-4

After electroporation, cuvettes were placed immediately on ice for about 20 min, and then the cells were incubated under normal conditions on mitomycin-treated EF-dishes (0.4 ml/dish) in 10 ml fresh medium.
On day 1 the medium was changed (no selection agent included) and on day 2 selection with G420 (0.3 mg/ml; PAN) started. During selection the medium was changed every day until

clones were visible around day 9. When clones were ready to be picked, they appeared round with a shiny well-defined border.

Four hundred clones were picked under the microscope in a sterile laminar flow and transferred to 96-well plates in 20 μl PBS. In order to get single cells from each clone, they were trypsinized for 10 min at 37°C and homogenized by gentle pipetting. Cells were incubated under normal conditions with regular medium change and, when confluent, they were transferred to 48-well plates. At this stage, duplicate plates were generated in order to freeze the "mother plates" and to expand the duplicates for DNA isolation and southern blotting.

When positive clones were identified, the mother plates were thawed to get those clones in culture. Clones were further expanded: an aliquot was frozen as back-up and the rest used for blastocyst injection (see section II.2.6.3.3).

II.2.3.2.5 Freezing EF/ES cells

When frozen in conventional cryotubes, cells were trypsinized, spun down and resuspended in freezing medium to a final concentration of at least $1x10^6$ cells/ml.

ES clones on 48-well plates ("mother plates") were washed twice with PBS and then 100 μl trypsin was added. After incubation at 37°C for 10 minutes, digestion was stopped with 100 μl fresh medium and further diluted 1:1 with 2x freezing medium (see Appendix A). Plates were wrapped and, in both cases, cells were incubated at -20°C for about 2 hours before being transferred to -80°C.

II.2.3.3 Generation of anti-fcnB antibody-producing hybridomas

DNA synthesis in mammalian cells proceeds through a main (*de novo*) pathway which requires glutamine and aspartate (as well as activated phosphate) as initial substrates for a series of reactions for the synthesis of purine-type (dATP and dGTP) and pyrimidine-type (dCTP and dTTP) dNTPs, respectively. Several of the reactions involved can be blocked by aminopterin or methotrexate, both analogues of dihydrofolate, which bind with very high affinity and block the enzyme dihydrofolate reductase. As a result, *de novo* synthesis of dATP, dGTP, dCTP and dTTP is blocked. Mammalian cells, however, survive in the presence of aminopterin because they can utilise two salvage pathways. The first converts hypoxanthine in IMP, a reaction catalysed by the enzyme hypoxanthine-guanine phosporibosyl transferase (HPRT). The second converts thymidine in dTMP, a reaction catalyzed by thymidine kinase (TK). Thus a mutation in either the HPRT or the TK gene would lead to normal growth in standard culture medium but to death in Littlefield's HAT medium containing hypoxanthine, aminopterin and thymidine. Since the SP2/O-Ag14 cell line

is HPRT deficient ($HPRT^-$), these cells die in the presence of aminopterin. However, hybrids produced between such a myeloma cell line and B cells would survive because they would utilise the normal HPRT gene of the B cell.

II.2.3.3.1 Preparation of splenocytes for fusion

Three days after the last boost with ficolin-B (see section II.2.6.1) the immunized rats were sacrificed, and the spleen was aseptically removed and placed in a culture dish containing 10 ml of pre-warmed serum-free RPMI medium. Contaminant tissue was trimmed off before the spleen was cut into pieces and gently strained through a metal sieve in order to obtain a cell suspension. Splenocytes were centrifuged at 300x*g* for 5 min and washed twice with serum-free medium. One fourth of the total amount of cells was further used for the fusion whereas the remaining cells were frozen down as a back-up.

II.2.3.3.2 Preparation of peritoneal exudate cells

Peritoneal exudate cells (PEC) were prepared for RNA isolation (see section III.4.2) or two days before the fusion to be used as feeder cells during the hybridoma selection. For this, C57BL/6 mice (1 mouse per 96-well plate) were injected with 1 ml sterile PBS i.p. and sacrificed on the following day by cervical dislocation. Cells were aseptically harvested by peritoneal lavage with 8-10 ml chilled complete RPMI medium, pooled, and washed twice with PBS (300x*g*, 5 min, 4°C). The cell number was checked to be between 1-2x10^6 cells/mouse before plating them on 15x 96-well plates in HAT medium (see Appendix A) and cultured at 37°C, 5% CO_2 (Littlefield, 1964). On the next day, feeder cells were observed under the microscope for any possible contamination.

II.2.3.3.3 Fusion

Splenocytes were mixed with the SP2/O-Ag14 myeloma cells (see section II.1.6) in a 3:1 ratio in serum-free medium and pelleted at 300x*g* for 5 minutes. After the supernatant was completely removed, the cell mixture was incubated for 1 minute at 37°C with gently stirring. Polyethylenglycol 1500 (PEG 1500) (1ml) was then added on the cells slowly within 1 minute with constant swirling and finally 10 ml of HAT medium was also slowly added to the cells (within 5 min) drop by drop under stirring. The fused cells were resuspended in a final volume of 150 ml HAT medium, plated on 15x 96-well plates (100 µl/well), and incubated at 37°C, 5% CO_2. As an aminopterin-sensitivity control of the myeloma cell line, the last 8 wells of the 15th plate contained only SP2/O-Ag14 cells.

II.2.3.3.4 Maintenance and expansion of clones

Approximately 10 days after the fusion the first clones were visible. Screening started as soon as they reached ≥ 60% confluence or when the culture medium turned yellow. In every case, when supernatant was collected for the screening (see section II.2.5.3), cells were given fresh medium and/or were split to larger wells for expansion (feeder cells were used in the first two transfers to favour cell viability). As long as the hybridomas were expanded, medium was gradually changed from HAT to HT and from HT to RPMI (see Appendix A). Once the clones were adapted to RPMI medium, they were regularly split in a 1:3 to 1:6 ratio, approximately every 4 days.

II.2.3.3.5 Single-cell cloning (subcloning)

Since a well of fused positive cells often contains more than one clone of hybridoma cells, it is necessary to perform single-cell cloning as soon as possible to avoid overgrowth of undesired hybrid cells and to ensure that the cells that produce the antibody of interest are truly monoclonal and that the secretion of this antibody can be stably maintained.
Single-cell cloning was carried out by limiting dilution in the presence of feeder cells. Cells from a positive well (tested by ELISA, see section II.2.5.3) were counted and plated on a 96-well plate by serial 1:2 dilution starting with ~25 cells/well and cultured under normal conditions. After ~10 days the subclones were visible and subjected to screening (see section II.2.5.3). This procedure was performed with 5 mother clones which were stably positive after 4 rounds of screening. To ensure that the hybridomas were stable and truly monoclonal, the subcloning procedure was repeated until every well tested was positive.

II.2.3.3.6 Freezing hybridomas

After propagation of single clone cultures to achieve the required cell number, hybridomas were frozen in liquid nitrogen as back-up at different stages. For this, cells were counted, resuspended in fresh medium and diluted 1:2 in 2x freezing medium (see Appendix A) at a density of 0.5-1x10^7 cells/ml.

II.2.3.4 Mycoplasma test

Every eukaryotic cell line used in this work was mycoplasma-free.
Cells were periodically checked for mycoplasma contamination by DAPI staining. For this, cells were cytospun, stained with DAPI (1:100) and observed under the fluorescent microscope. Mycoplasma positive cells appear surrounded by small fluorescent dots.

II.2.4 Protein-biochemical techniques

II.2.4.1 Protein purification

II.2.4.1.1 Purification of recombinant ficolins by ion-metal affinity chromatography

Due to the features of the pMT/BiP/V5-His A expression vector (see section II.1.3.2), 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 Ni-NTA (nickel-nitrilotriacetic acid). 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).

Ten days after induction, the conditioned medium was collected and cleared by centrifugation at 3000x*g* 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.2.4.2). 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.2.4.4)

II.2.4.1.2 Polyclonal antibodies

II.2.4.1.2.1 Coupling of recombinant ficolin B to CNBr-activated sepharose

Rabbit anti-ficolin B polyclonal antibodies (see section II.2.6.2) were purified from sera by affinity chromatography on rfcn-B. For this, the CNBr-activated Sepharose™ 4B resin (GE Healthcare) was coupled to S2-expressed rfcn-B according to the manufacturer's instructions. The CNBr medium is pre-activated for immobilization of ligands containing primary amines by the cyanogen bromide method.

To prepare 0.5 ml of medium, 150 mg of the Sepharose freeze dried powder was suspended in 1 mM HCl on a sintered glass filter to wash away the additives (the use of pH 2-3 preserves the activity of the reactive groups, which otherwise hydrolyze at high pH). Additionally, 3 mg of rfcn-B were dialysed overnight at 4°C against coupling buffer (0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl) ("coupling solution"). The coupling solution and the clean medium were mixed together and incubated for 1.5 hours at RT under gentle rotation, and then excess ligand was washed away with 5 vol of coupling buffer. In order to block any remaining active groups, the medium was transferred to 1 M ethanolamine pH 8.0 and incubated for 2 hours at RT. Finally, the medium was washed with three cycles of alternating pH with at least 5 vol of each buffer. Each cycle consisted of a wash with 0.1 M acetate buffer in 0.5 NaCl pH 4.0 followed by a wash with 0.1 M Tris-HCl in 0.5 NaCl pH 8.0.

II.2.4.1.2.2 Purification of antibodies by affinity chromatography

Collected rabbit serum was filtered and mixed with the CNBr-fcn-B coupled medium batchwise overnight at 4°C under rotation. All the following steps were performed at 4°C with pre-equilibrated buffers. The medium was poured on a column and washed with 10 ml PBS before elution. Specific antibodies were eluted by a change in the pH that alters the degree of ionization of charged groups at the binding sites, thus, weakening the antibody-matrix interaction. For this, an elution buffer containing 100 mM glycine in 100 mM NaCl pH 2.4 was used followed by immediate neutralization of the samples by the addition of 1M Tris pH 11. Samples were collected in 0.5 ml aliquots and analyzed by SDS-PAGE. Positive 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.2.4.4).

The medium was finally regenerated by three cycling washes with 2-3 column volumes of alternating high pH (0.1 Tris-HCl in 0.5 M NaCl pH 8.5) and low pH (0.1 sodium acetate in 0.5 M NaCl pH 4.5) buffers.

II.2.4.1.3 Monoclonal antibodies

Anti-ficolin B monoclonal IgG antibodies (see section II.2.3.3) were purified by affinity chromatography on a protein-G sepharose column, according to the following steps:

II.2.4.1.3.1 Sample preparation: ammonium sulphate precipitation

Antibodies were precipitated from the hybridoma supernatant with 313 g/L ammonium sulphate (50% saturation), which was added within 1 hour under gentle stirring at RT. This is based on the observation that proteins in solution form hydrogen bonds with water through their exposed polar and ionic groups. When high concentrations of small, highly charged ions such as ammonium or sulphate are added, these groups compete with the proteins for binding to water. This removes the water molecules from the protein and decreases its solubility, resulting in precipitation. The proteins were allowed to precipitate overnight at 4°C and pelleted at 3000x*g* for 30 minutes at 4°C. The pellet was dissolved in deionized water (5% of the initial volume) and dialyzed overnight against 100 vol PBS followed by another overnight dialysis step against 100 vol binding buffer (20 mM sodium citrate pH 6.25). The dialyzed antibody solution was cleared by centrifugation to remove any remaining debris before applying it on the protein-G column.

II.2.4.1.3.2 Purification over protein-G sepharose

Two millilitres of protein-G sepharose were packed into a column and attached to the BioRad Econo System device. The resin was pre-equilibrated with 4 vol of binding buffer at 0.5 ml/min before the antibody solution was passed through at the same flow rate. Unspecifically bound proteins were washed away with binding buffer until baseline UV absorbance monitored at 280 nm. Elution was performed at 0.3 ml/min with 3 vol of elution buffer (0.1 M glycine pH 3.1) and samples were immediately neutralized by the addition of 1M Tris pH 11. Samples were collected in 0.5 ml aliquots and analyzed by SDS-PAGE. Positive 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.2.4.4). The resin was regenerated in 6 M Urea and stored in 20% ethanol.

II.2.4.2 SDS-PAGE

Purification performance and protein characterization was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. In the first case, the strongly anionic detergent SDS is used in combination with a reducing agent (e.g.

β-mercaptoethanol or DTT/iodoacetamide) 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. For characterization purposes, however, recombinant ficolins were run in the absence of any reducing agents which allows the detection of high-molecular weight oligomeric structures.

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 (see Appendix A) at 25 mA in the stacking gel and 45 mA in the resolving gel. For characterization purposes, recombinant ficolins were resolved on a gradient (4-12%) Bis-Tris precast gel (Criterion™XT, BioRad) and compared to the "All blue" protein standard (BioRad). Purified elution fractions were analyzed on a 12.5% polyacrylamide gel (see Table II.1) and compared to the "Broad Range" protein marker (BioRad).

	Stacking gel (5%)	Resolving gel (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 (TEMED)	5 µl	10 µl
10% Ammoniumpersulphate (APS)	50 µl	100 µl

Table II.1: composition of a 12.5% PAA-gel.

II.2.4.3 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 (see Appendix A) 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 then soaked with 5% glycerol in 35% ethanol for 1 hour, wrapped with cellophane (soaked in the same solution) and fixed in an easy breeze gel drying frame for air drying overnight at room temperature.

II.2.4.4 Determination of protein concentration

The amount of protein in the elution fractions was measured with the BioRad *DC* Protein Assay Kit (BioRad). 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 the Folin reagent by the copper-treated protein. Colour development is primarily due to the amino acids tyrosine and tryptophan. Proteins induce a reduction of the Folin reagent by loss of 1-3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum and minimum absorbance at 750 nm and 405 nm, respectively.
In brief, 5 µl of standards and samples were pipetted into a clean microtiter plate and mixed sequentially with 25 µl of reagent A and 200 µl of reagent B. Absorbance values were read at 650 nm after 15 min in an *Emax microplate reader* (MWG Biotech) and the concentration of the samples calculated according to the standard curve. To obtain this curve, a protein of known concentration (standard) was included in each plate at different dilutions and the concentration was plotted versus the absorbance.

II.2.4.5 Protein sequencing

S2-expressed ficolins-A and -B were sequenced by MALDI-TOF to confirm the identity and integrity of the recombinant material. For this, samples (~5 µg) were diluted in reducing buffer (containing 1 mM DTT) and heated at 60°C for 15 minutes before adding iodacetamide (20 mM final concentration). Denatured samples were run on a PAA gel, cast with fresh and high-quality buffers to avoid contaminants which could interfere with the protein. The PAA-gel was stained with coomassie blue for 30 min, destained with 7% acetic acid for 3 hours and

washed with water overnight. Corresponding bands were excised with a clean scalpel, cut into cubic pieces (1 mm^3), and extracted for 30 min each with:

1) 50 mM NH_4CO_3
2) 50 mM NH_4CO_3/25% acetonitril
3) 25% acetonitril
4) 50% acetonitril

Extracted samples were lyophilized for one hour (Speedvac) and digested with trypsine (2 µg/100 µl gel volume) in 50mM HN_4HCO_3 overnight at 37°C. Samples were extracted again with 100 mM NH_4HCO_3 (two times) and 100 mM NH_4HCO_3/acetonitril (1:1), lyophilized, dissolved in water and lyophilized again to ensure the removal of NH_4HCO_3. Finally, samples were dissolved in 5–10 µl 0.05 % triflouracetic acid (TFA), 9 µl of matrix solution was added to 1 µl sample solution and 0.5 µl of this mixture was loaded on a MALDI target plate.

II.2.4.6 Gel permeation chromatography (GPC)

GPC is a procedure frequently used for fractionating proteins from a mixture. The protein mixture is applied onto a column packed with porous beads with a specific and defined pore size that allow small and medium sized proteins to enter, under exclusion of larger proteins. The proteins pass through the column by gravity flow or by applied pressure. Because large proteins, not arrested through the volume of the beads, exceed the fractionation range they elute first from the column (in the so-called void volume (Vo)). Smaller proteins that fully or partially enter the pores of the beads are slowed down in the column and, therefore, elute later (Johnstone and Thorpe, 1996). Proteins are fractionated according to size when assuming they attain a normal globular shape. Asymmetrically shaped proteins appear to elute with a much higher molecular weight than a globular shaped molecule of similar molecular weight (Stellwagen, 1990).

This technique was the method of choice to investigate the ability of recombinant ficolins to associate to recombinant human MASP-2 (rhMASP-2). If association took place, ficolins and MASP-2 would elute as a complex, resulting in a peak shift in the chromatogram.

Recombinant hMASP-2 supernatant (Stengaard-Pedersen *et al.,* 2003) (1 µg) was subjected to GPC in the presence or absence of recombinant ficolin-A or –B (10 µg) in TBS/ Ca^{2+}/emulfogen buffer (see Appendix A) over a Superose-12 column (10 mm x 300 mm, Amersham Bioscience). Samples were incubated for 1 hour at RT before applying to the column for the complex formation to take place. The column was previously equilibrated in the same buffer and calibrated with a range of proteins of known molecular mass: blue

dextran 2000 (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), IgG (normal rabbit immunoglobulin G) (150 kDa) and human serum albumin (67 kDa). Void volume of the column was 7.7 ml. The column was operated in the ÄKTA FPLC chromatography system (UPC-900/P920/Frac-950, Amersham Bioscience) at a flow rate of 0.5 ml/min.

Eluted proteins were collected in fractions of 250 µl in polystyrene microtiter plates (Nunc) previously blocked with TBS/Tween. Fractions were subsequently analyzed by TRIFMA (see sections II.2.5.4.1 and II.2.5.4.3)

II.2.4.7 N-acetylglucosamine/ficolin-B binding assay

This assay was performed as reported by Fujimori and co-workers for ficolin-A (Fujimori *et al.,* 1998). Briefly, 30 µg of rfcn-B were mixed with 100 µl N-acetylglucosamine (GlcNAc)-agarose (Sigma), rotated slowly for 2.5 hours at room temperature and washed five times with 1% Triton, 0.1% SDS, 0.5 M NaCl in 50 mM Tris-HCl (pH 7.4) (1 ml). Bound proteins were detached with SDS sample buffer and subjected to SDS-PAGE under reducing conditions.

II.2.5 Immunoassays

II.2.5.1 Western Blot

In order to identify and assess the size of the protein(s) of interest, samples were subjected to immunoblotting. After being resolved by SDS-PAGE, proteins were transferred from the PAA-gel to a PVDF membrane (Immobilon-P, Millipore) by a three-buffer semi-dry blotting system (Towbin *et al*., 1979). Accordingly, three pieces of Whatman 3MM paper were soaked in blot buffer A, B or C each (see Appendix A). The gel was washed and fixed in blot buffer B and the membrane rehydrated in methanol before being transferred to blot buffer B. Each component was positioned in a way that the membrane was placed on the side of the gel facing the anode. Air bubbles in-between the layers were carefully avoided. Proteins were transferred during 0.8 hours at 0.8 mA/cm^2.

Before proceeding to immunodetection, membranes were incubated overnight at 4°C in blocking solution (1% low fat dry milk powder in TBS/0,05%Tween). The first antibody, specific for the protein, was added for 2 hours at room temperature at the optimal concentration (see section II.1.4) in blocking solution. After three 5-minute washing steps with TBS/0,05%Tween, a horseradish-peroxidase (HRP)-coupled secondary antibody, detecting the isotype of the first antibody, was added for 1 hour at room temperature. After washing, the HRP-substrate (1:1 mixture of solution A and B from Nowa) was added for

about 1 minute. HRP oxidizes luminol (B) in the presence of hydrogen peroxide (A) and alkaline pH. Oxidized and activated luminol emits light for a short time. Blots were exposed to a autoradiography film (Hyperfilm™ ECL, Amersham) for 5 sec to 30 min depending on the signal intensities.

II.2.5.2 Dot blot

Dot blots were performed either to assess the ability of poly- and monoclonal antibodies in detecting non-reduced ficolin-B or to evaluate the binding affinity of ficolin-B to different organic compounds. In each case ficolin-B (in different concentrations) or the organic compound (1 μg) were spotted on a nitrocellulose membrane at the corresponding concentration and let air-dry for 30 minutes before blocking with 1% low fat dry milk powder in TBS/0,05%Tween. The following detection steps were identical to a Western Blot (see section II.2.5.1).

II.2.5.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA was the method of choice to screen the hybridomas for specific antibody production (see section II.2.3.3.4) and to assess antibody titer in blood from immunized animals (see sections II.2.6.1 and II.2.6.2).

