Characterization and functional analysis of mouse Ficolin B

Dissertation

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Unterschrift

Erklärung

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

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Unterschrift

When everything seems to be going against you, remember that the airplane takes off against the wind, not with it.

(Henry Ford - Founder of the Ford Motor Company)

To Lukas and my family.

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IV. Abbreviations

B-ME	β-Mercaptoethanol
18S	Subunit of the ribosomal RNA
AcBSA	Acetylated Bovine serum albumine
AcLDL	Acetylated low density lipoprotein
ANOVA	*analysis of variance*, statistical model
APS	Ammoniumpersulfat
BMDC	Bone marrow derived dendritic cells
BMDM	Bone marrow derived macrophages
BSA	Bovine serum albumine
C1INH	C1 inhibitor
C2	Complement factor 2
C3	Complement factor 3
C4	Complement factor 4
C4a	Complement factor 4a
C4b	Complement factor 4b
C4BP	C4 binding protein
cDNA	Complementary Desoxyribonucleic acid
CFA	Complete Freud's adjuvant
CLP	Cecal ligation and puncture
CLR	C-type lectin receptor
CpG	cytosine-phosphate-guanosine
CRD	Carbohydrate recognition domain
CRP	C-reactive protein
d	day
DAMPs	Damage associated molecular pattern molecules
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyldicarbonat or Diethylpyrocarbonat
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonukleosidtriphosphate
dsDNA	Double-stranded DNA
DS-2	Drosophila Schneider 2
DTT	Dithiothreitol
ECL	*enhanced chemiluminescence*
E. coli	*Escherichia coli* 0127:B8
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzymatic-linked immunosorbent assay
Em	Emission
Eu^{3+}	Europium
Ex	Excitation
FACS	Fluorescence-activated cell sorting
FcnA	Ficolin A
FcnB	Ficolin B
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanat
fMLP	N-Formylmethionyl-Lencyl-Phenylalanin
GBS	Group B *Streptococcus*

GalNAc	N-acetylgalactosamine
GlcNAc	N-Acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H	Hour
HAT	Hypoxanthine-Aminopterin-Thymidine
HBS	HEPES-buffered saline
His	Histidine
HT	Hypoxanthine-Thymidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IFA	Incomplete Freud's adjuvant
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMC	Immatute myeloid cell
IFNγ	Interferon γ
i.p.	Intra peritoneal
In vitro	"within glass"
In vivo	"within the living"
kDa	Kilo Dalton
LAMP-1	Lysosome-associated membrane protein 1
LB	Luria-Bertani
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MACS	Magnetic Activated Cell Sorting
Map19	Also called sMAP (small MBL associated protein)
MASP	Mannan-/Mannose-binding lectin associated serine protease
MBL	Mannan-/Mannose-binding lectin
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived Suppressor cells
min	Minute
mM	Millimolar
mRNA	Messenger Ribonucleic acid
NET	Neutrophil extracellular traps
NHS	N-Hydroxysuccinimid
Nm	Nanometer
OD	Optical density
O/N	Overnight
P. aerugniosa	*Pseudomonas aerugniosa*
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEC	Peritoneal exudate cells
PFA	Paraformaldehyd
PLL	Poly-L-lysine
PMA	Phorbol-12-Myristat-13-Acetat
PMN	Polymorphonuclear neutrophils
P/S	Penicillin/Streptomycin
qRT-PCR	Quantitative Real-time PCR

Rec	Recombinant
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Real-time Polymerase chain reaction
S. abortus equi	*Salmonella abortus equi*
S. aureus	*Staphylococcus aureus*
SCF	Stem cell factor
SD	Standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
ssDNA	Single-stranded DNA
sMAP	See Map19
TAE	buffer solution containing Tris base, acetic acid, EDTA
TBS	Tris-buffered Saline
TCC	Terminal complement complex
TLR	Toll-like receptor
TNF	Tumor-necrosis factor
TNFR2	TNF receptor type 2
TRIFMA	Time-resolved immunofluorimetic assay
TRF	Time-resolved fluorescence
Tw	Tween
U/ml	Units/milliliter
UV	Ultraviolet
VBS	Veronal buffer saline
WM	Whole molecule
µg	Microgram
µl	Microliter
µM	Micromolar