Maxisorp™ flat bottomed microtiter plates (Nunc) were coated with 1 μg/ml rfcn-B (100 μl) in coating buffer (see Appendix A) overnight at 4°C. Blocking of unspecific sites was performed with 0.1% HSA in TBS (200 μl) for 90 minutes at RT, and the same blocking buffer was used to dilute the reagents of the following steps. Samples were added undiluted (hybridoma supernatant) or in serial dilutions (rabbit/rat serum) in duplicates and incubated for 90 minutes at RT. For detection, goat anti-rat or anti-rabbit antibodies were used for the hybridomas screening or antibody titer measurement, respectively. In both cases, the antibodies (Sigma) were conjugated to alkaline phosphatase (AP) and used in a 1:1000 dilution for 90 minutes at RT. In order to develop the colour reaction, para-nitrophenylphosphate (“Sigma 104”) was added as the substrate diluted in AP buffer (see Appendix A) at 0.6 mg/ml. Absorbance was determined after about 15 minutes at 405 nm.

Washings between different steps were carried out with 200 μl TBS/0.05%Tween three times and plates were dried upside down on paper towels.

II.2.5.4 Time-resolved immunofluorometric assay (TRIFMA)

TRIFMA measures the fluorescence energy emitted from a fluorophore excited by a pulsed light source (Lovgren T.H.I. *et al.,* 1985). The fluorophore formed in this technique is an organic ligand chelated with europium ions (Eu^{3+}). The ligand absorbs light and transfers the energy to the europium ions which, in turn, emit the energy that is measured in the assay. The europium ions are used as a label and are bound in a non-fluorescent form to an immunoreactive component (e.g. an antibody or streptavidin). To measure the amount of antibody or streptavidin, the europium ions are dissociated into solution where highly fluorescent europium chelates are formed by adding enhancement buffer (see Appendix A) to the immunoreaction. The buffer creates the conditions necessary for the europium ions to dissociate and form the fluorescent chelate in solution (Lovgren T.H.I. *et al.,* 1985). In the various TRIFMA assays described below, 200 µl of enhancement buffer were added to each well and incubated with agitation for a minimum of five minutes before the fluorescent energy was measured using a DELFIA fluorometer (Delfia 1232, Wallac).
TRIFMA analysis was used to evaluate (i) the binding of recombinant ficolin-A and -B to an acetylated surface as already observed for L- and M-ficolin (Jensenius *et al*, unpublished observation), (ii) the ability of rfcn-A and –B to activate the complement system in association with rhMASP-2 resulting in C4b deposition, and (iii) to analyze the fractions from the GPC.

II.2.5.4.1 Ficolin-ligand TRIFMA analysis

The detailed description of the generation of acetylated ligands in microtiter wells will be described elsewhere (Larsen *et al*, unpublished). In brief, microtiter wells were coated with HSA (10 µg/ml) in coating buffer (see Appendix A) and the HSA molecules acetylated by incubation with acetic acid anhydride (0%, 0.1%, 0.2%, 0.5% $^{v}/_{v}$) in methanol. After washing thrice in TBS/Tween and blocking of residual binding sites with TBS/Tween containing 1 mg/ml HSA, the plates were incubated with rfcn-A or -B (1 µg/ml or 10 µg/ml) overnight at 4°C. To detect bound ficolin, the plates were incubated for 2 hours at room temperature with mouse anti-His antibody (BD) at 1 µg/ml in TBS/Tween/Ca^{2+}. Plates were washed with TBS/Tween/Ca^{2+} and incubated for 1 hour with 1 µg/ml biotin-conjugated rabbit-anti-mouse antibody in TBS/Tween/Ca^{2+} before addition of Eu^{3+}-streptavidin (Dako) (1:1000 in TBS/Tween/25 mM EDTA, for 1 hour) and readout of the signals as described above. In following assays, an acetylation with 0.2% acetic acid anhydride was performed as it showed the best signal (data not shown).

II.2.5.4.2 C4b-deposition TRIFMA analysis

The formation of complexes between the recombinant ficolin-A or -B and the recombinant human MASP-2 (from HEK-293S transfected cells) was analyzed by TRIFMA. Acetyl-HSA coated and blocked (see previous section) microtiter plates were incubated overnight at 4°C with ficolin/MASP-2-mixtures (1 or 10 μg/ml rfcnA/B and 0, 0.1 or 1 μg/ml rhMASP-2 in TBS/Tween/Ca^{2+}), washed with TBS/Tween/Ca^{2+} and incubated with purified human C4 (Dodds, 1993) (5 µg/ml in B1 Buffer (see Appendix A) at 37°C for 1.5 hours. After washing, the wells were incubated with a mixture of two biotinylated anti-C4 monoclonal antibodies (Hyb 161-1 and Hyb 161-2; Immunolex), each at a concentration of 0.25 µg/ml in TBS/Tween/Ca^{2+}. Detection was performed after the addition of europium-labelled streptavidin.

II.2.5.4.3 MASP-2/ficolin-detection TRIFMA analysis

Gel filtration fractions (see section II.2.4.6) were analyzed by TRIFMA in order to identify the different peaks obtained in the chromatogram.

For MASP-2 detection, microtiter plates were coated with 3 μg/ml of mouse anti-MASP-2 monoclonal antibody (8B5) in coating buffer overnight at 4°C. After washing and blocking as previously described, GPC elution samples were added in a 1:2 dilution in MASP-2 buffer (see Appendix A) and incubated overnight at 4°C. MASP-2 was detected with a biotin-conjugated mouse anti-human MASP-2 monoclonal antibody (6G12) in a 1:1500 dilution for 2 hours at RT followed by europium-labelled streptavidin and detected as described above.

For ficolin detection, fractions from the gel filtration were diluted and plated on plates coated with 0.2% acetylated-HSA and TRIFMA was performed as described in section II.2.5.4.1.

II.2.5.5 Immunohistochemistry on PEC

In order to verify the hypothesis that ficolin-B could be expressed in mouse phagocytes, peritoneal exudate cells (PEC) were isolated and subjected to immunohistochemistry (IHC). For this, male C57BL/6 mice received 1 ml PBS i.p. with or without LPS (1 μg) from *Salmonella abortus equi* (kindly provided by M. Freudenberg, Freiburg, Germany). As control peritoneal cells (PC) were also harvested from non-treated mice. After 16 hours the mice were sacrificed by cervical dislocation and a peritoneal lavage was performed with 10 ml of cold RPMI medium. Washed PEC and PC were seeded in Lab-Tek II 4-chamber slides (Nunc) ($5x10^5$ cells/chamber) and cultured at 37°C overnight. Cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0,1% Triton-X 100 for 5 min or not permeabilized at all. Fc receptors were blocked with the rat anti-mouse FcγRII/III 2G4.2

antibody (2 μg/ml in PBS) and with 10% goat serum in PBS for 1 hour each. Cells were stained with rabbit anti-ficolin B polyclonal antibodies (2 μg/ml in PBS) or with irrelevant antibodies (rabbit IgG, 2 μg/ml in PBS) for 1 hour, or incubated in the absence of antibodies as a control. As a secondary antibody for the specific ficolin-B staining, FITC-conjugated goat anti-rabbit-IgG antibody was used in a 1:100 dilution for 1 hour. Finally, cells were incubated for 20 min with DAPI working solution (1:100 in PBS) for staining of nuclei. All steps were performed at room temperature. Slides were covered and stored at 4°C in the dark until pictures were taken at the fluorescence microscope (Axiovert S100, Zeiss).

A peritoneal cell population rich in polymorphonuclear neutrophils (PMN) (~ 80%) was harvested in 10 ml RPMI 3 hours after i.p. injection of 1 ml PBS, and subjected to cytospin (800 rpm, 7 min) and the same staining protocol.

For co-localization experiments, cells were incubated with a FITC-conjugated anti-mouse Lamp-1 (CD107a) antibody (BD, 1:100) for 1 hour before the DAPI staining. In this case, TRITC-conjugated goat anti-rabbit-IgG antibodies (1:200) were used as secondary antibodies for the ficolin-B specific detection. Pictures were taken under the confocal microscope LSM 510 from Zeiss.

II.2.5.6 Immunoprecipitation

In order to confirm the presence of ficolin-B protein in adult mouse tissue, immunoprecipitation (IP) from lung, liver, spleen and bone marrow was performed using rabbit anti-ficolin B polyclonal antibodies. The whole procedure was carried out at 4°C with pre-equilibrated buffers.

C57BL/6 mice were sacrificed by cervical dislocation and the organs of interest removed and placed in RPMI medium immediately. Small pieces of tissue were cut and gently strained through a metal sieve in order to get a cell suspension. Cells were homogenized by pipetting, and spun down at 250x*g* for 10 min. Pelleted cells were washed with PBS and counted in a Neubauer chamber. 1-1.5x10^7 cells were lysed in 1 ml RIPA buffer (see Apendix A) for 15 minutes, vortexed and centrifuged for 10 minutes. The lysate was transferred to a clean tube and pre-cleared with 20 μl protein-G sepharose overnight under gentle rotation. After centrifugation (3000*g*, 5 min), the pre-cleared lysate was subjected to IP by the addition of 10 μg rabbit anti-ficolin- B polyclonal antibody (overnight under rotation) and further incubation with 20 μl protein-G sepharose (1 hour under rotation). Samples were washed three times with PBS and subjected to SDS-PAGE (see section II.2.4.2).

II.2.6 Animal experiments

II.2.6.1 Immunization of rats

Two young female Wistar rats were immunized with 50 μg of purified rfcn-B in adjuvants (Titermax Gold, Sigma) s.c. and boosted three times in three-week intervals with 20 μg rfcn-B s.c. for the first boost and 10 μg rfcn-B in PBS i.p. for the last two. One week after the first boost, the animals were anesthetized (8 mg xylazine + 37 mg ketamine/body weight in 0.2 ml PBS) and blood was drawn from the retroorbital plexus to analyze the antibody titer by ELISA (see section II.2.5.3). The best responder animal was sacrificed three days after the last boost and the spleen used in the fusion (see section II.2.3.3.3).

II.2.6.2 Immunization of rabbits

Female Chinchilla Bastard rabbits were purchased from Charles River and immunized with 50 μg of purified rfcn-B in adjuvants (Titermax Gold) s.c. and boosted 3 times at 3-week intervals with 20 μg of the recombinant material in adjuvants s.c. or PBS i.m. (for the last two times). Blood was collected 1 week after the last boost, and regularly every 2 weeks, from the central ear artery which was exposed to Gaultheria oil to promote vasodilatation. Blood was allowed to clot at RT for 1 hour and overnight at 4°C. Serum was obtained after centrifugation at 3200x*g* for 5 minutes at 4°C. Sera were stored at -20°C. Freezing/thawing cycles were avoided.

II.2.6.3 Mice used for gene targeting

II.2.6.3.1 Superovulation

In order to enhance the yield of embryos, 4 weeks old C57BL/6 mice were superovulated by injecting 5 U pregnant mare serum gonadotropin (PMSG; mimics follicle stimulating hormone) in 100 µl PBS i.p. on day 0 and 5 IU human chorionic gonadotropin in 100 µl PBS i.p. (hCG; mimics luteinizing hormone) 48 hours later (day 2). After administration of hCG these females were mated to male C57Bl/6 mice and the presence of vaginal plugs was checked the next morning (day 3.5). Plug positive mice were then caged separately. On day 6.5 (this means 2.5 days post coitum (dpc)), blastocysts were collected for injection (see section II.2.6.3.4).

II.2.6.3.2 Foster mice

To generate pseudopregnant foster mice, CD1 female mice (≥ 8 weeks old) were mated to vasectomized CD1 mice. Plug positive foster mice were used 3.5 dpc for embryotransfer.

II.2.6.3.3 Preparation of ES cells for injection

An ES cell clone that had undergone homologous recombination was cultured and split 2 days before the injection. At the day of injection (day 6.5) the cells were fed with fresh ES medium. After 2-3 hours, cells were washed with PBS and incubated with trypsin for 10 minutes at 37°C. The reaction was stopped by adding 6 ml of ES medium and clumps were disrupted by pipetting. Cells were then incubated at 37°C for 10-15 minutes to allow the feeder cells to adhere to the dish while the ES cells remain in the supernatant ("preplating"). ES cells were then carefully transferred to a sterile tube and centrifuged at 125x*g* for 5 min. Prior to injection ES cells were resuspended in ES medium containing Hepes (20 µl 1 M Hepes/ml ES medium) and further incubated on ice for 30 minutes in order to select living cells for the injection. Therefore, the supernatant (containing dead cells) was carefully discarded and sedimented cells resuspended in ES-medium containing Hepes.

II.2.6.3.4. Collecting Blastocysts for injection

Uteri from 1 or 2 superovulated C57Bl/6 mice were dissected and collected in a 6 cm dish containing 1 ml M2 medium (Sigma). Blastocysts were flushed out of both uterine horns using a 2 ml syringe and a 22 gauge needle. Blastocysts were washed 3 times in M16 medium (Sigma) and kept at 37°C until use.

II.2.6.3.5. Blastocysts injection

For injection, blastocysts were retransferred to M2 medium. The injection was performed using a microscope (Leica) equipped with two micromanipulators. Between 10 and 15 ES cells were injected into one blastocyst.

Before being transferred into the foster mother, injected blastocysts were washed 3 times in M2 medium. About 8 injected blastocysts were transferred into each uterine horn per foster mouse. After embryo transfer, foster mothers were kept in a quiet, separated environment in the animal facility as stress reduces the chance of embryo implantation and hence the number of successful pregnancies. Mouse weight was monitored every 2 days up to day 16 after blastocysts transfer to monitor pregnancy.

Chimeras were identified by coat colour (black and brown vs. black only) and southern blotting, and later mated to wild type (wt) C57BL/6 mice.

III Results

III.1 Cloning and expression of recombinant ficolin-A and -B

The generation of new tools like monoclonal and polyclonal antibodies against a protein of interest requires such a protein to be available for immunization purposes. It is quite often, however, that the natural product has not been isolated due to the lack of knowledge about its binding affinity and, thus, it is necessary to produce recombinant material. Such is the case with the two mouse ficolins (termed ficolin-A and –B), the genes of which have been sequenced (Fujimori *et al.,* 1998) (Ohashi and Erickson, 1998) and scanned in search of conserved domains and potential posttranslational modification sites. However, very little is known about the properties of the proteins they encode.

Therefore, the first aim of this work was the synthesis of V5/His-tagged ficolin-A and –B. The whole process towards the generation of the recombinant material consisted of (i) the cloning of the ficolin genes into an expression vector for (ii) protein production in insect cells and (iii) further purification by metal affinity chromatography.

III.1.1 Generation of ficolin-A/-B expression constructs

The ficolin-A gene contains a 1002-base pair (bp) open reading frame (ORF) encoding 334 amino acids, while ficolin-B is encoded as a protein of 314 amino acids from a 942-bp ORF. In order to clone both sequences into the pMT/BiP/V5-His A expression vector, primer pairs (numbered from 5 to 8 in section II.1.3.1) were designed to amplify the genes without their leader peptide region (Fig. III.1) because the plasmid contains the Drosophila BiP signal sequence for secretion of the recombinant protein into the culture medium. Therefore, a 942 bp and an 882 bp fragment were amplified from spleen cDNA by PCR for ficolin-A and –B, respectively (see section II.2.2.1.1). These fragments were first cloned into the pCR2.1-TOPO vector, which is an activated plasmid ready to accept PCR products as inserts, to generate the “TOPO-fcnA” and “TOPO-fcnB” constructs. Since the primers also contained endonuclease restriction sites at the 5’ ends these constructs were further digested with *Bgl*II/*Xho*I and *BamH*I/*Xho*I for ficolin-A and –B, respectively, and subcloned into the pMT/BiP/V5-His A vector, which was previously digested with the same enzymes, to generate the constructs called “pMTA-fcnA” and “pMTA-fcnB”. Figure III.2 shows a scheme of the described cloning process (see cloning protocols in section II.2.1). The resulting expression constructs were evaluated by restriction analysis and sequenced to confirm that the inserts were cloned in-frame with the V5/His-tags and lack any mutations before proceeding to transfection.

Fig. III.1: **Ficolin-A (left) and ficolin-B (right) nucleotide and deduced amino acid sequences.** The putative signal sequences (blue), collagen-like domains (yellow) and fibrinogen-like domains (red) are shadowed. Potential N-glycosylation sites (open boxes) as well as the start and stop codons (orange boxes) are indicated. The binding sites for the primers used to amplify both genes are underlined in green.

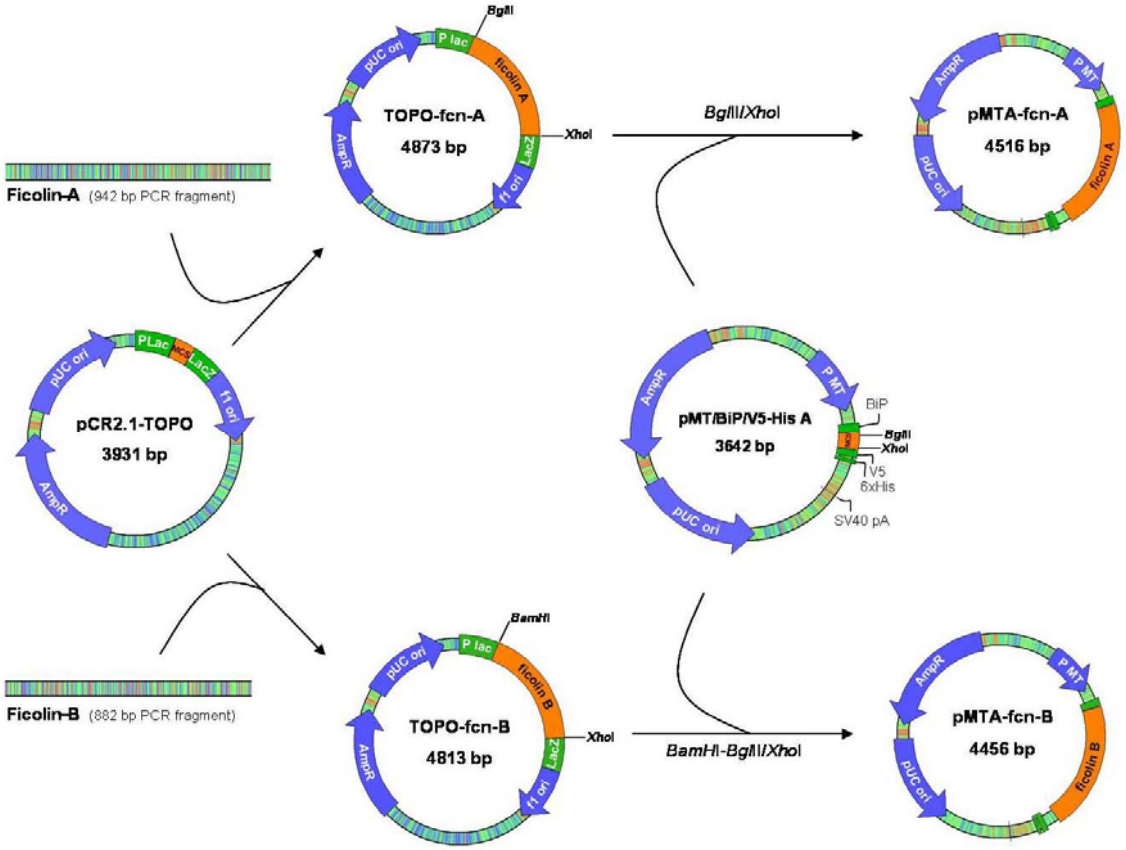

Fig. III.2: Scheme for the cloning of ficolin-A and –B genes into the pMT/BiP/V5-His A expression vector. The ficolin-A and –B genes were amplified by PCR and cloned into the pCR2.1 vector. Constructs were then digested with *Bgl*II/*Xho*I (ficolin-A) or *BamH*I/*Xho*I (ficolin-B) and further subcloned into the pMT/BiP/V5-His A expression plasmid. **AmpR**: ampicillin resistance gene; **LacZ**: α-fragment of the ß-galactosidase gene; **MCS:** multiple cloning site; **P Lac**: LacZ promoter; **P MT**: metallothionein promoter; **SV40 pA**: Simian virus 40 polyadenylation signal.

III.1.2 Stable expression of ficolin-A and –B in Drosophila Schneider 2 (S2) cells

S2 cells were co-transfected with the expression plasmid ("pMTA-fcnA" or "pMTA-fcnB") and the pCoHygro vector bearing the hygromycin resistance gene (see section II.2.3.1). After 4 weeks under hygromycin-B selection, stably transfected S2 cells were induced with 500 μM $CuSO_4$ (final concentration) for 3 days and harvested by centrifugation. The supernatant was evaluated for protein expression by Western blot (see section II.2.5.1). The migration of ficolin-A and –B bands through the 12.5% polyacrylamide (PAA) gel under reducing conditions was compared with a molecular weight (MW) standard (Fig. III.3). Both bands migrated between the carbonic anhydrase (31 kDa) and the ovalbumin (45 kDa) bands. As expected, the apparent MW of ficolin-A was slightly higher than the one of ficolin-B, which correlates with the present literature (Fujimori *et al.,* 1998), (Ohashi and Erickson, 1998).

In spite of these positive signals by Western blot, the amount of recombinant protein after 3 days of induction was clearly not enough for purification purposes since it was not possible to detect any protein band by Coomassie staining (data not shown). Therefore, for high yield production of the recombinant material, supernatants were collected at day 10 after induction and subjected to metal-affinity chromatography.

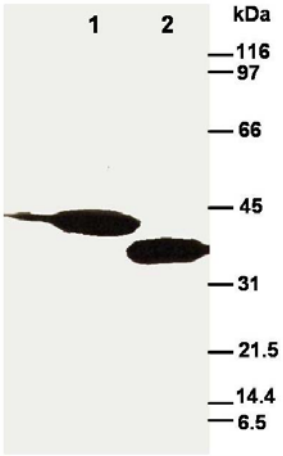

Fig. III.3: Ficolin-A and –B expression by Drosophila S2 cells. S2 conditioned medium containing secreted ficolin-A (lane 1) or –B (lane 2) was loaded onto a 12,5% PAA-gel and blotted. Membranes were incubated with a mouse anti-V5 antibody, followed by an HRP-coupled anti-mouse IgG antibody, and developed with the enhanced chemiluminiscent (ECL) system.

III.1.3 Purification of recombinant ficolin-A and –B by IMAC

The expression of recombinant proteins with histidine fusion tags is widely used to facilitate their purification from a variety of prokaryotic and eukaryotic expression systems (Ford *et al.,* 1991) (Schmitt *et al.,* 1993) by employing immobilized metal affinity chromatography (IMAC) methods. In addition, since the 6xHis tag is much smaller than other commonly used tags, it normally does not interfere with the structure and function of the recombinant protein and due to its poor immunogenicity the protein can be used as an antigen to generate antibodies without prior removal of the tag.

In order to purify the S2-expressed V5/His-tagged ficolins, a modified IMAC using a chelating sepharose was the method of choice (Lehr *et al.,* 2000). This resin has a capacity of approximately 5 mg His-tagged protein/ml resin, and mouse ficolins were purified from the S2 conditioned media as described before (see section II.2.4.1.1). Figure III.4 shows a representative elution profile obtained during the purification. This plot and the amount of

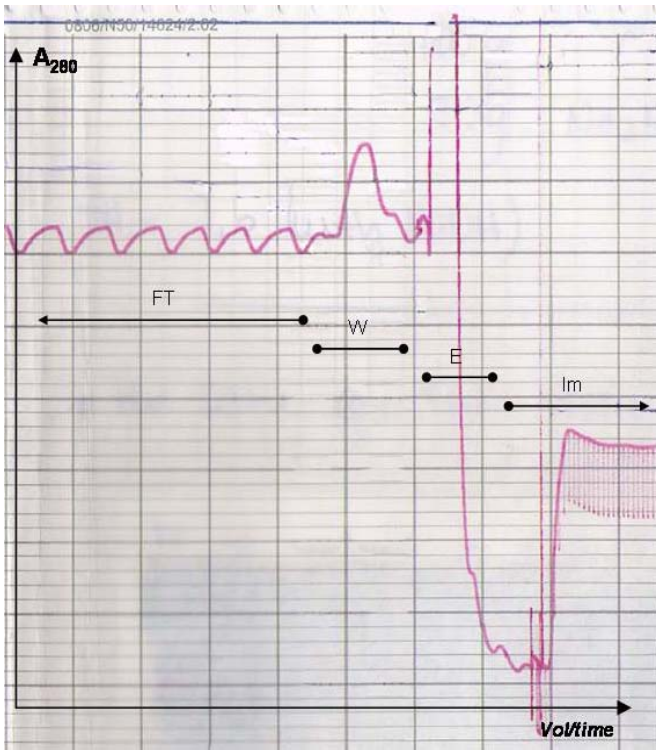

Fig. III.4: Representative purification profile for rfcn-A and –B by IMAC. S2 conditioned medium containing secreted fcn-A or –B was incubated batchwise overnight with the Chelating Sepharose resin. The washing and elution steps were performed in-column. His-tagged proteins were eluted with 250 mM imidazole in PBS. **FT:** flow-through, **W:** washings, **E:** elution, **Im:** imidazole absorbance.

Protein in each fraction (measured as stated in section II.2.4.4) correlated with the colour changes occurring in the resin: the sepharose, originally white, turned light blue after incubation with the S2 conditioned medium, due to the binding of copper ions, and stayed blue during the washing and elution steps. After stripping with EDTA, the resin regained its white colour indicating the successful removal of the metal ions off the sepharose. Flow-through (not bound proteins), washing and elution fractions were collected and analyzed by reducing SDS-PAGE and Coomassie blue staining (Fig. III.5) under reducing conditions. While ficolin-B only displayed one band in the elution fractions, ficolin-A revealed two co-purifying bands, one below and one above the 45 kDa band of the ovalbumin, the smaller one having the expected ficolin-A apparent MW. In order to evaluate if the second band was a contaminating protein, both bands were sequenced by MALDI-TOF (see section II.2.4.5). Sequences were blasted against the PubMed database and both bands were identified as ficolin-A, suggesting that the presence of the second band might be due to a distinct glycosylation pattern although this could not be confirmed. In addition, the identity of the unique ficolin-B band was also confirmed by sequencing.

Positive pure elution fractions were pooled and dialyzed overnight against PBS and yields were determined to be 3.6 and 6.7 mg of recombinant protein per liter of S2 conditioned medium for ficolin-A and –B, respectively.

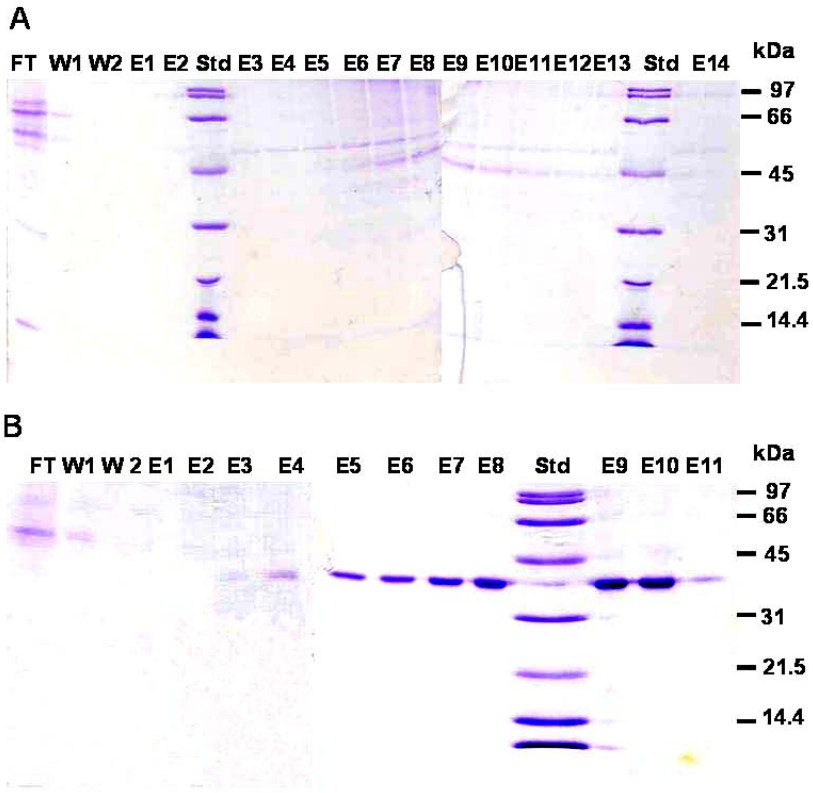

Fig. III.5: Recombinant ficolin-A and –B purification by IMAC. Coomassie-stained 12,5% SDS-PAGE gels for **A)** ficolin-A and **B)** ficolin-B purification. **FT:** flow-through, **W1:** PBS washing, **W2:** 10 mM imidazole in 0.5% NaCl/PBS washing, **E#:** elution fractions (250 mM imidazole), **Std:** molecular weight standard.

III.1.4 Characterization of the recombinant ficolin-A and –B

Ficolins represent a group of multimeric proteins composed of identical polypeptides. While the fibrinogen-like domains form the globular heads responsible for the carbohydrate binding activity, the collagen-like segments assemble the ficolin subunits into trimers and are involved in the ficolin/MASP-2 association. In order to investigate whether the S2-expressed recombinant ficolin-A (rfcn-A) and –B (rfcn-B) retain these characteristics different approaches were performed.

The ability to form large oligomers was tested by comparing the migration of the recombinant proteins on a 4-12% gradient SDS-PAGE (see section II.2.4.2) under reduced and unreduced conditions (Fig. III.6). Both ficolin-A and –B displayed bands of higher MW in the absence of reducing reagents, ranging from 75 kDa to over 250 kDa, suggesting a certain degree of oligomerization.

Although this tertiary multimeric structure is essential for ficolins to function as lectins, this finding was not sufficient to speculate about a possible ficolin/MASP-2 association, and the

lack of purified mouse MASP-2 did not allow to test it. However, the fact that recombinant human MASP-2 (rhMASP-2) binds recombinant mouse MBL (Jensenius *et al*., unpublished observation) raised the question whether it would also be able to interact with mouse ficolins and, consequently, provide a convenient way to characterize the recombinant material. Therefore, in order to elucidate whether rfcn-A and -B are able to form complexes with rhMASP-2, a C4b deposition assay by time-resolved immunofluorometric assay (TRIFMA) and gel permeation chromatography (GPC) were carried out.

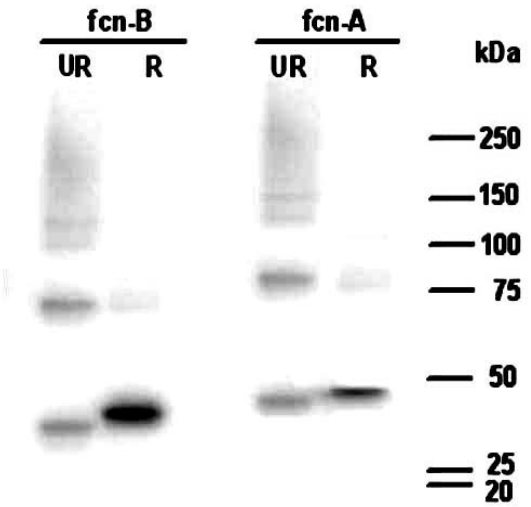

Fig. III.6: Western blot analysis of S2-expressed ficolin-A and –B. Recombinant fcn-A and –B (1 μg each) were resolved on a 4-12% gradient polyacrylamide gel under reduced (R) and unreduced (UR) conditions and blotted. Membranes were incubated with a mouse anti-His antibody followed by an HRP-conjugated anti-mouse secondary antibody and detection with the ECL system.

To perform the C4b deposition assay, microtiter plates were coated with acetylated HSA (Ac-HSA). Therefore, it was first necessary to evaluate the ability of rfcn-A and –B to bind to this coating. Once the positive binding of recombinant mouse ficolins to Ac-HSA was confirmed (see section II.2.5.4.1) (Fig. III.7a), the C4b deposition assay was performed as previously described (see section II.2.5.4.2). L-ficolin was used as a positive control. As seen in figure III.7B, no C4 cleavage by ficolin/MASP-2 was detected, indicating either that mouse ficolins do not activate the complement system or that they do not associate to the recombinant human MASP-2. In order to discriminate between these two possibilities, GPC was performed.

GPC was carried out by running rhMASP-2 supernatant and ficolin-A or -B on a Superose-12 column either alone or after each ficolin had been incubated together with rhMASP-2 for 1 hour in the presence of calcium (see section II.2.4.6). If ficolin/MASP-2 complexes were formed, they would behave as a bigger molecule, and elute earlier from the column. However, as figure III.8 shows, when elution samples were analyzed by TRIFMA for the presence of MASP-2 (see section II.2.5.4.3), no change was observed in the elution profile of this molecule, indicating its lack of interaction with the recombinant ficolins.

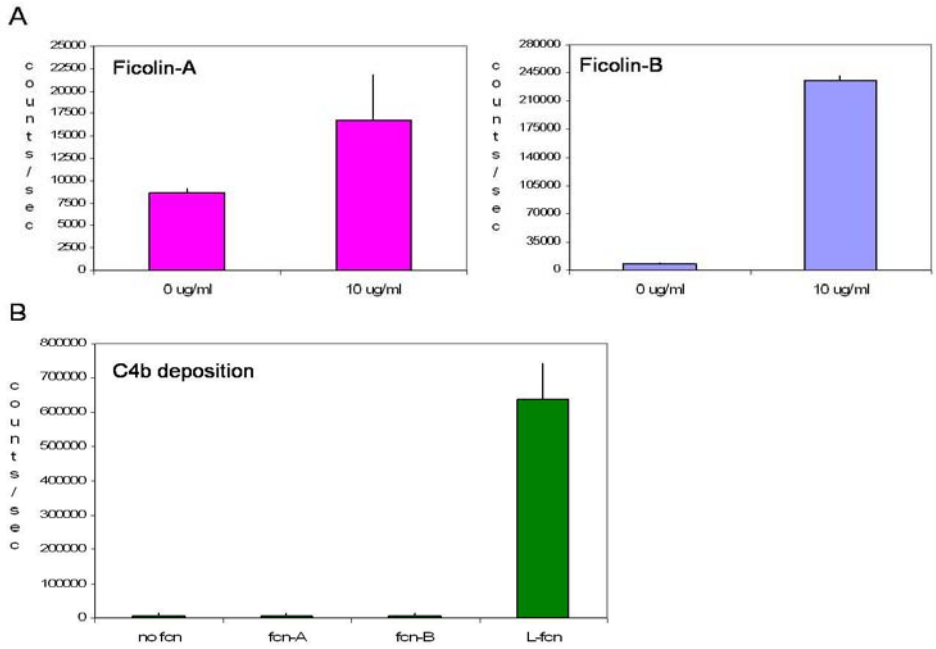

Fig. III.7: TRIFMA assays for the binding of recombinant mouse ficolins to acetylated-HSA and complement activation. A) Microtiter plates were coated with HSA (10 μg/ml) and subjected to acetylation. Ficolins (10 μg/ml) were added and detected with a mouse anti-His antibody, followed by a biotinylated anti-mouse antibody and Eu^{3+}-streptavidin. **B)** Purified C4 was added to Ac-HSA-bound ficolins and the split product C4b detected by the mixture of two anti-C4b antibodies (see section II.2.5.4.2). L-ficolin (1 μg/ml) was used as a positive control.

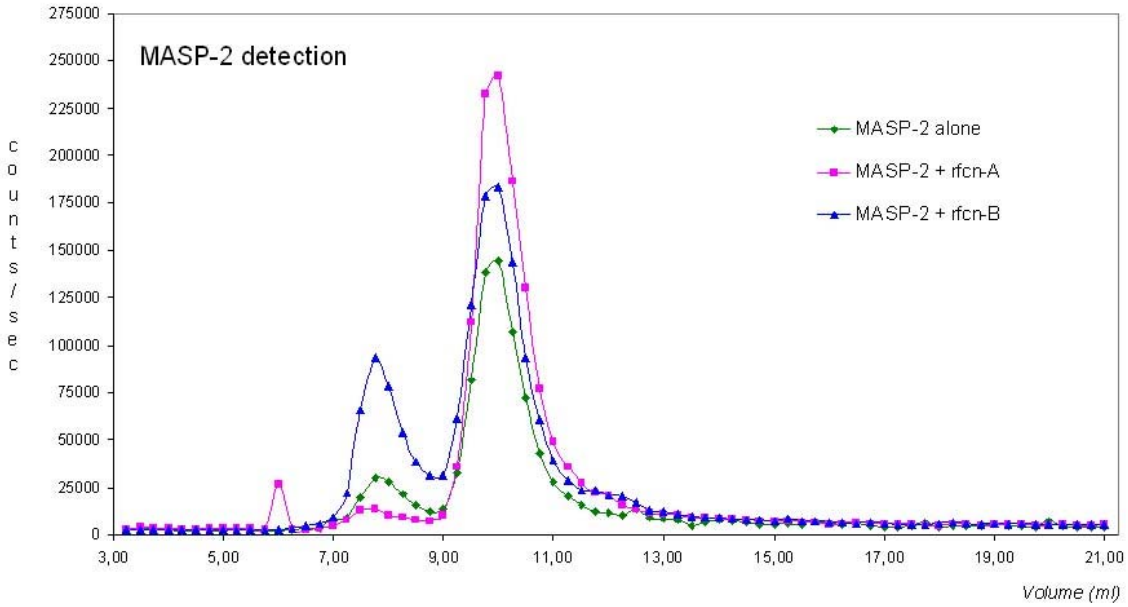

Fig. III.8: GPC elution fractions analyzed by TRIFMA for MASP-2 detection. Recombinant hMASP-2 (1 μg) was run over a Superose-12 column, either alone or together with rfcn-A or –B (10 μg), in the presence of calcium. Elution fractions were analyzed by TRIFMA for MASP-2 detection.

III.2 Generation of anti-ficolin-B antibodies

III.2.1 Generation of polyclonal antiserum

Upon immunization a large number of antibodies are produced that recognize different independent epitopes (the antibody binding site) on the antigen. Each specific antibody is produced by a different clone of plasma cells, making serum a very good source of polyclonal antibodies. These antibodies are commonly used as reagents in immunochemical techniques, either by taking the complete serum as the source of antibodies or after they are affinity-purified on solid phase antigen.

Due to the lack of specific reagents for the study of mouse ficolins, the main reason for the expression of the recombinant proteins was their use in the immunization of rabbits to produce specific anti-sera. Thanks to the high yield production of recombinant ficolin-B it was possible to purify the polyclonal antibodies by affinity chromatography on ficolin-B-coupled sepharose. In addition, these monospecific polyclonal antibodies were characterized in different immunoassays and further used for the detection of native ficolin-B.

III.2.1.2 Immunization and production of antiserum

For the production of anti-ficolin-B polyclonal antibodies two rabbits were immunized as described before (see section II.2.6.2). Blood was collected before the immunization started ("pre-immune serum" to be used as a negative control in every ELISA test), 1 week after the first boost, and regularly every 2 weeks after the last boost to collect the anti-serum. Assessment of the antibody titer after the first boost revealed that only one rabbit developed a strong immune response (data not shown). Therefore, only this animal was further boosted.

After each bleeding 1:2 serial dilutions of the obtained sera (see section II.2.6.2) were subjected to antibody-capture ELISA in order to assess the antibody titer (see section II.2.5.3). The titer of each sample was defined as the reciprocal of a serum dilution whose absorbance at 405 nm (A_{405}) was 50% of the maximum level. Figure III.9 shows the development of the antibody titer over 32 weeks. At week 23 rabbits were boosted for the fourth time with 20 μg of antigen in PBS due to the decline in the titer.

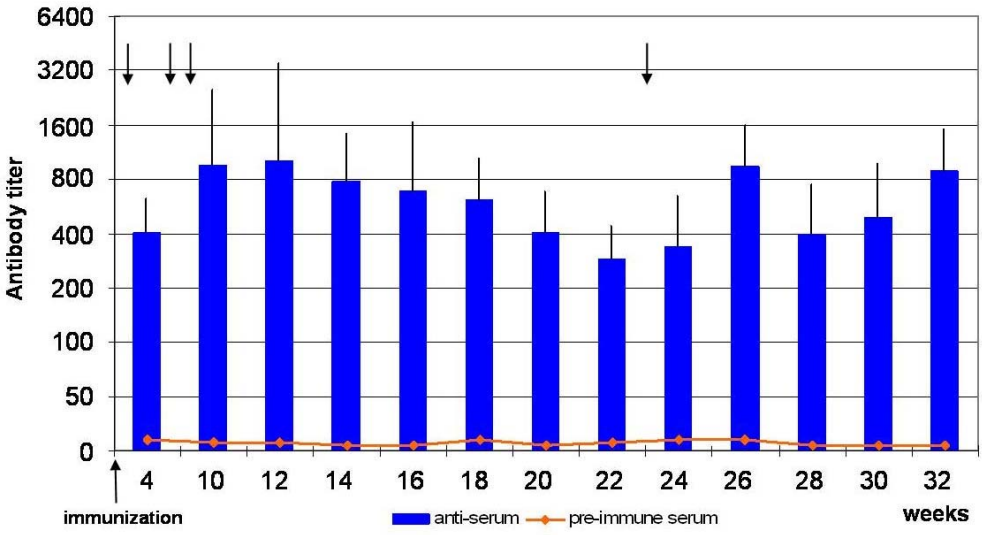

Fig. III.9: Anti-ficolin-B titer of rabbit anti-serum. Serum samples of an immunized rabbit were analyzed over 32 weeks by ELISA to assess the antibody titer. The course of the antibody production was monitored and enhanced by a 4th boost at week 23. Values were always compared to those obtained from the pre-immune serum. Arrows indicate the boosts at weeks 3, 6, 9, and 23.