1. The innate immune system

1.1 The complement system

The complement system forms the first innate defense mechanism against pathogens before adaptive immune responses takes place. In this function it helps to trigger the opsonization and neutralization of these pathogenic agents (Zundel et al., 2004). The term “complement” was introduced 1890 by Paul Ehrlich.
The complement system consists of more than 60 (Zipfel and Skerka, 2009) components including recognition molecules with effector or control-function (Krarup et al., 2004). Within this system, the molecules are arranged in complexes with proteolytic enzymes and non-enzymatic proteins (Wittenborn et al., 2010).
Three different pathways are able to activate the complement system: (I) the classical pathway, (II) the alternative pathway and (III) the lectin pathway.
All three pathways have in common that they activate the molecule C3 and subsequently form the C3 convertase (C4bC2a) (see Fig. 1).

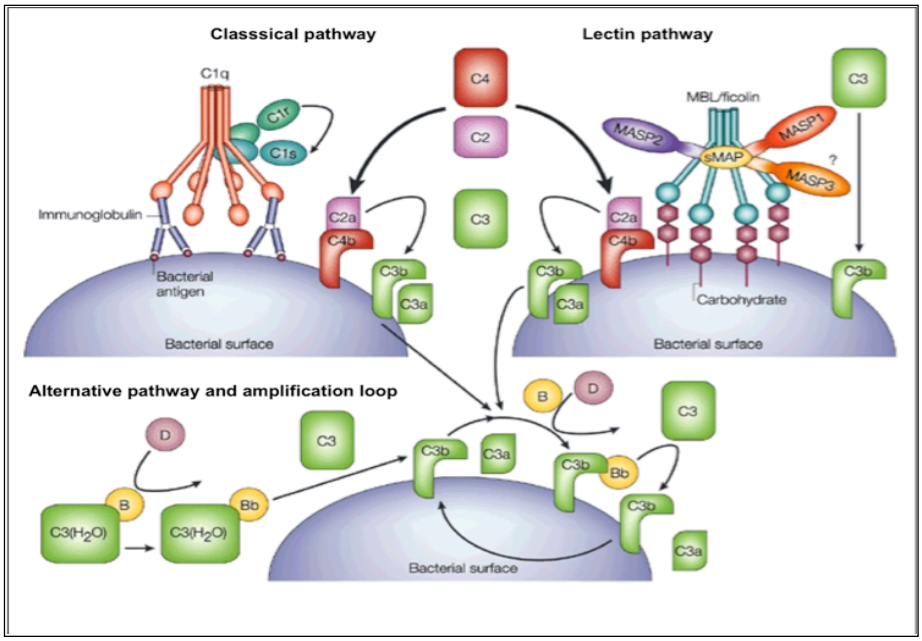

Figure 1: Overview complement pathways.

Three pathways of protein and proteases cascades are known to activate the complement system. These are the classical, the lectin and the alternative pathway of complement activation. All of them contribute to the clearing of invading pathogens (Modified after Fujita, 2002. *Nature Reviews Immunology,* 2, 346-353).

(I) Classical pathway

The classical pathway is characterized by the involvement of antibodies (adaptive immunity). Here an antigen is recognized by an antibody, which in turn is bound by the collectin C1q. C1q later activates the associated serine proteases, first C1r, than C1s. C1r mediates the internal activation of this new built C1 complex, which starts the complement cascade. When C1s is activated, C4 and C2 can be cleaved to generate C3 convertase (C4bC2a) (Zundel et al., 2004). This convertase cleaves the molecule C3. As a result C3 becomes activated and the resulting C3b fragment initiates the formation of the final membrane attack complex, consisting of the complement factors C5 to C9. Besides this function, C3b also acts as an opsonin, through directly marking the pathogen itself (Miller et al., 1968; Levinsky et al., 1978; Matsushita, 2010).
Interestingly, C1q also shows binding to C-reactive protein (CRP), which in return is bound to the surface of microorganism. Thereby the complement system is also activated via the classical pathway.
The classical pathway is regulated by C1 inhibitor (C1INH), via binding to the serine proteases C1s and C1r. This inhibitor also is known to bind to the serine proteases of the lectin pathway - MASP-1 and MASP-2 - but not to MASP-3 (Petersen et al., 2000).