III.2.1.3 Purification of monospecific anti-ficolin-B polyclonal antibodies

Collected sera were cleared by centrifugation and filtration before proceeding to the purification step. Anti-ficolin-B polyclonal antibodies were made monospecific by affinity chromatography on ficolin-B-coupled CNBr-activated sepharose (see section II.2.4.1.2). Elution fractions were analyzed by SDS-PAGE where both the heavy and the light chain of the immunoglobulins could be identified at 50 kDa and 25 kDa, respectively (Fig.III.10). Positive fractions were pooled and dialyzed against PBS overnight, resulting in a yield of 90 μg of monospecific antibody per milliliter of serum.

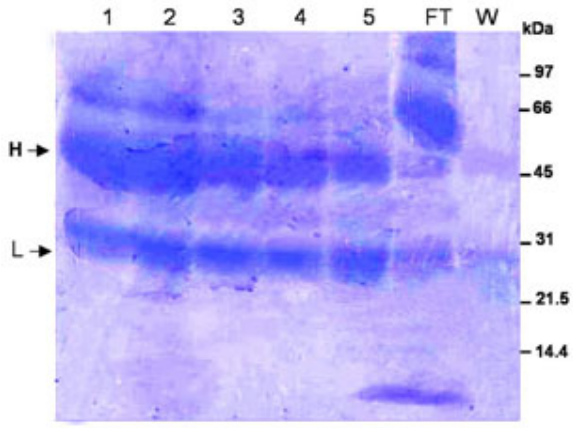

Fig. III.10: Purification of specific anti-ficolin-B antibodies by affinity chromatography. Elution (lanes 1-5), flow-through (FT) and washing (W) fractions were resolved on a 12,5% PAA-gel under reducing conditions and stained with Coomassie blue. **H:** heavy chain; **L:** light chain.

III.2.1.4 Characterization of the anti-ficolin-B monospecific polyclonal antibodies

In order to characterize the monospecific anti-ficolin-B polyclonal antibodies, they were tested in different assays such as ELISA and Western blot.

For the antibody-capture ELISA test, microtiter plates were coated with ficolin-B in serial two-fold dilutions starting at 1 μg/ml. Polyclonal anti-ficolin-B antibodies were then added in different dilutions and detected with an AP-conjugated goat anti-rabbit IgG antibody (see section II.2.5.3). While high concentrations of antibody (i.e. 1:100 and 1:500) gave high background signals (even in the absence of ficolin), a dilution of 1:2000 was found to be optimal regarding the signal/background ratio (Fig. III.11). Therefore, 1:2000 was chosen as the working dilution for the following experiments.

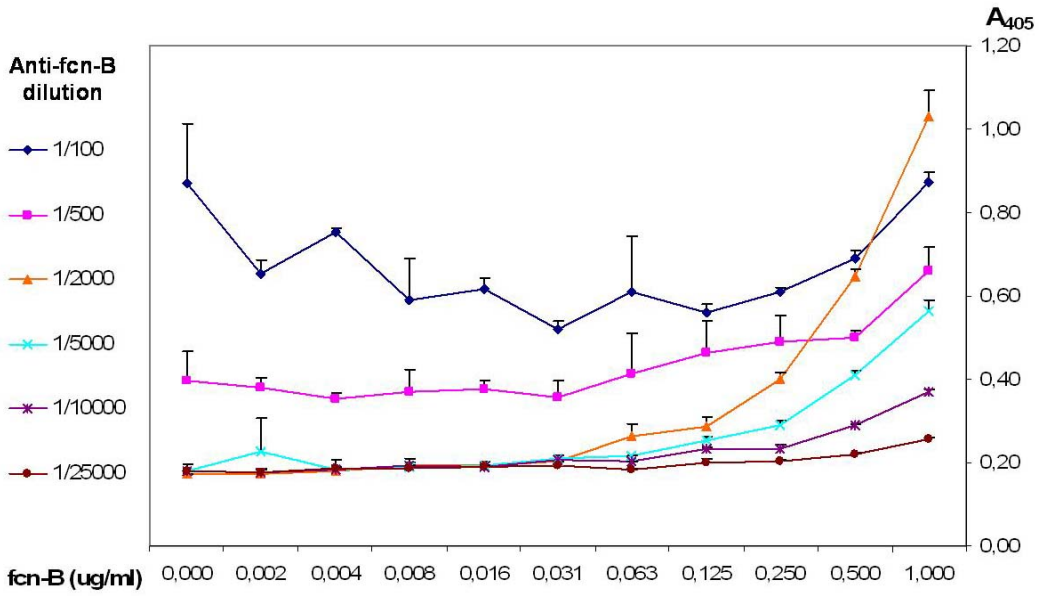

Fig. III.11: Antibody-capture ELISA of purified anti-ficolin-B polyclonal antibodies. Different dilutions of anti-ficolin-B antibodies were tested on microtiter plates coated with 1:2 serial dilutions of rfcn-B starting at 1 μg/ml. An irrelevant rabbit IgG (rIgG, 1 μg/ml) was used as an isotype control.

The ability of these monospecific antibodies to recognize ficolin-B under reducing conditions was checked by Western blot. Moreover, in the same assay their cross-reactivity with ficolin-A was also determined. Accordingly, ficolin-A and -B were resolved on a 12,5% PAA-gel, blotted and detected with the anti-ficolin-B monospecific antibodies. As Fig. III.12 shows, the antibodies are able to recognize reduced ficolin-B on a membrane, with little cross-reactivity with the reduced ficolin-A.

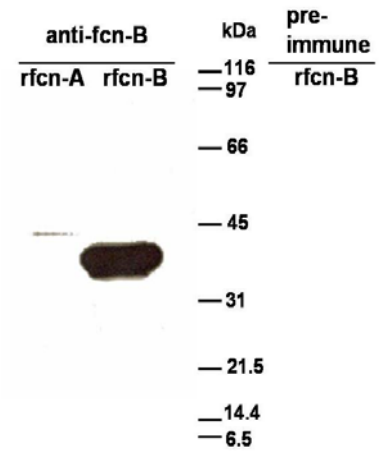

Fig. III.12: Anti-ficolin-B polyclonal antibodies recognize ficolin-B but not ficolin-A under reduced conditions. Ficolin-A and –B (1 μg) were resolved on a 12,5% PAA-gel and blotted. Membranes were incubated with either the purified antibodies (1:2000) or with pre-immune rabbit serum as a control, and an HRP-conjugated anti-rabbit IgG as a secondary antibody.

III.2.2 Generation of monoclonal antibodies

Although polyclonal antibodies can be very useful for a variety of immunological assays, serum contains many different types of antibodies that are specific for different antigens and for different epitopes on the same antigen. Even in hyperimmune animals, normally not more than one-tenth of the circulating antibodies are specific for one antigen (Harlow and Lane, 1988). Therefore, the use of these mixed populations of antibodies might create a variety of problems in some immunochemical techniques due to polyspecific binding. In this work, anti-ficolin-B polyclonal antibodies were made monospecific by affinity chromatography on ficolin-B-coupled CNBr sepharose (see section II.2.4.1.2.1). However, the preparation of homogenous antibodies with a defined specificity for one epitope on ficolin-B was also aimed and, thus monoclonal antibodies were generated.

III.2.2.1 Immunization schedule and fusion

For the generation of anti-ficolin-B monoclonal antibodies two young Wistar female rats were immunized with rfcn-B and boosted three times in three-week intervals (see section II.2.6.1). One week after the first boost, blood was taken to determine the antibody titer by ELISA (see section II.2.5.3). Only the animal with the highest titer of ficolin-B-specific antibodies (rat “A”) was boosted for a second and a third time before being sacrificed. Figure III.16 shows the antibody titer of both rats after the first boost compared to normal rat serum.

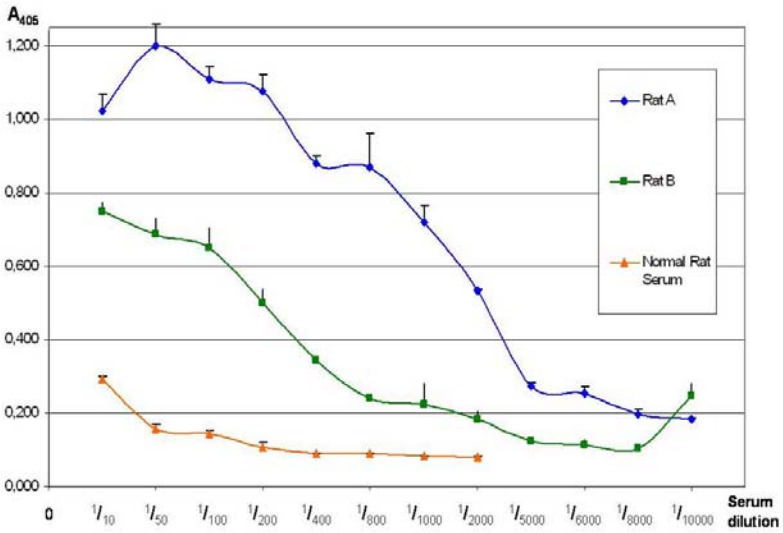

Fig. III.16: Anti-ficolin-B antibody response of Wistar rats ("A" and "B") after the first boost injection. Serum taken from rats immunized and boosted once with purified rfcn-B was tested in ELISA for antibody production.

Three days after the last boost the rat was sacrificed and the spleen was removed. Splenocytes were isolated (see section II.2.3.3.1) and fused to SP2/O-Ag14 cells for the generation of antibody-secreting hybridomas (see section II.2.3.3.3). Hybridomas were screened three times by antibody-capture ELISA (see section II.2.5.3) before the expansion was started. In order to screen only for the IgG-producing hybridomas the assay was performed with a γ–chain-specific secondary antibody. In this way, 20 positive hybridomas were identified in the first round, 11 in the second, and only 7 were still positive in the third round. After subcloning, 4 out of 7 hybridomas were monoclonal and selected for scaled-up production, namely 6B5D6, 7G6E7, 15H2D3 and 15H2H6.

III.2.2.2 Purification of anti-ficolin-B monoclonal antibodies by chromatography

By the time this thesis is being written, only the anti-ficolin-B monoclonal antibody 15H2D3 has been purified.

Supernatant from the hybridoma cell line secreting 15H2D3 was concentrated by ammonium sulphate precipitation and applied to a protein-G sepharose column for purification by affinity chromatography (see section II.2.4.1.3). Fractions of every step were analyzed by SDS-PAGE and Coomassie blue staining of the gel (Fig. III.17).

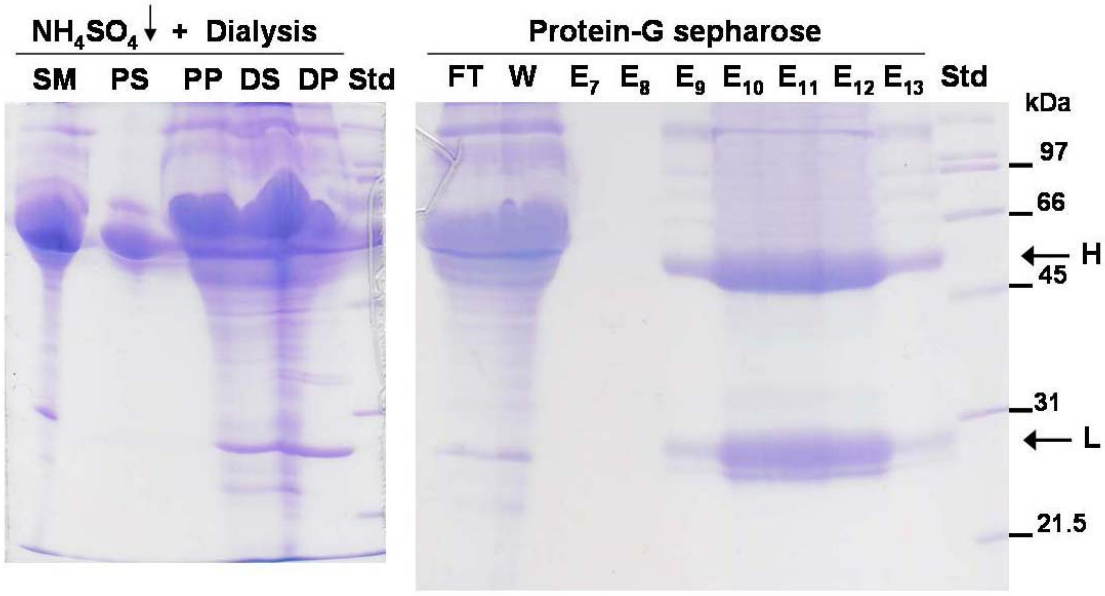

Fig. III.17: Purification of the anti-ficolin-B monoclonal antibody 15H2D3 by affinity chromatography. The hybridoma supernatant was subjected to ammonium sulphate precipitation and extensive dialysis before purification on a protein-G sepharose column took place. Fractions were collected at every step and analyzed by 12,5% SDS-PAGE and Coomassie blue staining. **SM:** starting material; **PS:** precipitation supernatant; **PP:** precipitated proteins; **DS:** supernatant after dialysis; **DP:** precipitate after dialysis; **FT:** flow through; **W:** washing; **$E_{\#}$:** elution fractions; **Std:** molecular weight standard. **H:** heavy chain; **L:** light chain.

Due to the presence of fetal calf serum in the culture medium of the hybridomas, albumin was the main contaminant, even after ammonium sulphate precipitation. When concentrated proteins were sequentially dialyzed against PBS and binding buffer (see section II.2.4.1.3.1) the generation of a white precipitate was observed. Although most of the antibody of interest was still soluble, some immunoglobulins could also be detected in the precipitate. Albumin was efficiently removed by affinity chromatography on protein-G sepharose. Finally, the pure monoclonal antibody was eluted and it was mainly found in elution fractions 9 to 13. Fractions of similar concentration were pooled, and after dialysis the purification yield was determined to be 7.2 mg/l hybridoma supernatant.

III.2.2.3 Characterization of the anti-ficolin-B monoclonal antibody 15H2D3

As with the polyclonal antibodies, it was of interest to evaluate the usability of the anti-fcn-B monoclonal antibody 15H2D3 and establish the optimal conditions in distinct assays.

To optimize ELISA conditions, microtiter plates were coated with ficolin-B in several concentrations and incubated with different dilutions of 15H2D3. As shown in figure III.18, a dilution of 1/1000 was found to be optimal for detection of ficolin-B down to 0.25 μg/ml.

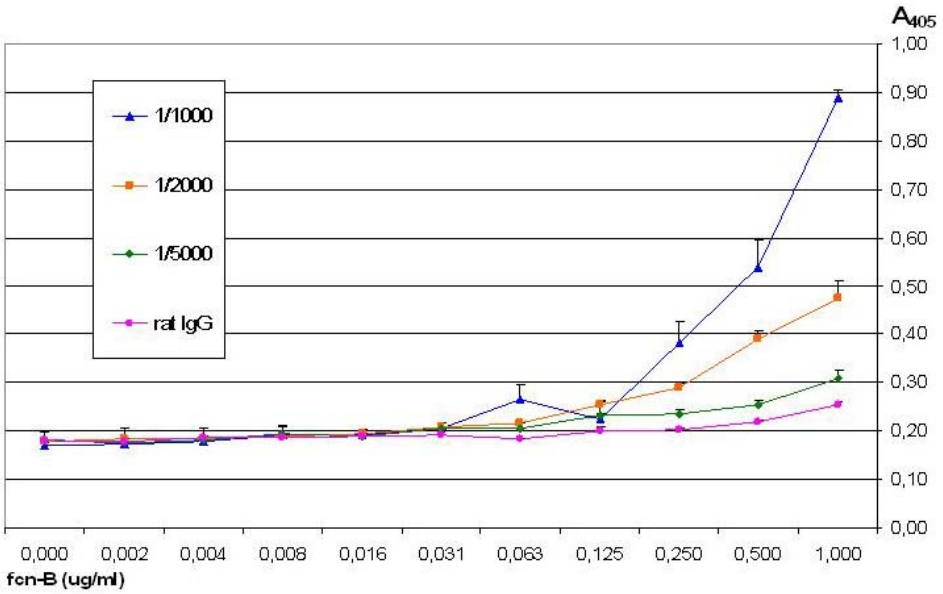

Fig. III.18: Antibody-capture ELISA with purified anti-ficolin-B monoclonal antibody 15H2D3. Different dilutions of 15H2D3 were tested on a microtiter plate coated with 1:2 serial dilutions of rfcn-B starting at 1 μg/ml. An irrelevant rat IgG (1 μg/ml) was used as an isotype control.

For Western blot analysis ficolin-B (1 μg) was run under reducing conditions, and detected with different dilutions of 15H2D3 ranging from 1:500 to 1:5000. However, no signals were obtained in any case (data not shown), suggesting that 15H2D3 is not able to detect the denatured form of ficolin-B.

In order to confirm that the antibody is able to detect the native form of the recombinant protein, a dot blot assay was performed (see section II.2.5.2). Recombinant ficolin-B was spotted in different amounts ranging from 3.5 to 500 ng and detected with 15H2D3 in a 1:1000 dilution. The antibody was able to recognize the rfcn-B when dotted in a 30 ng/μl concentration as shown in figure III.19. The same assay was performed with the monospecific anti-ficolin-B polyclonal antibodies with similar results (data not shown).

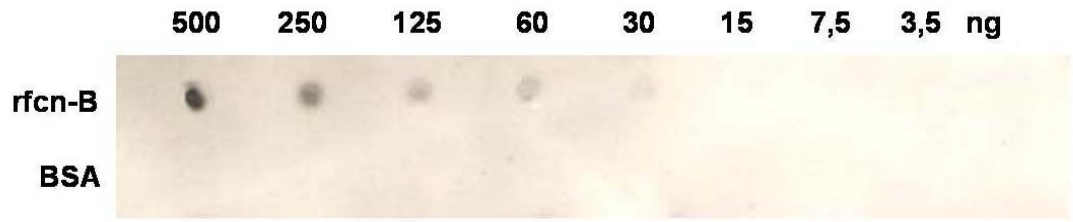

Fig. III.19: Dot blot for the detection of non-reduced ficolin-B with the monoclonal antibody 15H2D3. Recombinant ficolin-B was spotted (1 µl) on a nitrocellulose membrane at the specified concentrations and incubated with the anti-ficolin-B antibody 15H2D3 in a 1:1000 dilution. An HRP-conjugated goat anti-rat IgG antibody was used for detection. BSA was also spotted at different concentrations as a negative control.

III.3 Binding studies on recombinant ficolin-B using the monospecific anti-ficolin-B polyclonal antibodies

Since it was shown that human ficolins as well as mouse ficolin-A share binding specificity for N-acetylglucosamine (GlcNAc) (Le *et al.,* 1998), (Fujimori *et al.,* 1998), (Teh *et al.,* 2000), it was of particular interest to assess the ficolin-B specificity for this molecule. The assay was performed as Fujimori and co-workers reported in 1998 for ficolin-A (see section II.2.4.7). As shown in figure III.13, bound rfcn-B was recovered in the SDS sample buffer, indicating the binding specificity of ficolin-B to GlcNAc.

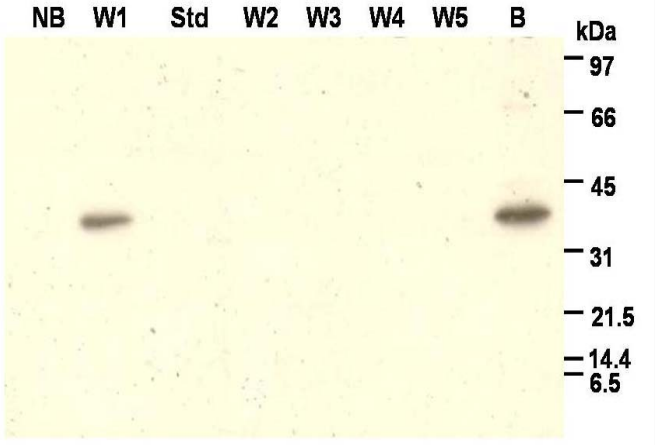

Fig. III.13: Binding of ficolin-B to GlcNAc. Recombinant fcn-B was mixed with GlcNAc-agarose and rotated for 2.5 h at RT. After extensive washings, bound proteins were eluted with SDS sample buffer and subjected to Western blot developed with anti-fcn-B polyclonal antibodies. **NB:** not bound proteins; **W#:** washings; **B:** bound proteins; **Std:** molecular weight standard.

As a first attempt to investigate the binding specificity of ficolin-B further, the recombinant protein was used in dot blot studies in which different organic compounds were spotted on nitrocellulose membranes and exposed to rfcn-B. Detection was performed by making use of

the monospecific anti-fcn-B polyclonal antibodies and an HRP-conjugated anti-rabbit-IgG secondary antibody (see section II.2.5.2).

Samples of soluble peptidoglycan (sPG) extracts from *Staphylococcus aureus* (containing a terminal GlcNAc-MurNAc residue, prepared according to Rosenthal and Dziarski, 1994), LTA from *S. aureus*, and LPS from *Yersinia enterocolitica* (containing a rough-type LPS with a terminal GlcNAc) were obtained from Prof. U. Zähringer, Borstel, Germany. The peptidoglycan samples sPG7 and sPG9 consisted of different batches of water soluble substances collected from supernatant of penicillin G treated *Staphylococcus aureus* (lyophilized supernatants). Interestingly, in a preliminary assay, sPG9 but not sPG7 was positive for ficolin-B binding (data not shown). Therefore, a new batch (sPG5) was tested after GPC fractioning in order to obtain more information about the binding material. As shown in figure III.14, only the first three GPC fractions were positive for ficolin-B binding. Again sPG9 but not sPG7 gave a positive signal while neither LTA nor LPS seemed to be recognized by ficolin-B.

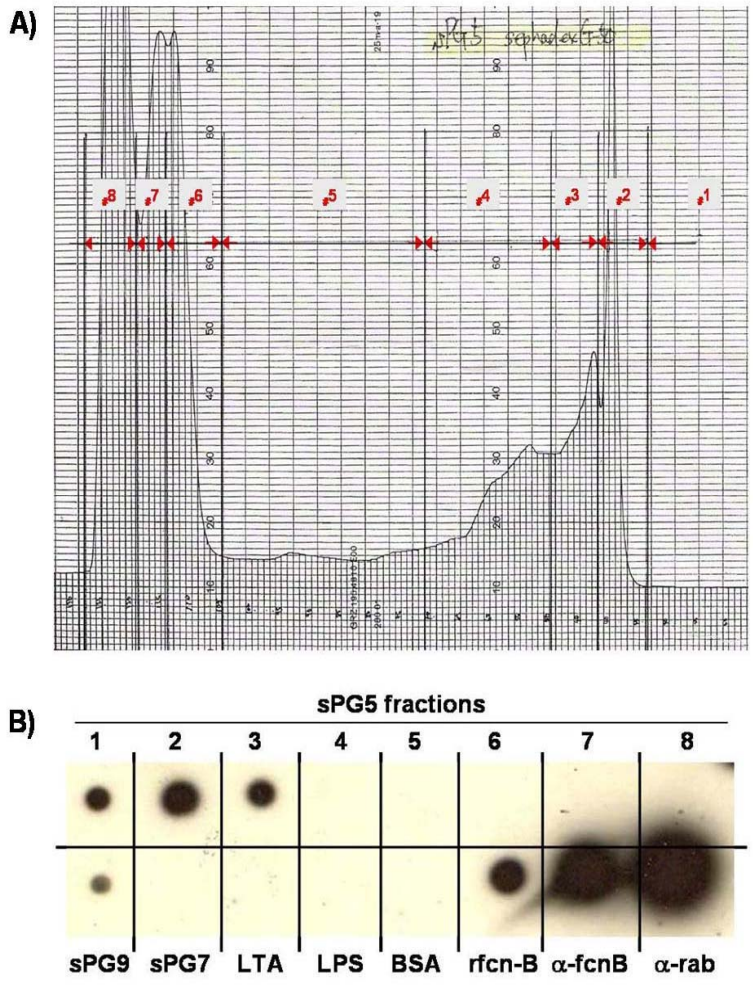

Fig. III.14: Binding specificity of recombinant ficolin-B. Soluble peptidoglycan GPC fractions (sPG5 from #1 to #8) **(A)** as well as the crude extracts sPG7 and sPG9 from *S. aureus*, LTA from *S. aureus* and LPS from *Y. enterocolitica* were spotted on nitrocellulose membranes and incubated with rfcn-B (1 μg/ml). Upon incubation with anti-fcn-B polyclonal antibodies, detection was carried out with an HRP-conjugated anti-rabbit-IgG antibody **(B)**. BSA was spotted as a negative control while rfcn-B and both the primary and the secondary antibodies were spotted as positive controls.

In order to elucidate the nature of the interaction between the samples and fcn-B, i.e. a protein-protein or protein-sugar, sPG5 fractions (#1 to #4) as well as a new non-penicillin-treated *S. aureus* extract (SaLP4) were subjected to mild periodate treatment which promotes oxidation of carbohydrate structures. Figure III.15 shows that no binding of ficolin-B could be detected after the samples were treated with periodate.

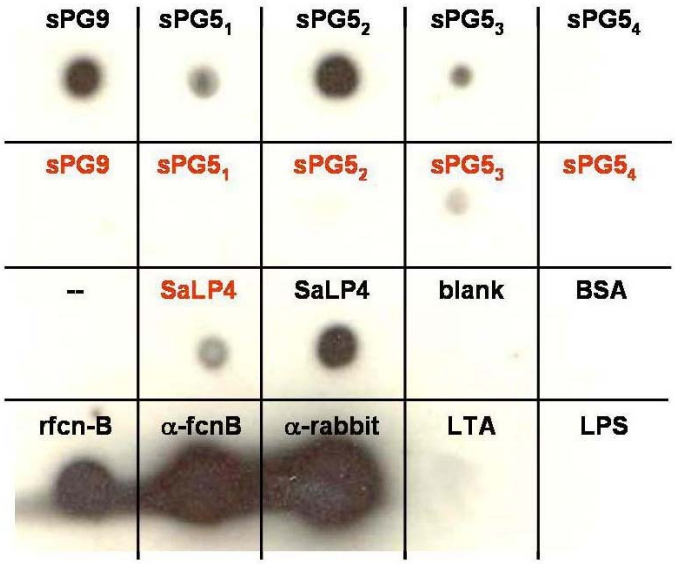

Fig. III.15: Ficolin-B binding affinity before and after periodate treatment. sPG samples before (black) and after (red) periodate treatment were subjected to dot blot for ficolin-B binding as stated before. BSA and periodate treated water ('blank') were spotted as negative controls. Recombinant fcn-B and both the primary and the secondary antibodies were spotted as positive controls.

III.4 Studies on the expression and localization of native ficolin-B using the monospecific anti-ficolin-B polyclonal antibodies

As already mentioned in the introduction, the different reports on ficolin-B expression performed up to date were either on DNA or RNA level (Ohashi and Erickson, 1998) (Endo *et al.,* 2004) (Liu *et al.,* 2005a). This means that, in spite of all these approaches, ficolin-B protein has never been localized, probably due to the lack of specific reagents. Therefore, after succeeding in the generation of monospecific polyclonal antibodies, we aimed to elucidate the ficolin-B expression pattern at the protein level.

III.4.1 Tissue expression pattern of ficolin-B

In order to determine the tissue distribution of ficolin-B, mRNA was extracted from different mouse tissues (see section II.2.2.3) and subjected to RT-PCR (see section II.2.2.1.3) with ficolin-B specific primers ("ficolinB-EcoRI-ATG-forw" and "ficolinB-XhoI-Stop-rev", section II.1.3.1). In accordance with previous reports, ficolin-B was detected in spleen and bone marrow but not in lung or liver (Fig. III.20a). Therefore, in a first attempt to detect ficolin-B protein, an immunoprecipitation from different mouse tissues was performed by making use of the purified monospecific anti-ficolin-B polyclonal antibodies (see section II.2.5.6). Ficolin-B was immunoprecipitated from supernatants of cell lysates of disrupted tissue from lung, liver, spleen, kidney, and bone marrow and detected by Western blot. As expected, spleen and bone marrow, but not lung, kidney or liver, were positive for ficolin-B, displaying a band of approximately the same size (35 kDa) as the recombinant ficolin-B (Fig. III.20b).

III.4.2 Cellular and subcellular localization of ficolin-B protein

The next goal was to define the cell types in which ficolin-B was expressed. Since the human M-ficolin and the porcine ficolin-ß, orthologues of ficolin-B, are expressed in monocytes and neutrophils, respectively, mouse peritoneal exudate macrophages (PECs) were tested for ficolin-B expression. PECs were elicited by intraperitoneal injection of PBS (see section II.2.3.3.2) or simultaneously activated by LPS in PBS 16 hours prior to peritoneal lavage. In addition, resident peritoneal cells (PCs) were harvested. From the adherent cell populations (mainly macrophages) an enriched messenger RNA fraction was isolated (see section II.2.2.3) and the presence of ficolin-B mRNA was confirmed by RT-PCR (see section II.2.2.1.3) (Fig. III.21). Although all three macrophage populations were positive for ficolin-B mRNA, there was an apparent higher expression of ficolin-B in the PECs cultures, especially after LPS activation.

In order to localize endogenously expressed ficolin-B protein, PECs treated as described above were subjected to immunostaining. For this purpose cells were either permeabilized for intracellular staining or left untreated for membrane staining (see section II.2.5.5). Surprisingly, ficolin-B was only detected when the cells were made permeable, indicating that ficolin-B is localized inside the cell (Fig. III.22a).

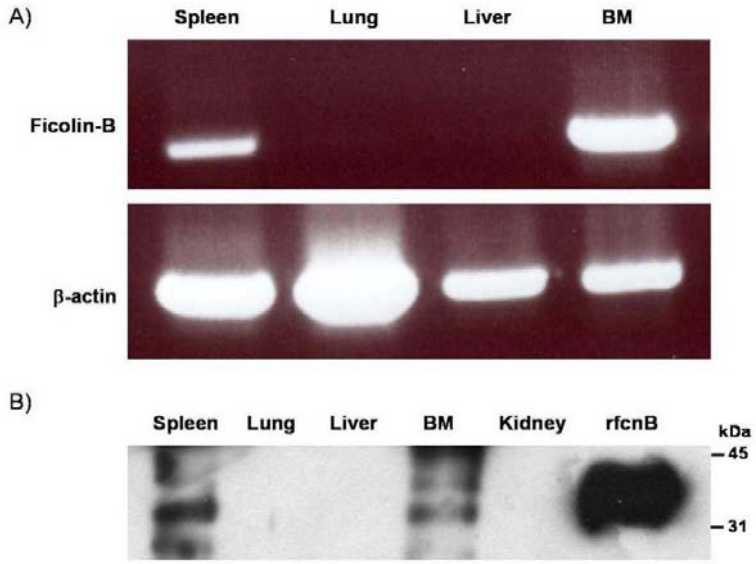

Fig. III.20: Ficolin B is expressed in bone marrow and spleen at RNA and protein level. A) RNA from spleen, lung, liver, and bone marrow (BM) was subjected to RT-PCR with ficolin-B specific primers. The products were resolved on a 1% agarose gel containing ethidium bromide. β-actin primers were used as controls. **B)** Immunoprecipitation with anti-ficolin-B antibodies was performed from cell lysates from spleen, lung, liver, BM, and kidney and subjected to Western blot analysis with the purified monospecific anti-fcn-B antibodies. Recombinant ficolin-B (rfcn-B) was included as a positive control.

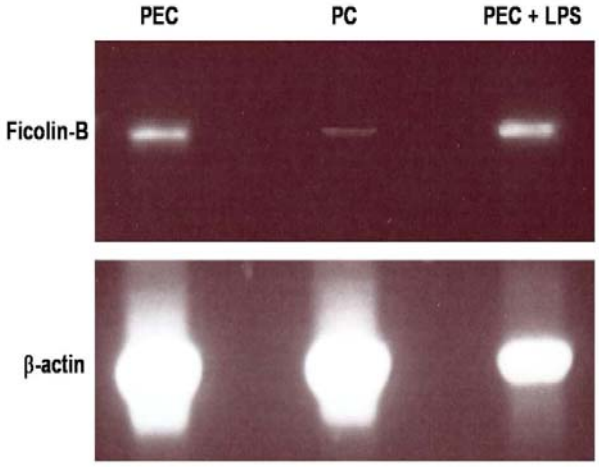

Fig. III.21: Peritoneal macrophages express ficolin-B mRNA. RNA from adherent peritoneal cells of either untreated mice (PC) or mice elicited with PBS (PEC) or PBS containing LPS (PEC + LPS) 16 hours earlier was isolated and subjected to RT-PCR with ficolin-B specific primers. Amplification products were resolved on a 1% agarose gel containing ethidium bromide. β-actin primers were used as controls.

When staining of PCs and PECs with or without previous LPS stimulation was compared, positive staining for ficolin-B seemed to be increased upon stimulation (Fig. III.22b). In contrast, polymorphonuclear neutrophils (PMNs) from a peritoneal cell population harvested

three hours after PBS injection were found to be negative for ficolin-B staining (data not shown).

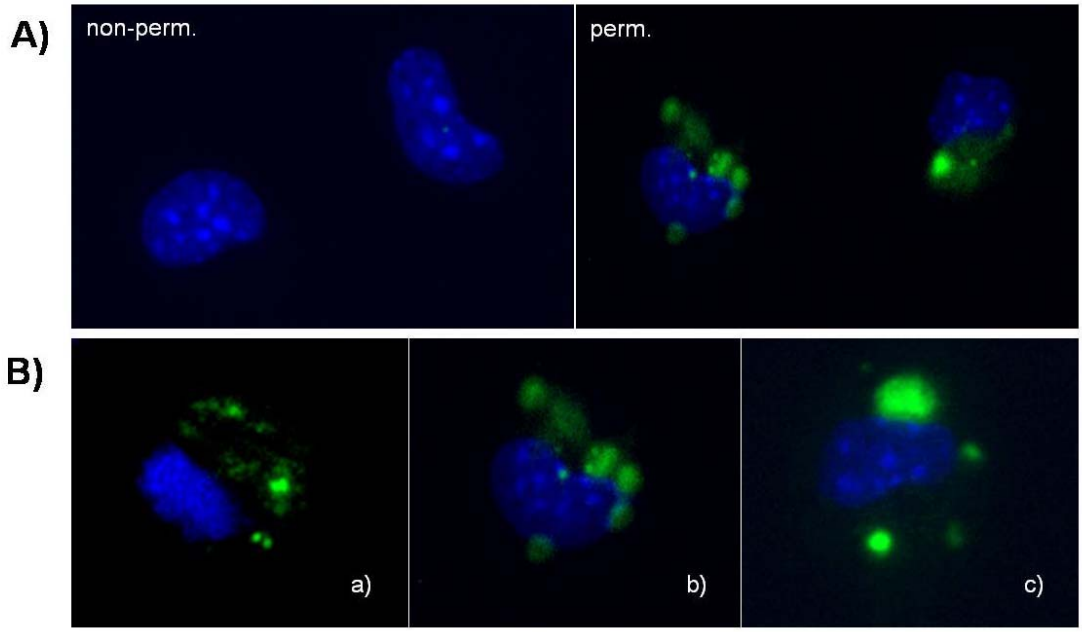

Fig. III.22: Peritoneal macrophage-expressed ficolin-B is localized inside the cells and is upregulated upon stimulation. A) PECs elicited with PBS were either permeabilized or not, and subjected to ficolin-B immunostaining with monospecific polyclonal antibodies. **B)** PCs (a) and PECs elicited with PBS (b) or with PBS + LPS (c) were made permeabilized and immunostained for ficolin-B. Nuclei were stained with DAPI (blue) and ficolin-B detected with monospecific polyclonal antibodies followed by an anti-rabbit-FITC antibody (green). Magnification: 40x.

Since ficolin-B was found to localize inside the cell, the next aim was to define the subcellular compartment harboring the protein. Due to the shape of the stained spots and the possible role of ficolin-B in macrophages, the endosomal/lysosomal compartment was analyzed for ficolin-B expression. For this, PECs elicited by injection of PBS were double-stained with FITC-labeled anti-Lamp-1 and rabbit anti-ficolin-B, detected with TRITC-labeled anti-rabbit antibodies (see section II.2.5.5). As shown in figure III.23, confocal microscopy revealed a clear co-localization of ficolin-B with the lysosome associated membrane protein 1 (Lamp-1) in the analyzed macrophages. Microscopical pictures are representative of three independent experiments. Negative controls (i.e. without the primary or secondary antibodies) confirmed the specificity of the results (data not shown).

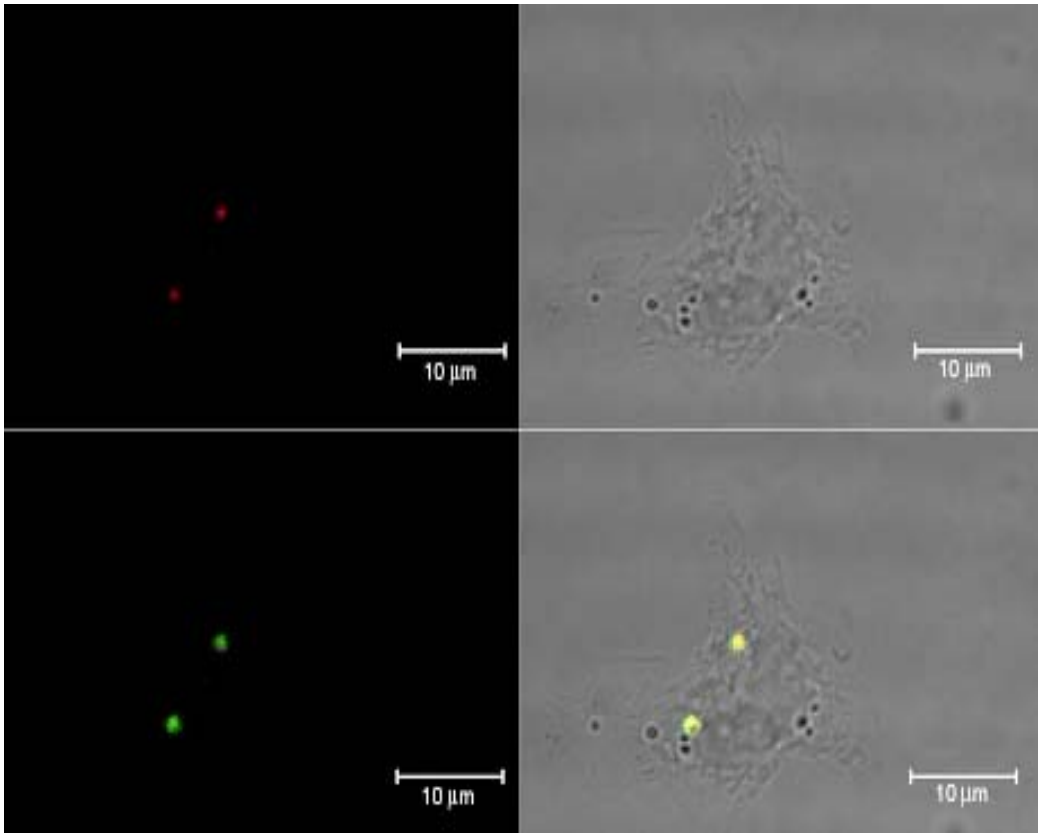

Fig. III.23: Ficolin-B co-localizes with lysosomes in the PEC. PBS-elicted PECs were double-stained with monospecific anti-ficolin-B/anti-rabbit-TRITC antibodies (red, upper left panel) and anti-Lamp-1-FITC (green, lower left panel). Overlay of the single images revealed a positive co-localization of ficolin-B and Lamp-1 (yellow, lower right panel). Upper right panel: macrophage morphology. Magnification: 63x.

III.5 Generation of ficolin deficient mouse lines

Embryonic stem (ES) cells retain the pluripotency of early embryo-derived cells and, as such, have the potential to direct the development of a mouse. Coupled with the ability to identify homologous recombination events within these cells, the ability to manipulate ES cells has revolutionized the way biological questions are approached. The generation of genetically altered mice with specific deficiencies has made it possible to study the function of genes *in vivo* and, therefore, offers a powerful tool for genetic research.

Another aim of this work was the design of targeting strategies to abrogate the genes encoding for ficolin-A and –B and, thus the production of novel tools for the functional study of these mouse genes. Figure III.24 outlines the entire procedure towards the generation of knock-out mice.