(II) Alternative pathway

The theory of this alternative complement pathway, additional to the classical pathway, was proposed by Pillemer (Klein, 1982).
The alternative pathway of the complement system is characterized by activation in the absence of immunoglobulins.
The molecules of the alternative pathway are C3, properdin, factor B, factor D, and proteins for regulation (Matsushita, 2010). So far it seems that MBL and MASPs (e.g. MASP-1) activate not only the lectin pathway, but also the alternative pathway. In this case C2 is not involved (Selander et al., 2006).

(III) Lectin pathway

The lectin pathway of the complement system is initiated via binding of ficolins (lectins) or MBL (collectin) to carbohydrate or other patterns on microorganism. Binding of MASPs to these recognition molecules leads to phagocytosis and killing of these pathogens by the complement system. This activation is triggered by different

complex formations between ficolins or MBL and MASPs (Endo et al., 2010). This pathway of the complement activation was first discovered by the interaction of MBL with MASPs (Matsushita, 1996; Gadjeva et al., 2001; Fujita, 2002). MBL leads to opsonization of the pathogen and thereby initiates further clearance by neutrophils (Miller et al., 1968; Levinsky et al., 1978).
Also, ficolin forms complexes with serine proteases. Binding of ficolin (in human: M-, L- and H-ficolin, in other mammalians: Ficolin A and B) to patterns on microbial surfaces and subsequent binding of MASPs leads to activation of the protease (Schwaeble et al., 2002). Activated MASP further initiates the complement cascade of the lectin pathway via binding to complement factor C4.
Historically, the lectin pathway was discovered later than the classical and alternative pathways. The existence of a third pathway was assumed when recognizing that pathogens are also detected, when antibodies (classical) were absent, and mechanisms unrelated to the alternative pathway were observed (Schwaeble et al., 2002). Previously, this new complement pathway was called MBL pathway because MBL was the first protein discovered to activate this pathway. Later when also ficolins were found to interact with MASPs, it was more generally named the lectin pathway (Wallis et al., 2010).

1.2 Activation of the complement system

The classical pathway of complement is activated when C1 proteins bind to an antibody-antigen complex. C1 protein is the first molecule to initiate this antibody-induced complement pathway. C1 protein consists of the collectin C1q and the serine proteases C1s and C1r (Bally et al., 2009). Thereby one C1q molecule is in complex with two molecules C1r and two molecules C1s (Basiglio et al., 2009). C1q is also able to bind directly to surfaces of pathogens and thereby activating the classical pathway independently of antibodies. Also CRP (C-reactive protein), a member of the pentraxin family, binds directly to C1q and is therefore also an important activator of the classical pathway (Fabian et al., 2006).
The alternative pathway is activated by the complement factor C3 that is spontaneously cleaved into C3a and C3b. C3b acts as an opsonin and binds to the surface of pathogenic agents. On this foreign surface C3b stays active and is able to bind complement factor B to form the C3 convertase of the alternative pathway. This complex is very weak and has to be stabilized by binding through properdin. Binding of properdin initiates the alternative pathway, which is therefore also called the properdin-pathway of complement activation. The lectin pathway of the complement system is activated via binding of MBL or ficolin to one of three serine proteases (MASP), that in turn initiate the lectin pathway of the complement system. Each

complement pathway contributes to the defense against invading microbes, as well as to the clearance of dead cells via building a terminal membrane attack complex (MAC). This complex was also formerly known as terminal complement complex (TCC), leading to cell lysis. Beyond that complement is involved in other non-immune functions.