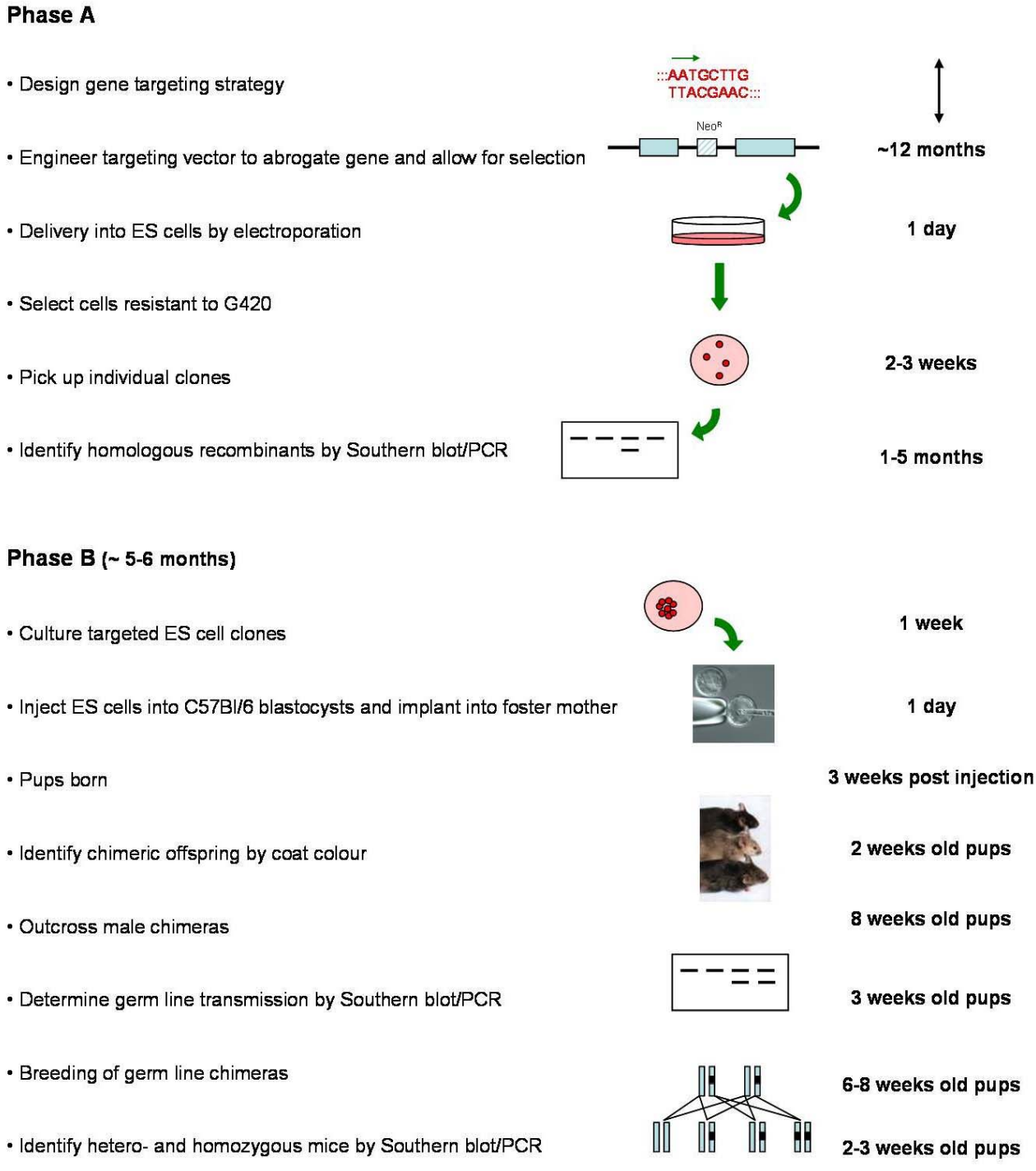

Fig. III.24: Stages of the generation of a gene deficient mouse line.

III.5.1 Gene targeting strategy

Both ficolin-A and –B genes are localized on chromosome 2 of the mouse genome at position 2A3 (Contig, NT-039206), and consist of ten and nine exons, respectively. In both cases the first exon encodes a signal peptide, the following three the collagen-like domain, the fifth and sixth (in the fcn-A gene) or the fifth (in the fcn-B gene) the neck sequence, and the last four exons the fibrinogen-like domain. The fcn-B gene is located between the collagen type Vα1 (Col5a1) gene and the olfactomedin-related ER localized protein (Olfm1) gene. The fcn-A gene is located at a distance of 2.3 Mb from the fcn-B gene, between the lipocalin 8 (Lpcn8, 9230106L18Rik) and the 4921530D09Rik genes (Endo *et al.,* 2004).

The targeting strategy developed in this work was the same for both ficolin genes. It consisted of the generation of a replacement vector containing a short and a long arm of homology to the target locus, a positive selection marker (e.g. the neomycin resistance (Neo^R) cassette), and a linearization site outside the homologous sequences of the vector. In this way, the final recombinant allele can be effectively described as a consequence of double homologous recombination which takes place between the vector and the endogenous locus after the delivery of the plasmid into ES cells (Fig. III.25), leading to the genome insertion of all the vector components that are flanked by the homologous sequences (e.g. Neo^R cassette).

A targeting strategy must also include the design of the screening method that will be used to identify homologous recombinants. Normally Southern blot or PCR are the techniques of choice, and for this project both methods were applied.

For Southern blot analysis it is important to generate a probe that will anneal outside the homology sequence in order to identify only those mutants that arose from homologous recombination. It is also necessary to scan the genome sequence for restriction sites which result in a different band pattern after targeting. In this way, *BamH*I and *EcoR*I were found to be the optimal enzymes for fcn-A and –B analysis, respectively. Both sites are introduced once in the targeted locus by the insertion of the Neo^R cassette, at a distance from the endogenous sites that allows for discrimination in size by Southern blot (Fig. III.25).

For PCR analysis of the targeted ES clones it is also important to design a primer pair in a way that allows for amplification of the expected product only if homologous recombination has occurred. This is achieved by making one primer complementary to the genomic region outside the homology sequence, and the other primer complementary to the Neo^R cassette (Fig. III.25). In this way only those clones bearing the Neo^R cassette as a consequence of homologous recombination (and not due to random insertions) will give a PCR product.

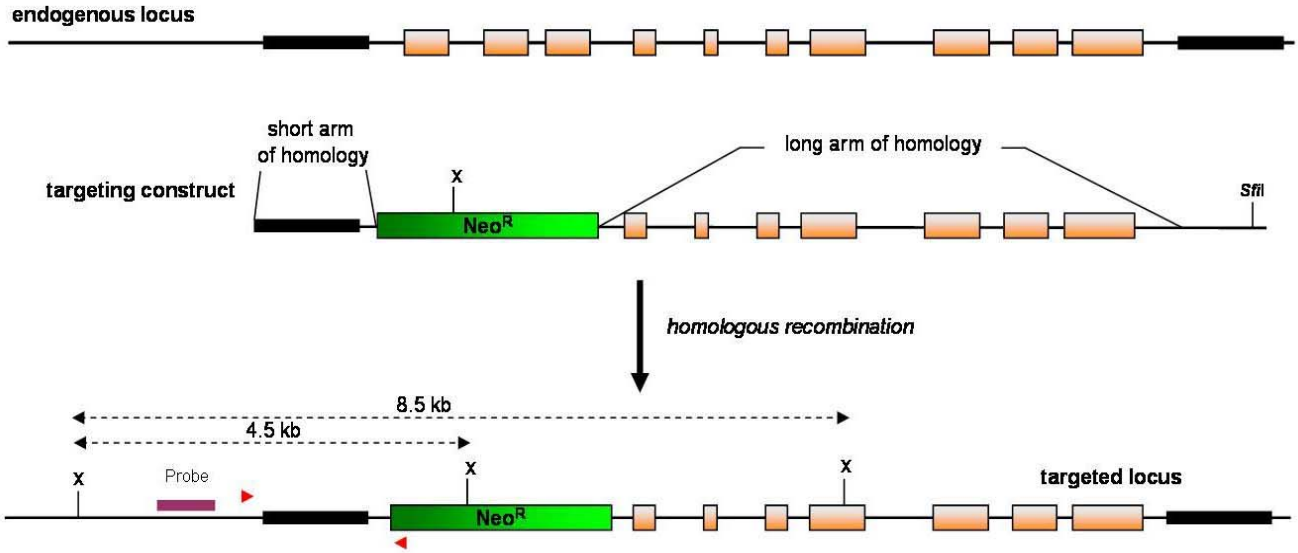

Fig. III.25: Scheme of the targeting strategy to abrogate the ficolin genes. A targeting vector was designed to contain a positive resistance cassette (Neo^R, green) flanked by a short and a long arm of homology to the endogenous ficolin locus, and a linearization site outside the homology sequence (*Sfi*I). In the resulting mutant locus after homologous recombination the first three exons would be replaced by the Neo^R cassette. Southern blot analysis would be performed after digesting the genomic DNA with the enzyme "X" (*BamH*I for fcn-A and *EcoR*I for fcn-B) and detection with a probe binding 5' outside the homology sequence (purple). The dashed arrows indicate the different fragments expected on the blot. For PCR analysis the primers (red arrow heads) were designed to anneal 5' outside the homology region and at the 3' end of the Neo^R cassette. Exons are shown as orange boxes.

III.5.2 Generation of targeting vectors for ficolin-A and –B

For both fcn-A and –B targeting vectors, the short (1.2 kb) and the long (4 kb) arms of homology were amplified from mouse genomic ES cells DNA by PCR (see section II.2.2.1.1) using the primers numbered from 9 to 14 (fcn-A) and from 19 to 24 (fcn-B) (see section II.1.3.1). Due to its length, the long arm consisted of two PCR products cloned together (termed "2nd fragment" and "3rd fragment"). In this way the short arm ("1st fragment") and the 2nd and 3rd fragments were first cloned separately into the pCR2.1-TOPO vector (see section II.2.1.6.1). In order to build the long arm, the 2nd and the 3rd fragments were subcloned together into the pBluescript II KS+ vector (pBS) by *Hind*III/*Xho*I and *Hind*III/*Spe*I digestion (see section II.2.1.7) of the "TOPO-2nd fragment" and "TOPO-3rd fragment", respectively (Fig. III.26). Once the long arm was generated, it was subcloned into the pSL301 plasmid (which was chosen as the backbone of the targeting vector due to its large multiple cloning site) by *Xho*I/*Spe*I-*Nhe*I digestion, taking advantage of the fact that *Spe*I and *Nhe*I generate compatible ends.

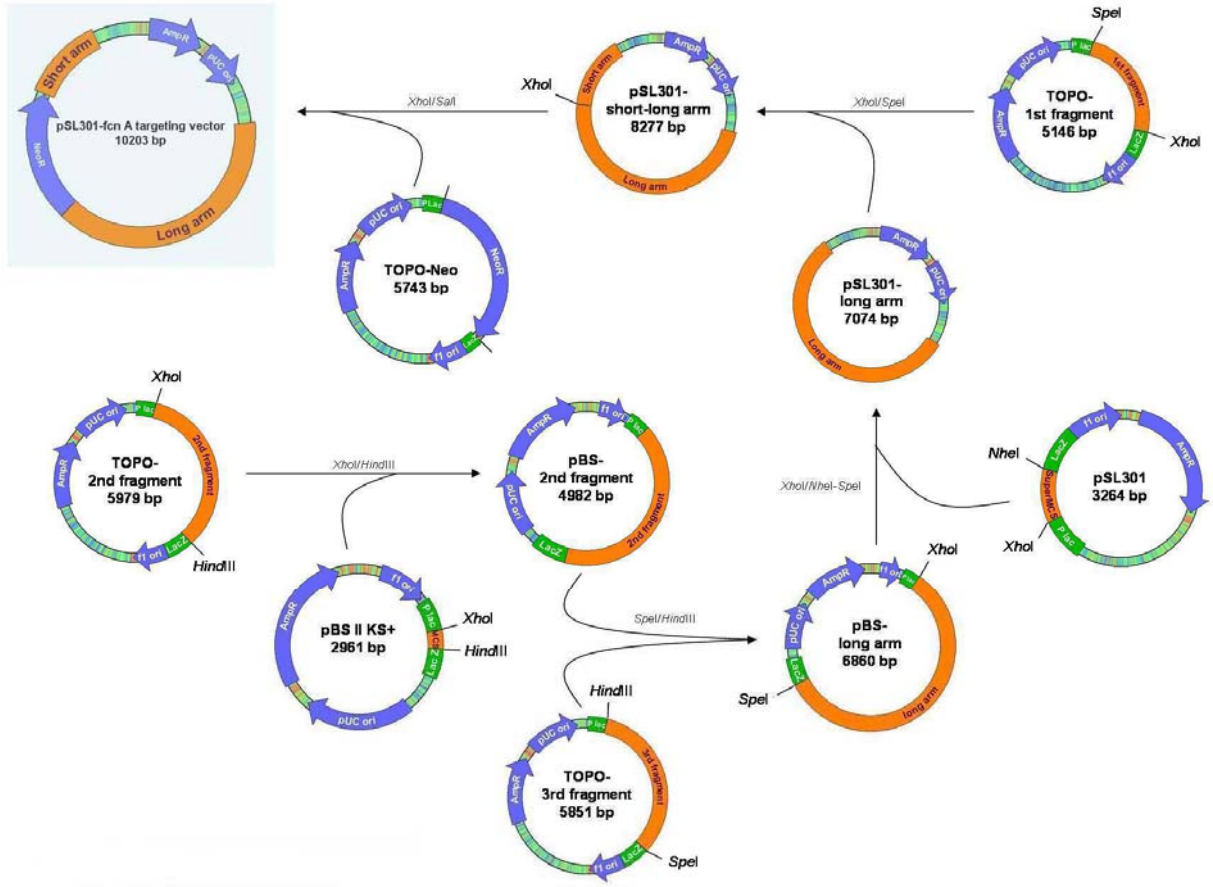

Fig. III.26: Cloning strategy of the fcn-A targeting vector. The short and the long arms of homology were amplified by PCR from mouse genomic DNA and cloned into the pCR2.1 TOPO vector. Subcloning into the pSL301 vector was performed as indicated in the scheme and in the text. **AmpR**: ampicillin resistance gene; **LacZ**: α-fragment of the ß-galactosidase gene; **P Lac**: LacZ promoter.

The next step was the insertion of the short arm into the vector backbone already containing the long arm. For that purpose, the short arm was excised from the "TOPO-1st fragment" construct by *Spe*I/*Xho*I digestion and ligated with "pBS-long arm", previously digested with the same enzymes. As the last step, the Neo^{R} cassette was excised by *Xho*I/*Sal*I digestion from "TOPO- Neo", and inserted between the short and the long arm of homology by *Xho*I digestion and further dephosphorylation of the "pSL301-short-long arm" construct (see section II.2.1.8). Once the targeting vector was ready, all its fragments were finally analyzed by sequencing and restriction analysis (see sections II.2.1.7 and II.2.1.10). Figure III.26 outlines the entire cloning process.

The enzymes described in the text and in the scheme were particularly used in the generation of the fcn-A targeting vector. Although the general procedure for the cloning of the fcn-B targeting vector was basically the same, the enzymes *Hind*III and *Nhe*I were replaced

by *Xba*I and *BamH*I, respectively, due to the difference in the sequences of the fcn-A and –B genes. Whereas the fcn-B targeting vector was sent to Prof. W. Schwaeble's laboratory (Leicester, UK), the mice deficient in fcn-A were generated as part of this work.

III.5.3 Production of targeted embryonic stem cells

As outlined in figure III.24 the next step in the generation of a ficolin-A deficient mouse line after completion of the targeting vector was its delivery into embryonic stem (ES) cells by electroporation (see section II.2.3.2.4). After antibiotic selection, 354 clones were picked (Fig. III.30) and analyzed by Southern blot (see section II.2.2.2.2). Briefly, genomic DNA was isolated from duplicate clones, digested with *BamH*I and resolved on a 0,8% agarose gel. In order to be used as a hybridization probe, the PCR fragment amplified with the primers "5'beginProbeA" and "3'beginProbeA" (see section II.1.3.1), was radioactively labeled and hybridized to the blotted DNA. Figure III.27 shows one of the Southern blots obtained in this way. Only 21 of the 354 picked clones were positive for the targeted fcn-A locus displaying a band of 4.5 kb (additional to the wild type band of 8.5 kb), and only 11 positive clones survived after thawing the mother plate.

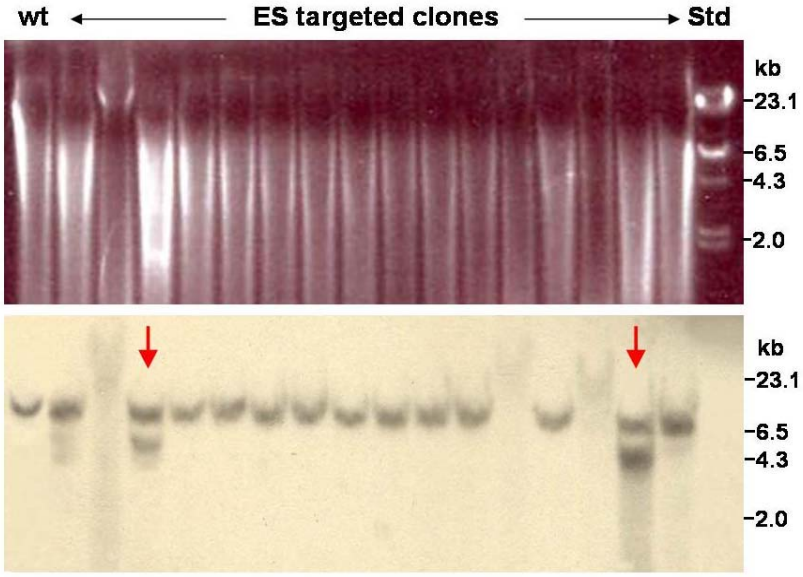

Fig. III.27: Southern blot analysis of targeted ES clones. Genomic DNA was isolated from 354 ES targeted clones, digested with *BamH*I and resolved on a 0,8% agarose gel. After blotting the membrane was subjected to hybridization with a ficolin-A specific radioactive probe. After extensive washings the membranes were exposed to a radiographic film at -80°C and developed. Upper panel: agarose gel. Lower panel: film. Positive clones are marked with red arrows. **wt:** wild type, **Std:** molecular weight standard.

These 11 clones (named 2A5, 2A8, 4A1, 4C1, 4F1, 4A2, 4E8, 6A2, 6F2, 8B1 and 8B8) were cultured under normal conditions for expansion, frozen as a back up, and analyzed again by touchdown PCR (see section II.2.2.1.2) with the primers “5’ES-PCR fcnA” and “3’Neo Out” (see section II.1.3.1). Sequencing of the PCR products amplified by this method confirmed that the targeting of the ficolin-A locus occurred by homologous recombination (Fig. III.28).

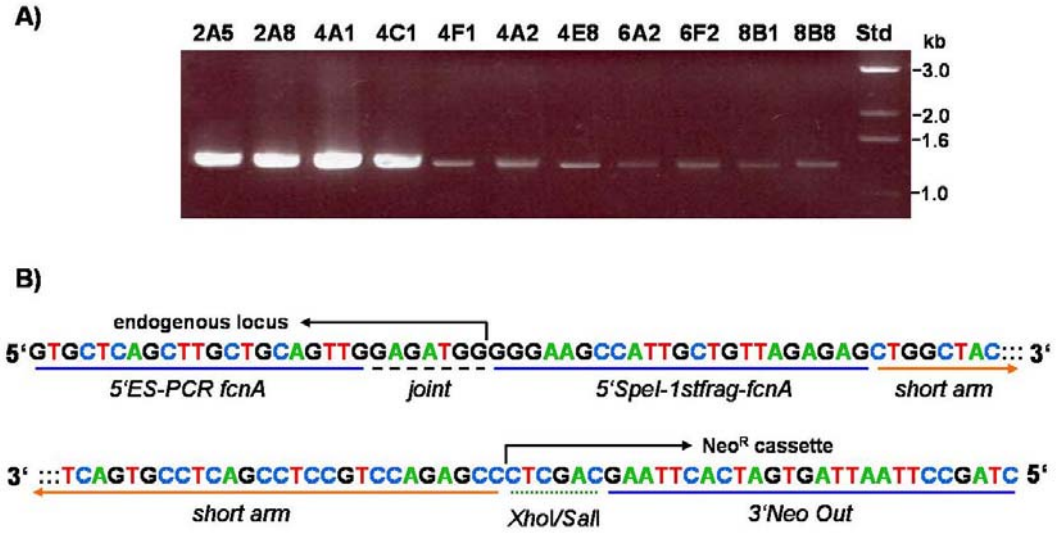

Fig. III.28: Analysis of targeted clones by touchdown PCR. A) Genomic DNA from all the 11 clones was isolated and subjected to touchdown PCR. Products were resolved on a 1% agarose gel and stained with ethidium bromide. **B)** Fragments were purified from the gel, cloned into the pCR2.1 TOPO vector and sequenced. All the sequences revealed a perfect joint between the endogenous ficolin-A locus and the targeting vector after homologous recombination.

III.5.4 Generation of ficolin-A chimeras and knock-out mice

Phase B in the generation of a gene-deficient mouse line (Fig. III.24) starts with the microinjection of the targeted ES cells into mouse embryos. Accordingly, blastocysts were collected from a pregnant C57Bl/6 mouse and injected with 10-15 cells of clone 4C1 (see section II.2.6.3). Blastocysts were reimplanted into a pseudopregnant mouse and the gestation allowed to complete. After several attempts three chimeras were identified by coat color. (Fig. III.29).

Fig. III.29: Ficolin-A chimeras born after blastocysts injection. Blastocysts from C57Bl/6 mice were injected with 10-15 cells from clone 4C1 and reimplanted into a pseudopregnant CD1 foster mother. Three weeks after the injection pups were born and chimeras identified by their black and brown fur colour.

IV Discussion

In order to elucidate the biological functions of mouse ficolins the generation of new specific tools is required. Therefore, the main aims of this work involved the production of poly- and monoclonal antibodies for the detection and analysis of ficolin expression, and the generation of gene-targeted mice for the future study of ficolin deficiencies in different infection scenarios. In the following sections, the obtained results and a future working plan will be discussed.

IV.1 Production of recombinant ficolin-A and –B

The first aim of the present work was the production of recombinant ficolin-A and -B by using the Drosophila Expression System. The construction of both vectors and the characterization of the purified protein after each co-transfection with the resistance-bearing vector (i.e. pCoHygro) demonstrated that the system is suitable to generate stably transfected S2 cell lines producing high quantities of recombinant mouse ficolins. In addition to the co-transfection experiments, a single transfection with a plasmid bearing both the ficolin-B gene and the hygromycin resistance cassette was also efficient (data not shown). Furthermore, the amount of expressed protein (3.6 and 6.7 μg/ml culture medium for ficolin-A and -B, respectively) exceeded the yields normally obtained by yeast and mammalian expression systems (Deml *et al.,* 1999), indicating that not only the speed and the simplicity but also the high yield production of exogenous proteins are the main advantages of the system. Although both co-transfections were performed with a fixed vector ratio (10:1), the difference in yield comparing ficolin-A and -B could be due to the fact that the number of integrated gene copies is only one of the several possible factors regulating an efficient expression of foreign genes. For example, the chromosomal position of the integrated transfer vectors within transcriptionally more or less active sites was reported to be an important variable for an efficient gene overexpression (Deml *et al.,* 1999).

Purification of ficolin-A and –B over a chelating sepharose column was successfully performed as shown in figure III.5. The use of serum-free S2 culture medium reduced the presence of contaminants facilitating the production of pure protein samples. In this way, ficolins could be purified in a one-step protocol by IMAC. Interestingly, while ficolin-B displayed only one band (37 kDa) in the elution fractions, ficolin-A revealed two co-purifying products. According to their migration on SDS-PAGE, the smaller band correlated with the expected apparent molecular weight of 40 kDa. In order to characterize the second band, more stringent reducing conditions were first tested since the upper band was considered to be an inefficiently reduced structure. However, no changes were observed after this

treatment (data not shown). Therefore, the most direct approach to identify this second product was protein sequencing. In fact, both bands displayed the same sequence identified as that of ficolin-A by data base comparison. The reason for the slower migrating behaviour of the second product through a polyacrylamide gel could not be elucidated. Distinct glycosylation patterns could be considered as a reasonable explanation for the difference in the apparent molecular weight of both products, since it is known that insect cells are able to perform this kind of posttranslational modifications (Benting *et al.,* 2000). Furthermore, the presence of doublets in SDS-PAGE due to a difference in the glycosylation was also observed for recombinant pig ficolin-α expressed in a mammalian system (Ohashi and Erickson, 2004). The use of N-glycosydases would allow to determine if this is also the case with the S2-expressed ficolins although it will not address the question why only ficolin-A and not ficolin-B was differently glycosylated, even though both proteins have a potential glycosylation site in their amino acid sequence (Fig. III.1).

It was also observed that the molecular weights of both recombinant ficolins were slightly higher than those published by other groups. In fact, this difference was expected and is due to the presence of the V5-tag at the C-terminal end of the protein (approximately 3.6 kDa). The biological consequence of this additional sequence cannot be predicted in advance. Nevertheless, previous reports on recombinant ficolins suggested that this modification does not seem to affect the structure and function of the protein (Frederiksen, 2005).

IV.2 Characterization of recombinant ficolin-A and –B

Although both ficolins were mainly produced for the immunization of rats and rabbits to generate mono- and polyclonal antibodies, respectively, a basic characterization of the recombinant proteins was of particular interest in order to use them in further biochemical studies without the need of isolating the native products. In this respect, the first question to answer was whether the S2-expressed mouse ficolins were able to form higher oligomeric structures. It is known that ficolin trimers as well as the trimer-based multimers are linked by intermolecular disulfide bonds, where the cysteine residues in the N-terminal domains appear to be the major players (Ohashi and Erickson, 2004). Comparison of recombinant mouse ficolins under reducing and non-reducing conditions revealed a certain degree of oligomerization (Fig. III.6). In the absence of a reducing agent, both ficolin-A and –B showed not only the 40 and 37 kDa monomeric products, respectively, but also dimers (at 80 and 74 kDa), and a ladder of higher molecular weight oligomers, which unfortunately displayed a smear rather than discrete bands. In the presence of β-mercaptoethanol, however, all the high oligomers were reduced to the monomeric form, which migrated slightly slower than under non-reducing conditions due to a more relaxed and extended structure.

The fact that both recombinant ficolins formed multimers led to the question whether these proteins were able to activate the complement system. A convenient and easy method to test this function *in vitro* is the C4b deposition assay, where the breakdown product of C4 is detected after incubation of the lectin together with the protease MASP-2 on a specific binding surface. It was already known for human L- and M-ficolin that they bind to acetylated-HSA-coated plates (Frederiksen, 2005) and, therefore, the same surface was tested for the recombinant mouse ficolins. As shown in figure III.7A, both ficolins showed affinity for the acetylated surface, although they did it to a markedly distinct extent. This could be explained either by an intrinsic difference in affinity between ficolins or by the fact that one of the glycosylated forms of ficolin-A might be inactive.

Once the binding of the mouse ficolins to acetylated-HSA was confirmed, the C4b deposition assay could be performed. Recombinant L-ficolin was used as a positive control as it was proven to cleave C4 in complex with the recombinant human MASP-2 (Frederiksen, 2005). Surprisingly, TRIFMA results for both ficolin-A and -B were negative (Fig. III.7B). These data, however, do not necessarily indicate that native mouse ficolins do not associate with MASP-2 and, consequently, are not able to activate the complement cascade. The assay was performed with recombinant material that differs from the native forms in several aspects. First, as mentioned before, these engineered proteins contain tags at the C-terminal end which might affect their folding and/or function. Second, the fact that these proteins were expressed in an insect system cannot be disregarded. It has been shown that insect cells are able to perform posttranslational modifications such as N- and O-glycosylation which are important for the proper folding and maturation of the recombinant proteins (Benting *et al.,* 2000). Because ficolins are multimeric molecules that undergo several grades of clustering during assembly, an eukaryotic expression system was preferred and due to its simplicity and low-cost handling the Drosophila system was the method of choice. However, even when most of the modifications performed by the insect cells are comparable to those occurring in a mammalian system, it is known that different lepidopteran cells generate distinct N-glycosylation patterns. N-glycans of these cells have been found to consist of shortened high-mannose structures that lack terminal galactose and sialic acid, although the synthesis of complex N-glycans has been observed (Kubelka *et al.,* 1994). Whether these observations are true for the recombinant mouse ficolins and have an effect on their lectin behaviour remains unknown.

Disregarding the presence of C-terminal tags and the potential differences in the post-translational modifications, the negative results could still be explained by a species incompatibility since the recombinant ficolins used in this assay were murine whereas the zymogen was recombinant human MASP-2. It has been shown that recombinant mouse MBL is actually able to activate the complement cascade in the presence of human MASP-2

(Jensenius *et al.,*, unpublished data). However, this observation does not seem to be true for mouse ficolins. Whether this is due to the recombinant nature of the proteins or to an intrinsic property of mouse ficolins could not be elucidated.

Although unexpected, the possibility that the S2-expressed ficolins effectively associate with the recombinant human MASP-2 but still do not mediate the C4 cleavage cannot be discarded either. In fact, very recently Wallis and co-workers described this phenomenon for a recombinant mutant variation of rat MBL and proposed a model for defective MASP-2 activation (Wallis *et al.,* 2005). In order to assess the formation of a complex between mouse ficolins and the human MASP-2 in a non-functional test, gel permeation chromatography (GPC) was performed. If the conditions required for ficolin-MASP-2 complex formation (i.e. calcium concentration) are maintained during the run, the elution profile is expected to be different from those generated by each protein alone (i.e. earlier elution). However, as shown in figure III.8, MASP-2 detection of the elution fractions by TRIFMA did not reveal any association between the proteins, which was consistent with the lack of C4b deposition observed in the previous assay.

Therefore, it can be concluded that particularly the S2-expressed ficolin-A and –B, but not necessarily mouse ficolins in general, neither associate with MASP-2 nor are able to activate the complement system.

IV.3 Generation of anti-ficolin-B antibodies

The recombinant ficolin-A and -B proteins were used to immunize rats and rabbits for the generation of mono- and polyclonal antibodies, respectively. Since specific anti-ficolin-A antibodies are being produced at the moment this thesis is being written, only the production and characterization of anti-ficolin-B antibodies is discussed.

Two rabbits were immunized with recombinant ficolin-B for the generation of antiserum. Rabbits were chosen because they are easy to handle, can be safely and repeatedly bled, and produce antibodies that are well characterized and easily purified. In addition, laboratory rabbits are normally outbred and, although little is known about the genetics of the immune response in this species, they have a wider range of MHC class II proteins than inbred animals, thus responding better to an immunization schedule (Harlow and Lane, 1988).

However, even in genetically identical animals a single preparation of antigen will elicit different antibodies. Therefore, since the amount of purified ficolin-B was not limiting, two animals were immunized and screened separately. After the first boost, the antibody titer was assessed by ELISA and found to be high only for one rabbit. This better responder was further boosted and the antibody titer monitored over weeks (Fig. III.9). After 23 weeks the antibody titer fell below 50% of the maximum titer obtained after the first three boosts and

was, therefore, enhanced by a fourth booster injection. Positive signals by ELISA were detected up to a dilution of $1x10^5$ (data not shown).

Collected rabbit serum was purified on a ficolin-B-coupled affinity sepharose column. In this way, a pool of monospecific polyclonal antibodies (i.e. recognizing several different epitopes on ficolin-B) were separated from the huge diversity of immunoglobulins present in serum. The purified monospecific antibodies were characterized by ELISA to establish optimal conditions for ficolin-B detection. As shown in figure III.11, low dilutions (1:100 and 1:500) generated a high background (positive signal even in the absence of coated antigen) while a dilution of 1:2000 was found to be optimal as it gave the highest signal/noise ratio. In addition, different detergent concentrations (0.05%, 0.1%, 0.5% and 1%) in the washing buffer were tested to reduce the unspecific binding in the absence of antigen and at different antibody dilutions. However, low dilutions of the antibody produced a high background even in the presence of 1% detergent (data not shown). Therefore, a dilution of 1:2000 was the concentration chosen for the next assays. Furthermore, since immunoglobulins are glycosylated proteins and ficolin-B is a lectin, a possible interaction between both molecules through the fibrinogen-like domain of the ficolin instead of the antigen binding site of the antibody could not be excluded. Thus, in an independent ELISA assay, coated ficolin-B was pre-incubated with or without GlcNAc before detection with the monospecific antibodies. Similar binding profiles were obtained both in the presence and absence of GlcNAc, indicating that the interaction between ficolin-B and the antibodies does not involve the lectin domain (data not shown).

Optimal conditions for Western blot were also established for the monospecific anti-ficolin-B polyclonal antibodies. As shown in figure III.12, reduced recombinant ficolin-B was successfully recognized as a single band on the blot. In addition, since ficolin-A and –B share a 60% identity at the amino acid level (Fig. I.8), it was of interest to determine the presence of cross-reacting antibodies in the sample. However, only a faint signal was obtained for recombinant ficolin-A under the same conditions, indicating a very low cross-reactivity. Moreover, the presence of specific antibodies raised against the V5- and His-tags was excluded by testing irrelevant proteins containing these epitopes in Western Blot. In this experiment no products were detected with the anti-ficolin-B antibodies (data not shown).

In parallel, two rats were immunized with recombinant ficolin-B for the generation of monoclonal antibodies. Although both animals showed a good immune response, one rat showed a markedly higher antibody titer and thus was further boosted and sacrificed (Fig. III.16). After cell fusion, the screening procedure was performed by antibody-capture ELISA using anti-IgG γ-chain specific antibodies to ensure the selection of IgG-producing hybridomas. The disadvantage of this screening methodology is based on the fact that the antigen is immobilized by direct coating on a plastic surface, which leads to partial

denaturation of the protein. Therefore, antibodies recognizing the denatured antigen are also inevitably positively selected. This inconvenience could be avoided by performing an antigen-capture assay where the plastic surface is coated with a specific antibody against the antigen. However, such an antibody was not available for ficolin-B. In addition, the use of anti-V5 or anti-His-tag antibodies for coating of plates as well as the use of Ni^{2+}-chalate plates were proven to be not only expensive but also inefficient (Frederiksen, 2005).

In this thesis the purification and characterization of the anti-ficolin-B monoclonal antibody 15H2D3 is described. Concentration by ammonium sulphate precipitation followed by affinity chromatography over protein-G sepharose was found to be efficient to obtain purified antibodies. However, the presence of antibodies in the precipitate formed after dialysis (Fig. III.17) obviously reduced the overall yield of the purification process. Resuspension of the ammonium sulphate precipitated immunoglobulins in a larger volume prior to dialysis and sufficient space for expansion of the antibody solution in the dialysis tubing should be considered for the next attempt.

Optimal conditions for ELISA assays were established as already described for the polyclonal antibodies, and a dilution of 1:1000 (equivalent to 0.12 μg/well) showed the highest signal/noise ratio. However, when tested in Western blot, 15H2D3 failed to detect the reduced ficolin-B. In order to exclude a possible loss of activity during the purification procedure, the antibody was used in a dot blot assay were native recombinant ficolin-B was spotted on a nitrocellulose membrane. As shown in figure III.19, 15H2D3 detected the unreduced protein, suggesting that the antibody recognizes a conformational epitope of ficolin-B.

IV.4 Binding affinity of recombinant ficolin-B

Binding to terminal N-acetyl-D-glucosamine (GlcNAc) seems to be a common feature among ficolins as it was reported for the human (Sugimoto *et al.,* 1998) (Matsushita *et al.,* 1996) (Teh *et al.,* 2000) and the porcine (Brooks *et al.,* 2003a) ficolins as well as for the mouse ficolin-A (Fujimori *et al.,* 1998). However, to date nothing was known about the binding properties and specificities of mouse ficolin-B. Therefore, in this work recombinant ficolin-B was used in different assays in order to characterize its carbohydrate binding affinity.

As expected, ficolin-B showed specific affinity for GlcNAc (Fig. III.13). The cell wall of Gram-positive bacteria, including that of *Staphylococcus aureus*, has a multilayered three-dimensional glycan matrix. The major cell wall component (~90% $^{w}/_{w}$) of these bacteria is peptidoglycan (PG), which consists of repeating arrays of disaccharides with β-1,4-linked GlcNAc and N-acetyl-muramic acid (MurNAc) (Nadesalingam *et al.,* 2005). In *S. aureus* a six amino acid-long peptide chain is covalently attached to the MurNAc moiety, whereas the

GlcNAc end of the disaccharide is either free or forms a β-1,4 glycosidic bond with the MurNAc of the adjacent disaccharide unit (Nadesalingam *et al.,* 2005). As being a structural motif which is only expressed in bacteria, PG is one of the outmost candidates to be a pathogen-associated molecular pattern which can act as an ideal ligand for pattern recognition proteins. Lipoteichoic acid (LTA) is the second most abundant molecule (~10% $^{w}/_{w}$) present on the surface of Gram-positive bacteria. This molecule is a single-chain polymer made of phosphate-linked repeating units of alcohols such as glycerol or ribitol and is anchored to the plasma membrane by its acyl chains. Frequently, carbohydrate moieties such as glucose, GlcNAc, small amino acids such as D-alanine, or short peptides are linked to the polyglycerol backbone. Although LTA can induce certain disease symptoms, PG is known to provoke pro-inflammatory reactions via the intracellular Nod receptors (Nod1 and Nod2) during Gram-positive bacterial infections, leading to the activation of NF-κB and finally to the secretion of pro-inflammatory cytokines (e.g. TNF, IL-1, IL-6). (Girardin *et al.,* 2003).

In a recent report, MBL was shown to bind both the insoluble and soluble forms of PG with high affinity via its carbohydrate recognition domains (CRDs) by typical C-type lectin-carbohydrate interactions (Nadesalingam *et al.,* 2005). In addition, MBL seems to preferentially recognize the GlcNAc moiety rather than MurNAc present in PG. Furthermore, the production of chemokines by PMA-activated macrophages is increased by MBL (Nadesalingam *et al.,* 2005). These findings established that PG is a biologically relevant ligand for MBL, and that this innate immune collectin helps to recruit phagocytes to clear the bacteria upon binding to PG.

In order to investigate if PG is also a ligand for ficolin-B, dot blot assays with soluble peptidoglycan extracts (sPG) and LTA from *S. aureus* as well as LPS from *Yersinia enterocolitica* were performed. Although it is known that the sPG samples are not pure PG, they do not contain any contaminating traces of LTA. In addition, the LTA sample was negative for ficolin-B binding, thus suggesting that the binding substance must be PG or a PG-associated protein (e.g. an LPTXG anchored protein). This is different to the human L-ficolin, which was shown to bind to LTA (Lynch *et al.,* 2004). Since preliminary studies revealed that different batches of sPG gave different results (data not shown), a new sPG extract was fractionated by GPC and fractions corresponding to distinct peaks in the chromatogram (Fig. III.14A) were analyzed for ficolin-B binding. In this way, the first three GPC fractions were identified as the ones containing the binding substance which is recognized by ficolin-B (Fig. III.14B), suggesting it is a molecule of a large size. In addition, both penicillin-treated and non-treated samples were recognized by ficolin-B, which was expected since the penicillin used to produce sPG inhibits the biosynthesis of the peptide cross-connection of the glycan strains in the PG and does not affect the basal structure of the polymer itself. Mild periodate treatment is known to promote oxidation of carbohydrate

structures, thus producing PG with low activity in inducing complement consumption (Wilkinson *et al.,* 1981). As shown in figure III.15, the binding affinity of ficolin-B for the substance present in the PG extract was lost after periodate oxidation, indicating that a carbohydrate is involved in the binding (as it was expected for a lectin). Although these data suggest that ficolin-B is binding to PG, it is interesting that GPC fractions 4 to 8, which represent smaller PG fragments, did not show any binding. Therefore, it seems conceivable that either other molecules or PG in different physical forms are present in the first high molecular weight fractions. For instance, it was reported by Wilkinson and co-workers that purified cell walls (containing PG and LTA covalently attached) showed higher complement consumption than each component alone and, more interestingly, than both purified components combined together. This suggests that the arrangement of the components plays a critical role in the recognition (Wilkinson *et al.,* 1981). In the same report it was also shown that not only chemical but physical factors may affect the activities of cell wall fractions in complement activation (e.g. sonication stimulated the activity of *S. aureus* PG in complement consumption). However, lyophilization of PG had no discernible effect on its activity in complement activation, although it is known to destroy the phage-binding properties of group A streptococcal PG (Wilkinson *et al.,* 1981). In addition, the use of whole bacterial cells should be considered for future binding assays since there may be supra-molecular effects and contributions both from the cell wall matrix and from material released extracellularly from the organisms with respect to complement activation. For the innate immune system, this seems reasonable since some components of the PG (e.g. GlcNAc) are also part of many glycoconjugates of host cells whereas the recognized pathogen pattern must be of unique bacterial origin.

IV.5 Localization of native ficolin-B

In accordance with previous studies (Ohashi and Erickson, 1998) (Liu *et al.,* 2005a), ficolin-B mRNA expression was found to be positive in bone marrow and spleen (Fig. III.20A). However, up to date the localization of ficolin-B protein had not been determined. In the present work, ficolin-B was detected for the first time at the protein level by immunoprecipitation with monospecific antibodies (Fig. III.20B). Unfortunately the amount of precipitated material was not sufficient to confirm the identity of the band by sequencing. However, the size and tissue distribution of the product strongly indicates that the immunoprecipitated protein is ficolin-B. The use of a second anti-ficolin-B antibody (e.g. the monoclonal 15H2D3) will allow to validate these results.