1.3 Regulation of the complement system

Via complement activation a strong immune response is initiated that needs to be tightly regulated and stopped after successful clearance to avoid overreaction and cell- and tissue-damage due to inflammation. During complement activation pro-inflammatory mediators are activated, the anaphylatoxins, that need to be controlled. Dysfunctions in complement regulation can lead to diseases. Activation and inhibition must be strictly balanced. These regulators and inhibitors engage at different stages in the complement cascade (Zipfel and Skerka, 2009) (see Fig. 2).
The complement system itself is able to regulate its response by adjusting the reaction to each appropriate situation. Hence, it is able to modulate the intensity of the reaction in a self-controlled manner (Zipfel and Skerka, 2009). One way by which the complement can be efficiently regulated are inhibitors that bind directly to the component that first initiates the cascade. C4b-binding-protein (C4BP) and factor H behave like this (Sjöberg et al., 2008). Some complement activators, like CRP, also interact directly with complement regulators. For example CRP is found to cooperate with inhibitors like factor H and C4BP (Sjöberg et al., 2008). Factor H and C4BP are so called fluid phase inhibitors of the complement system. They are also associated with dying host cells and found to bind to apoptotic cells. This is important, because these dying cells down-regulate their membrane bound complement inhibitors to signal for phagocytosis. Normally, each host-cell expresses more or less membrane-bound molecules on its surface to inhibit unwanted complement activation. Such a molecule is for example CD46. Binding of inhibitors does not totally block the complement activity but enables opsonization and dampens the immune response to avoid massive complement activation and cell lysis (Sjöberg et al., 2008).
An important control to avoid destruction of intact host cells by complement is managed by a combination of integral proteins, as well as surface attached and fluid-phase control molecules (Zipfel and Skerka, 2006). Most pathogens however prevent detection by the complement system through binding to complement inhibitors or they protect themselves by capturing C4BP and factor H (Zipfel and Skerka, 2006). Summarized, complement regulators can be divided into three groups: (I) fluid-phase, (II) surface-attached and (III) membrane-integral regulators. Factor H, FHL1 and properdin (itself also an activator protein) belong to the group of

fluid-phase control proteins (Zipfel and Skerka, 2006). Responsible for partly inactivation of the anaphylatoxins is Carboxipeptidase N. C1-Inhibitor (C1INH) is a regulator protein for the classical- and the lectin pathway. The same is the case for C4BP (Zipfel and Skerka, 2006). Regulator proteins that interfere during the terminal processes are CFHR1, clusterin and vitronectin (Zipfel and Skerka, 2006). Fluid phase regulators are more specific, compared to e.g. the membrane integral proteins by controlling either the classical, the alternative, or the lectin pathway. Membrane-integral proteins are CR1, CR2, CD55 and CD46. These molecules are inhibitors for all three complement pathways. CR1 and CD46 for example inactivate C3 and C4. Some fluid phase regulators are also shown to act as surface-attached regulators. These are factor H, FHL1, C4BP, CFHR1, clusterin, and vitronectin. (Zipfel and Skerka, 2006).

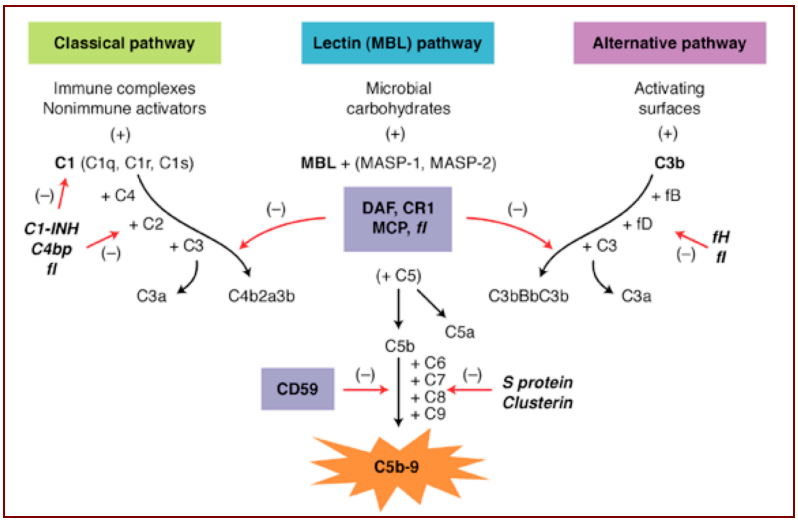

Figure 2: Activation and regulation of the complement system.

The complement system is a highly specific and strong system to eliminate pathogens. Due to this, activation must be strictly regulated by different effector molecules. (Modified after Francis et al., 2003).

1.4 Complement-dependent diseases

When activation and/or regulation of the complement cascade are disturbed complement diseases can occur (see Fig. 3). Many of them occur when one or more complement factors are deficient. Many diseases are connected with failures of the regulator proteins factor H and C1-inhibitor or with problems concerning the basic complement components C2, C3, and C4. Diseases that are often connected with

dysfunctional complement components are autoimmune diseases, cancer, or problems with infectious pathogens (Zipfel and Skerka, 2006).

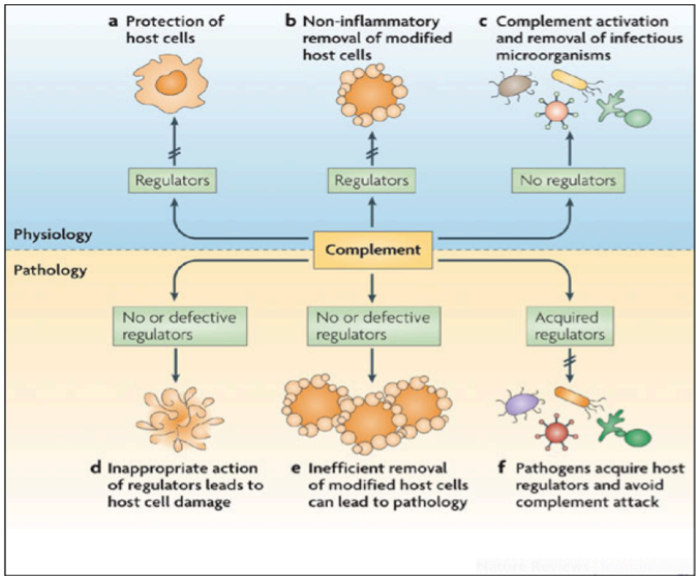

Figure 3: Complement regulators and failures in regulation.

Failures in complement activation and/or complement regulation often trigger complement diseases. (Modified after Zipfel and Skerka, 2009).

1.5 Cell surface receptors

Receptors expressed on the surface of cells, also called membrane or transmembrane receptors, are specialized in recognition of pattern-like structures and represent a connection between cell-inside and cell-outside.

1.5.1 Scavenger receptors

Scavenger receptors (see Fig. 4), or lipoprotein receptors, are a group of proteins that function in recognition of low density lipoprotein (LDL). These receptors are widely expressed on surfaces of cells (Dieckmann et al., 2010). They bind to molecules with a negative charge or to modified LDLs. They possess a role in cleaning (also called “scavenging”) by taking up and removing these molecules. These molecules include for example proteases or protease inhibitors from cell surfaces or from the environment. In this way they are also important in sensing the extracellular environmental situation (Dieckmann et al., 2010).

With regard to their structure, they are divided into group A, B-, and C-scavenger receptors. They are also distinguished by their uptake or non-uptake of the lipoprotein. The first group internalizes the lipoproteins and the second group promotes lipid exchange at the plasma, without taking it up (Dieckmann et al., 2010).

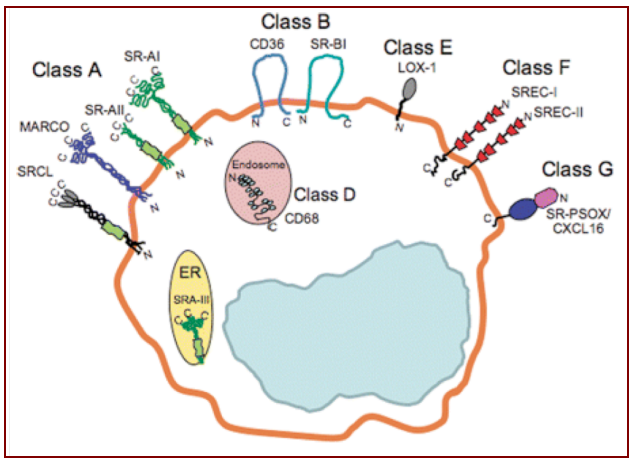

Figure 4: Overview the scavenger receptor family members.

Molecules of the scavenger receptor family contribute to immune responses against pathogens by recognition of patterns associated with these pathogens. (Modified after Moore and Freeman, 2006).

1.5.2 C-type lectin receptors (CLRs)

C-type lectin receptors are a heterogeneous group of molecules, located on the membrane of cells (see Fig. 5). Myeloid cells often express these receptors.
The name “C-type” came up by the characteristics of these proteins to be calcium-dependent binding lectins, compared to other lectins, which are Ca^{2+}-independent (Zelensky and Gready, 2005). Now the term C-type lectin is more generally used for proteins with a carbohydrate recognition domain (CRD) (Cambi et al., 2007), a structural feature common for members of these group. Proteins belonging to this group of recognition receptors are able to detect carbohydrates in a calcium-dependent or calcium-independent manner (Cambi et al., 2007). Protein families belonging to these receptors are defensins as well as collectins including the well-known member MBL.
The first discovered receptors to bind to bacteria, fungi and viruses were molecules of the mannan-receptor family (Ley and Kansas, 2004), DEC-205 and Langerin (Zelensky and Gready, 2005). Other groups within the C-type lectins are the selectins (Ley and Kansas, 2004) and the collectins, with selectins more responsible for cell-cell functions and collectins, e.g. MBL, specialized in pathogen recognition.