Regarding cellular distribution, ficolin-B orthologues (i.e. human M-ficolin and pig ficolin-β) were found to be expressed in cells of the myeloid lineage (Brooks *et al.,* 2003b) (Liu *et al.,*

2005b). In addition, immunohistochemical analysis of spleen sections using anti-ficolin-B antibodies showed a clear distribution of the protein throughout the red pulp where macrophages, monocytes, lymphocytes and blood cells are found (data not shown). These findings suggested that ficolin-B could be expressed in myeloid cells. Therefore, peritoneal resident cells (PCs) as well as peritoneal exudate macrophages (PECs) and polymorphonuclear neutrophils (PMNs) were stained for specific ficolin-B detection. Positive staining was validated by ficolin-B mRNA detection by RT-PCR (Fig. III.21). Surprisingly, ficolin-B protein was detected only after permeabilization of the cells, indicating an intracellular expression of the lectin (Fig. III.22A). This has also been shown for its homologues ficolin-M and ficolin-β, which were found in the cytoplasm of monocytes and neutrophils, respectively (Liu *et al.,* 2005b) (Brooks *et al.,* 2003b). However, while both ficolin-M and ficolin-β were also shown to be membrane-bound and secreted by the stimulated cells, ficolin-B could not be detected neither on the membrane of macrophages nor in the culture medium upon LPS stimulation (data not shown). Nevertheless, this could be due to either wrong or insufficient stimulation or a low sensitivity of the detection method. Therefore, different quantitative and qualitative stimuli as well as an optimized detection system are required for further studies. In addition, the signals obtained by both immunostaining and RT-PCR seem to indicate an increased synthesis of ficolin-B after LPS stimulation (Fig. III.22B). However, since none of the used techniques allowed quantitative analysis, it is not possible to conclude that ficolin-B expression is upregulated upon stimulation, and the proper techniques (e.g. real time PCR) must be established in order to validate these observations.

The intracellular localization of ficolin-B and the size and shape of the fluorescent spots suggested that the compartments harboring ficolin-B were round vesicles. Since some of the macrophage organelles involved in the killing of pathogens are those related to phagocytosis, co-localization of ficolin-B with late endosomes/lysosomes was performed. For this, a specific antibody for the detection of the lysosome associated membrane protein 1 (Lamp-1) was used. Results showed a clear co-localization of ficolin-B and Lamp-1 (Fig. III.23), indicating that ficolin-B was present in lysosomes. However, the low amount of stained vesicles called for caution. Double staining of optimally stimulated macrophages will probably be of help to obtain more detailed information on the localization of ficolin-B.

In order to elucidate the role of ficolin-B inside the cell further work is clearly required. However, one could hypothesize that ficolin-B serves as an intracellular pathogen recognition receptor in analogy to other known molecules. For example, it is known that the nucleotide-binding oligomerization domain protein 2 (Nod2) activates the NF-κB pathway following intracellular recognition of bacterial PG through the muramyl dipeptide (MDP), which is the minimal motif found in all PGs of both Gram-positive and -negative bacteria (Girardin *et al.,*

2003). As another example, during phagocytosis of yeast, TLR2 (which also senses bacterial PG) is recruited to phagosomes, presumably to screen the contents for its ligands and subsequently to trigger signaling via MyD88 (Ozinsky *et al.,* 2000). Furthermore, CpG-DNA recognition requires endocytosis of the pathogen followed by its Rab5-dependent transport to the endosomal/lysosomal compartment where intracellular TLR9 recruits MyD88 to initiate signal transduction (Ahmad-Nejad *et al.,* 2002). In this case, this seems a logical event since, in contrast to cell-wall components, bacterial DNA is liberated during processes affecting the structure of the pathogen, which take place in the endosomal/lysosomal compartment. Thus, for bacterial DNA this scenario favors a model where triggering of signal transduction occurs at a later stage of endosomal maturation. In this context, a very interesting recent report by Palaniyar and co-workers described nucleic acids as a novel ligand for the innate immune collectins SP-A, SP-D and MBL (Palaniyar *et al.,* 2004). Whether ficolins are able to bind DNA is unknown and still remains to be elucidated.

It is difficult to imagine, however, that by acting as an intracellular recognition molecule ficolin-B is able to activate the complement system. In this regard, it is not known if ficolin-β, for example, is able to initiate the complement cascade after being secreted by neutrophils at local sites of infection. In the case of M-ficolin, Liu and co-workers succeeded in showing association with proMASPs *in vitro* (Liu *et al.,* 2005b). Whether this is also true *in vivo* remains to be elucidated.

Therefore, a method to detect secreted ficolin-B would not only pose a source of native protein but would also allow to perform specific assays to establish whether ficolin-B plays a role in the complement system.

Finally, the generation of ficolin-B-deficient mouse lines will provide the proper negative control for future assays on ficolin-B localization and an excellent model for functional studies.

At the time this discussion is being written, a new study on recombinant ficolin-A and –B has been reported by Endo and co-workers (Endo *et al.,* 2005). Comparable to the results obtained in this thesis, no association of ficolin-B to mouse MASP-2 and subsequent C4 cleavage could be observed. In contrast, ficolin-A was found to bind to MASP-2 and activate the complement system. This controversial result stresses the need for further comparative analysis on the functional properties of recombinant and native ficolin-A.

IV.6 Ficolin-deficient mouse lines

In the present work, the strategy and procedure to disrupt both ficolin-A and -B genes in independent approaches were described. Unfortunately, this technique is highly time-

consuming and by the time this thesis is being written, the homozygous ficolin deficient mouse lines are still in process.

Nevertheless, based on the general knowledge on ficolins and the results obtained with MBL-null mice (MBL-$A^{-/-}$ x MBL-$C^{-/-}$) used in different infection models, it is possible to hypothesize about the phenotype and susceptibility to infections of the future ficolin-A/-B deficient mice.

Like MBL-null mice (Shi *et al.,* 2004), ficolin-deficient mice are expected to be healthy and fertile, and not to present any obvious developmental defect under normal conditions (i.e. in the absence of pathogens) since ficolins do not seem to be involved in any process related to embryonic development.

Although one could expect that the lack of an innate immune lectin will be detrimental for the organism to fight an infection, reports on MBL deficiency were not always in agreement with their original hypothesis. For example, it has been demonstrated that MBL-null mice are more susceptible to *S. aureus* infection (Shi *et al.,* 2004) whereas the MBL-$A^{-/-}$ mice were shown to be protected during acute septic peritonitis (Takahashi *et al.,* 2002). Therefore, it is difficult to predict the consequences of ficolin-A/-B deficiency during the course of an infection. It seems that MBL and ficolins have overlapping functions regarding recognition of microorganisms and activation of the complement system. However, elucidation of the non-redundant activities of each lectin will lead to a better understanding of the whole system. Thus, together with the existing MBL-null mice, ficolin-deficient mouse lines will offer a powerful tool to dissect the lectin pathway of the complement system.

V Future work

Recombinant ficolin-A and -B

The S2-expressed recombinant ficolin-A and -B were very useful for the immunization of rats and rabbits, and for preliminary biochemical characterization of both lectins. However, the generation of another source of recombinant material may be important for many comparative studies. The use of an eukaryotic cell line (e.g. CHO cells) will allow to produce proteins with the right posttranslational modifications, and the use of the antibodies already generated in this work will provide a useful tool for their purification, thus avoiding the need of fusion tags (i.e. V5 or His-tags). This will offer a less 'manipulated' system closer to the natural scenario. The new recombinant proteins will also be tested for MASP-2 association and subsequent C4 cleavage. However, it will be very useful to have recombinant mouse MASP-2 at disposal to exclude the possibility of a species incompatibility.

In addition, further binding and competition assays will be performed with the recombinant ficolins. Dot blots with different carbohydrates and acetylated compounds are to be tested in order to define the ligand affinity of both ficolin-A and –B in detail. Furthermore, the sPG extracts from *S. aureus* need further biochemical characterization to isolate the binding substance which is being recognized by ficolin-B. Binding assays with whole bacterial cells of *S. aureus* and other pathogenic Gram-positive as well as Gram-negative bacterial strains will also provide a detailed insight on the binding affinities and possible function of ficolin-B.

For the reasons discussed above, it will be of great interest to test the ability of mouse ficolins to recognize DNA motifs as well as apoptotic cells.

Anti-ficolin antibodies

As already mentioned, at the time this thesis is being written, both anti-ficolin-A monoclonal and polyclonal antibodies are being generated. Therefore, the next immediate aim is to purify and characterize them in a similar way as it was performed with the anti-ficolin-B antibodies. Once the antibodies are tested for their agonistic or antagonistic activity, they will be used in animal experiments to neutralize serum ficolin-A prior to the establishment of an infection, e.g. in a CLP model. The outcome (i.e. survival) and the pathophysiological signs will be analyzed, and in this way, the role of ficolin-A in fighting against pathogens will be elucidated.

In addition, since the anti-ficolin-B 15H2D3 monoclonal antibody is not able to recognize the reduced form of the protein, other anti-ficolin-B monoclonal antibodies need to be characterized since it might be useful to have specific reagents at disposal for utilization in different techniques (e.g. Western blot, sandwich ELISA).

Native ficolin-A and -B

Although the use of recombinant proteins provides an unlimited source of material, the isolation of native ficolin-A and -B will offer a better insight into the natural scenario. Co-precipitation studies will additionally provide a convenient tool to determine the real association of mouse ficolins with MASPs and their ability to activate the complement cascade.

Ficolin-A is a soluble protein and attempts to purify it from mouse sera are already being performed by affinity chromatography over a GlcNAc sepharose column. However, further purification techniques are required in order to remove contaminant MBL and immunoglobulins from the samples.

In this work, ficolin-B has been identified as an intracellular protein expressed in peritoneal exudate macrophages. Therefore, a purification methodology from cell lysates needs to be established. A wider knowledge about ficolin-B binding affinity will be useful in order to design this strategy. In addition, a more sensitive method to detect secreted ficolin-B may be important not only to dispose of a better source of native protein but also to elucidate the function of ficolin-B in the peritoneal cavity. In this regard, the use of specific markers for other subcellular organelles (e.g. Ag-1 for early endosomes, and SCAMP for secretory vesicles) will be helpful to investigate the intracellular trafficking of ficolin-B. Furthermore, phagocytosis assays with fluorescent bacteria are being established at the moment in an attempt to co-localize the internalized microorganism together with ficolin-B and the particular subcellular compartment in a triple-staining assay.

In order to further investigate the apparent upregulation of ficolin-B expression upon stimulation of the PECs, a detailed quantitative study of its mRNA expression by real time PCR is being established. The results of these quantitative assays will offer a better understanding of the possible function of ficolin-B in the peritoneal cavity.

Ficolin-deficient mouse lines

To define the role of ficolins in the activation of the complement system *in vivo*, homozygous deficient mice are being generated and further brought to a C57Bl/6 background before starting with the infection experiments. Once these genetically modified mice are ready to be tested, they will be subjected to different infection models. Since only little is known about murine ficolins and their role in the innate immune defense, the choice of these challenging assays will be based upon what is known about MBL and ficolins in the human system. In this way, we will be able to generate a proper mouse model of infection which will then allow us to study each particular syndrome to elucidate its molecular mechanisms and potential treatments.

A cecal-ligation and puncture model of sepsis (CLP) is already established at the Institute of Immunology, Regensburg. CLP is considered a clinically relevant model of septic peritonitis

from which mice are saved by antibiotics, demonstrating that animals die as a consequence of the bacterial infection (Echtenacher *et al.,* 2001). Ficolin-A/-B deficient mice undergoing CLP will be monitored and compared to wild type littermates. The readout will be the survival and the cytokine production will also be measured.

Moreover, in collaboration with Prof. M. Freudenberg, Freiburg, it will be investigated whether ficolins are involved in combating *P. acnes* infection. This is a well-established experimental model for studying the development of LPS hypersensitivity in mice which, after seven days of priming, develop splenomegaly, extramedullary hematopoiesis, and are hypersensitive to the cytokine-inducing and lethal activity of LPS and TNF (Gumenscheimer *et al.,* 2002).

It is known that mast cells can sense bacterial infections not only via TLRs or CD48 but also through signals generated by the host in response to invading bacteria, e.g. activated complement components. A number of observations from recent and ongoing studies indicate that mast cells also contribute to natural host defense in murine models of bacterial infections that allow extrapolation to the human system (i.e. skin infections) and that these responses are not limited to bacteria but may extend to other pathogens such as parasites and viruses (Prodeus *et al.,* 1997). In order to investigate whether ficolins play a role in fighting skin infections, experiments on infection models involving *Pseudomonas, Staphylococci* and *Streptococci* will be performed in collaboration with Dr. M. Maurer, Berlin.

Furthermore, it was recently described that both MBL and L-ficolin may play a role in protection from microorganisms that cause recurrent respiratory infections in children (Atkinson *et al.,* 2004). Therefore, it is becoming an interesting possibility to investigate the ability of ficolin-deficient mice at combating this kind of disorders. These experiments will be performed in collaboration with Dr. Gulbins, Essen, whose *P. aeuriginosa* model induces apoptosis in lung epithelial cells (Grassme *et al.,* 2000).

All these infection studies are planned to be performed on both ficolin-A and ficolin-B deficient mice. In parallel, a double knock-out mouse line (ficolin-null mice) will be generated and subjected to similar experiments in the future.

VI Appendix A

VI.1 Buffers and solutions

Competent Bacteria

FSB:	10 mM KAc
(pH 6.4)	45 mM $MnCl_2.4H_2O$
	10 mM $CaCl_2.2H_2O$
	100 mM KCl
	3 mM Hexaminecobaltchloride
TFB:	10 mM MES
(pH 6)	45 mM $MnCl_2.4H_2O$
	10 mM $CaCl_2.H_2O$
	100 mM KCl
	3 mM hexaminecobaltchloride

ELISA

AP Buffer:	9.8% ($^{v}/_{v}$) diethanolamin
(pH 9,8)	24 mM $MgCl_2$
Blocking Buffer:	0.1% HSA
	in TBS
Coating Buffer:	15 mM Na_2CO_3
(pH 9.6)	35 mM $NaHCO_3$
Washing Buffer:	0.05% Tween 20
	in TBS

GPC

TBS/ Ca^{2+}/emulfogen (running buffer):	5 mM $CaCl_2$
	0.01% emulfogen
	in TBS

Lysis Buffers

Cell lysis:	100 mM Tris·Cl 5 mM EDTA 200 mM NaCl 1% SDS
RIPA buffer (modified): (pH 7.4)	50 mM Tris·Cl 150 mM NaCl 1 mM EDTA 1% Triton X-100 1% Nonidet P40 0.5% sodium-desoxycholat Complete™ EDTA-free tablet (Roche)

Molecular Biology

6x DNA gel loading buffer:	300 mM NaOH 6 mM EDTA 18% ($^{w}/_{v}$) Ficoll 0.15% ($^{w}/_{v}$) bromocresol green 0.25% ($^{w}/_{v}$) xylene cyanol
TAE buffer:	40 mM Tris acetate 1 mM EDTA
10x TE buffer: (pH 7.4)	100 mM Tris·Cl 10 mM EDTA
PBS: (pH 7.4)	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4
TBS: (pH 7.4)	25 mM Tris Base 140 mM NaCl 2 mM KCl

SDS-PAGE

2x SDS gel loading buffer:	100 mM Tris·Cl 4% (w/v) SDS 0.2% (w/v) bromophenol blue 20% (w/v) glycerol 200 mM β-mercaptoethanol.
Coomassie staining solution:	40% methanol 10% acetic acid 0.2% Coomassie Brilliant Blue R250
Coomassie destaining solution:	40% methanol 10% acetic acid

Southern Blot

20x SSC: (pH 7)	3 M NaCl 300 mM $Na_3C_6H_5O_7.H_2O$
Denaturation buffer:	1.5 M NaCl 0.5 M NaOH
Neutralization buffer: (pH 7)	0.5 M Tris Base 1.5 M NaCl

TRIFMA analysis

B1 Buffer: (pH 7.4)	Vironal Buffer Saline 2 mM $CaCl_2$ 1 mM $MgCl_2$
Blocking Buffer:	0.1% Tween 20 in TBS
Coating Buffer: (pH 9.6)	15 mM Na_2CO_3 35 mM $NaHCO_3$

Enhancement Buffer:	0.57% ($^{v}/_{v}$) CH_3COOH 0.1% ($^{v}/_{v}$) Triton X-100 1% ($^{w}/_{v}$) PEG-6000 15 μM NTA 50 μM TOPO
MASP-2 Buffer:	0.05% Tween 20 10 mM EDTA 885 mM NaCl 100 μg/ml ΔhIgG in TBS
Streptavidin-Eu^{3+} Buffer:	0.05% Tween 20 25 μM EDTA in TBS
Washing Buffer:	0.05% Tween 20 in TBS

Western Blot

Buffer A: (pH 10.4)	0.3% Tris Base 10% methanol
Buffer B: (pH 10.4)	25 mM Tris Base 10% methanol
Buffer C: (pH 9.4)	25 mM Tris Base 40 mM ε-amino-n-capronic acid 10% methanol
5x Lämmli Buffer:	120 mM Tris Base 0.95 M glycine 0.5% SDS

VI.2 Culture Media

Bacterial cells

LB medium:	10 g tryptone
(pH 7)	5 g yeast extract
	10 g NaCl
LB-agar:	LB medium
	15g/l bacto agar
SOB medium:	20 g tryptone
(pH 7)	5 g yeast extract
	0.5 g NaCl
	2.5 mM KCl
	10 mM $MgCl_2$
SOB_{MgSO4}:	SOB medium
	20 mM $MgSO_4$
SOC medium:	SOB medium
	20 mM glucose

Eukaryotic cells

2x Freezing Medium:	20% DMSO
	80% FCS
50x HAT:	5 mmol/l hypoxanthine
	0.02 mmol/l aminopterin
	0.8 mmol/l thymidine
	in RPMI medium
50x HT:	5 mmol/l hypoxanthine
	0.8 mmol/l thymidine
	in RPMI medium

EF medium:	5% FCS 100 U/ml Pen/Strep 50 μM ß-mercaptoethanol 2 mM glutamine in DMEM medium (4500 mg/ml glucose)
ES medium:	15% FCS 100 U/ml Pen/Strep 50 μM ß-mercaptoethanol 2 mM glutamine 1% LIF in DMEM medium (4500 mg/ml glucose)
RPMI: (pH 7.2)	10.4 g medium powder (RPMI 1640) 2 g $NaHCO_3$ 0.1% gentamycin
S2 freezing medium:	45% fresh S2 medium 45% conditioned medium 10% DMSO.
S2 medium:	Insect media (Insect X-press, Cambrex) 100 mg/L kanamycin

VI.3 Companies

Ambion	Cambridgeshire, UK
Applied Biosystems	Darmstadt, Germany
Bachofer	Reutlingen, Germany
BD Becton Dickinson Biosciences	Heidelberg, Germany
Biochrom AG	Berlin, Germany
Biomol	Hamburg, Germany
BioRad	Munich, Germany
Boehringer Mannheim	Mannheim, Germany
Cambrex	Verviers, Belgium
Charles River	Sulzfeld, Germany
Dako	Glostrup, Denmark
Difco	Heidelberg, Germany
GE/Amersham/Pharmacia/Osmonics Inc.	Freiburg, Germany
Immunolex	Gentofte, Denmark
Invitrogen/Gibco	Karlsruhe, Germany
Merck	Darmstadt, Germany
Metabion	Martinsried, Germany
Millipore	Schwalbach, Germany
MoBiTec	Göttingen, Germany
MWG Biotech	Ebersberg, Germany
NEB (New England Biolabs)	Beverly, MA, USA
Nunc	Wiesbaden, Germany
PAA Laboratories	Linz, Austria
PAN Biotech	Aidenbach, Germany
PE Applied Biosystems	Darmstadt, Germany
Promega	Mannheim, Germany
QIAGEN GmbH	Hilden, Germany
R&D Systems	Wiesbaden, Germany
Roche Applied Science	Mannheim, Germany
Carl Roth	Karlsruhe, Germany
Sigma-Aldrich Chemie	Taufkirchen, Germany
Stratagene	Amsterdam, Netherlands
Wallac Biochemical Laboratory	Turku, Finland
Zeiss	Oberkochen, Germany

VII Appendix B

The results of the present work were partially published in:

Localization of the mouse defense lectin ficolin B in lysosomes of activated macrophages. Valeria L. Runza, Thomas Hehlgans, Bernd Echtenacher, Ulrich Zähringer, Wilhelm J. Schwaeble, and Daniela N. Männel. J. *Journal of Endotoxin Research*. Accepted for publication November, 2005.

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