Soluble collectins are structurally characterized by forming trimers, which in turn form higher oligomers. This multimerization helps to effectively bind patterns on microbes (Cambi et al., 2005). These receptors are able to act as endocytic recognition receptors to mediate the uptake of the pathogen or of altered host-cells. Upon this, they can initiate destruction of the particle or antigen presentation to T cells (Osorio and e Sousa, 2011). C-type lectins were found to sense cell death by binding to products from these cells. C-type lectins are localized on the cell membrane or they are found as soluble recognition receptors (Cambi and Figdor, 2009). Members of the transmembrane receptor group are divided into two subgroups, depending on the orientation of their N-terminus. Type-I transmembrane receptor proteins are characterized by the N-terminus pointing outwards of the cytoplasm. In the case of Type-II transmembrane receptor proteins the N-terminus points inwards in the cytoplasm (Cambi and Figdor, 2009).

With regard to pathogen recognition C-type lectins often collaborate with other receptors, such as the Toll-like receptors (TLRs). Besides functioning as a pattern recognition receptor C-type lectin receptors are also involved in dampening or alterating myeloid cell activation (Osorio and e Sousa, 2011).

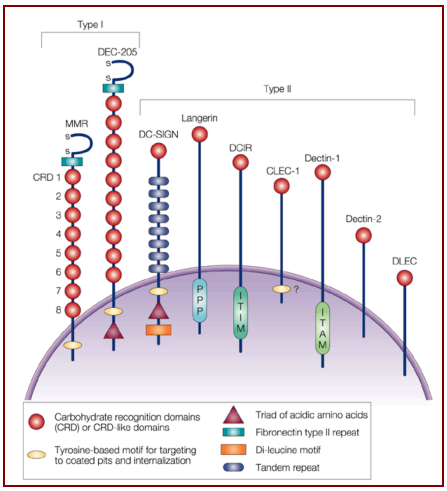

Figure 5: C-type lectin receptors on dendritic cells and Langerhans cells.

C-type lectin receptors are a group of molecules contributing to pathogen recognition and trigger thereby the elimination of microorganisms (modified after Figdor et al., 2002).

1.5.3 Toll-like receptors (TLRs)

Toll-like receptors are key molecules of the innate and the adaptive immune system recognizing patterns on invading pathogens and thereby initiating signal transduction cascades that subsequently lead to the expression of appropriate genes. These signaling pathways are regulated by TIR domain-containing adaptors such as MyD88, TRIF and TRAM (Takeda and Akira, 2005). The name TIR-domain is based on the similarity of the cytoplasmic tail between the members of the TLR- and the IL-1-family (Takeda and Akira, 2005). The term Toll-like receptor developed from the recognition molecule Toll in *Drosophila melanogaster* (Tukhvatulin et al., 2010), which binds to fungi (Lemaitre et al., 1996). Later homologues of this receptor were discovered in mammalians and named Toll-like-receptors. This first characterized mammalian TLR was termed TLR4 (Takeda and Akira, 2005). The mammalian TLR family consists of at least 11 structurally related proteins, with TLR1 to TLR9 rather conserved between human and mouse. TLR10 is assumed to be non-functional in the mouse and TLR11 seems to be active in the mouse but, due to a stop-codon, without function in the human system. Individual TLRs are able to recognize patterns on pathogens like bacteria, fungi, protozoa and viruses (Takeda and Akira, 2005).
TLR1 is expressed on the cell surface and recognizes structures of Gram-positive bacteria (Takeuchi et al., 2002; Lien et al., 2002).
TLR2 binds to lipoproteins from various microbes, as well as peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Hirschfeld et al., 2001; Werts et al., 2001; Smith et al., 2003). It also recognizes LPS from non-enterogenic bacteria. This is interesting because this form of polysaccharide differs from the LPS detected by TLR4 (Netea et al., 2002). TLR2 is structurally related to TLR1 and TLR6 and forms heterophilic dimers with these TLRs but also with others. TLR2 is also expressed on the cell surface.
TLR3, which is expressed in intracellular compartments, recognizes double-stranded RNA (dsRNA) and viruses. However, TLR3-independent mechanisms of dsRNA recognition exist (Takeda and Akira, 2005).
Essential for detection of bacterial lipopolysaccharide (LPS) is TLR4 (Poitorak et al., 1998; Hoshino et al., 1999). Expression of TLR4 is found on cell surfaces. Due to the fact that LPS is a very potent activator of an inflammatory immune reaction, only a small amount of LPS is necessary to trigger a response.
The receptor TLR5 binds to flagellin, which is a monomeric constituent of bacterial flagella (Hayashi et al., 2001). TLR5 is expressed by epithelial cells (Gewirtz et al., 2001; Maaser et al., 2004).

TLR7 and human TLR8, both expressed in intracellular compartments, recognize a nucleic acid-like structure on viruses, more specific guanoside or uridine-rich single-stranded RNA (ssRNA), e.g. from human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV), and influenza virus (Heil et al., 2004; Diebold et al., 2004; Lund et al., 2004). Host-derived ssRNA is not detected because this RNA is not transported to the endosome, where both TLRs are expressed.
TLR9 is a receptor for both, bacterial and viral CpG-rich DNA (Hemmi et al., 2000). Due to the recognition of the chromatin structure, TLR9 seems to be involved in autoimmune diseases. Also, TLR9 expression is found in intracellular compartments.
TLR11 is a functional receptor in mice even if its ligand is not identified yet (Takeda and Akira, 2005). The human homologue to mouse TLR11 is non-functional and became maybe lost during evolution (Zhang et al., 2004).
TLRs are also able to sense danger signals via recognition of alarmins, e.g. heat shock proteins, whose concentration is increased in the intracellular space in this situation (Tukhvatulin et al., 2010). Alarmins belong to the group of damage-associated molecular patterns (DAMPs).

1.5.4 Soluble pattern recognition receptors (PRR)

Soluble or secreted pattern recognition receptors are molecules that do not remain associated with the cell expressing them.

1.5.4.1 MBL and MASPs

1.5.4.1.1 Human MBL

Mannan binding lectin (MBL, see Fig. 6 and 7) is an archetypical molecule for recognition of invading pathogens (Jensenius et al., 2009). It is named Mannan- (or also Mannose-) binding lectin, because the protein was first discovered through its interaction with this yeast polysaccharide (Kawasaki et al., 1978; Kawasaki et al., 1983). Mannan is a polymer of the sugar mannose. But MBL is not particular selective for this sugar (Jensenius et al., 2002); it also binds to D-mannose, N-acetyl-D-glucosamine, D-glucose, L-fucose, but not to D-galactose (Weis et al., 1992; Wallis, 2002).
In humans, two MBL genes are known: MBL-1 and MBL-2, where active MBL is encoded by MBL-2 gene and MBL-1 is a pseudo gene. Additionally MBL protein is divided in two classes respective to its oligomerization: a lower-oligomer (MBL-I) and a higher oligomeric form (MBL-II). MBL belongs to the acute phase proteins, but the

increase in this phase is not more than threefold compared to normal MBL-concentrations (Holmskov et al., 2003). Further, MBL belongs to the family of collectins due to its collagen domain, while ficolins are lectins.
Other collectins are the surfactant proteins SP-A and SP-D, CL-P1, CL-K1, Cl-L1 and conglutinin (Kilpatrick, 2007). All collectins recognize cell wall components in bacteria: lipoteichic acid in Gram-positive and lipopolysaccharide in Gram-negative bacteria (Polotsky et al., 1996; Holmskov et al., 2003).
MBL reacts with pathogens like bacteria, fungi, viruses, and protozoa (Turner and Hamwas, 2000). Some bacteria seem to avoid their recognition by MBL or lectins with the help of their capsule (Holmskov et al., 2003).
The broad binding specificity of MBL is due to the fact that it binds to repeating sugar structures found on these pathogens (Miller et al., 1968; Levinsky et al., 1978) but not on mammalian cells (Eisen and Minchinton, 2003; Sorenson et al., 2005). MBL is also able to detect viruses like HIV-1 and HIV-2 (Haurum et al., 1993) and binds to parasites like leishmania promastigotes (Green et al., 1994). Oligomerization of MBL is crucial for its biological function and activation of the complement system (Larsen et al., 2004; Jensen et al., 2005; Teillet et al., 2005). MBL-II in humans is the higher oligomeric form of MBL, which seems to mainly interact with MASP-2 and activates thereby the complement (Dahl et al., 2001).

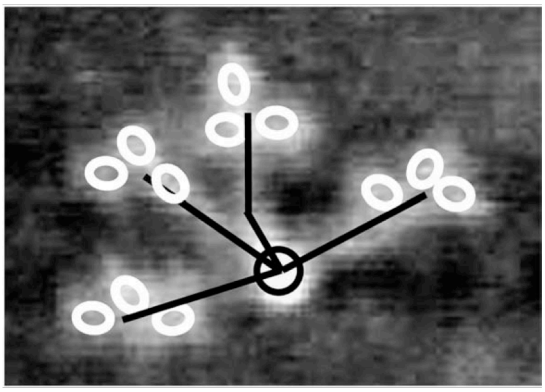

Figure 6: CRDs and MBL structure.
(modified after Jensenius et al., 2009**)**

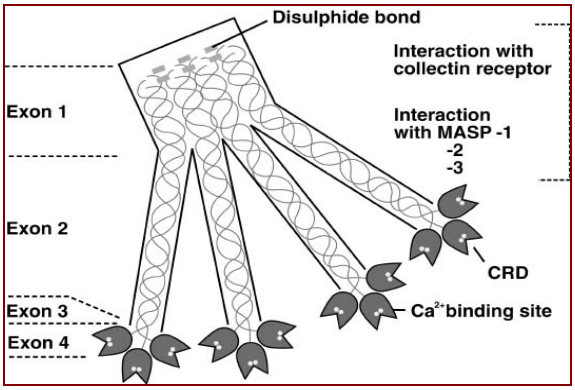

Figure 7: Tetrameric strcuture of human MBL.

(Modified after Turner, 2002**)**

1.5.4.1.2 Mouse MBL

In contrast to the human system, in mice two forms of MBL exist, namely MBL-A and MBL-C (Ihara et al., 1991; Hansen and Holmskov, 1998). Both proteins are distinguished by their distribution: MBL-A is found in serum, while MBL-C is found in the liver (Oka et al., 1998). In the year 2000, Hansen et al. described that both forms are detectable in serum (Hansen et al 2000). Structurally it is assumed that mouse MBL-C forms higher oligomeric forms compared to MBL-A (Liu et al., 2001). Human MBL was shown to be an acute phase protein. Investigations in the mouse system revealed that mouse MBL-A shows a mild acute phase response, whereas mouse MBL-C had no effect (Liu et al., 2001). MBL-C is highly sensitive to proteolytic degradation (Hansen et al., 2000). Both forms of mouse MBL are able to activate complement factor C4 to mediate complement activation via the lectin pathway (Hansen et al., 2000).

1.5.4.1.3 MBL-associated serine proteases (MASPs)

Because only discovered in 1992 (Matsushita and Fujita, 1992) together with MBL these proteases are called MBL-associated serine proteases (MASPs) (Schwaeble et al., 2002). The MASP family consists of MASP-1, MASP-2, MASP-3 and additionally MAp19. MAp19 is a non-enzymatic protein of 19kDa, also called sMAP. All members of the MASP family show a domain structure similar to other factors of the complement system: C1r and C1s. MASP-1 and MASP-3 originate from a common MASP-1/-3 gene and are produced by alternative splicing. In the same manner, MASP-2 and MAp19 evolved as different splice-products from the MASP-2

gene. All MASPs exist circulating in serum in two forms: in their inactive and their active form. The pro-enzymes, also named “Zymogens”, are proteolytically inactive until they are bound to recognition components. After this, they convert to the active forms. The site of their enzymatic activity is found in the β-chain of the serine proteases (Schwaeble et al., 2002). Pro-enzymes appear in Western blot analysis as one polypeptide chain (one band) compared to the active form with two polypeptide chains (two bands). The zymogens are mainly found in complexes with MBL or ficolins. Complexes between MASPs and ficolins are more often seen than MASP-MBL connections. It is discussed that MASPs are more bound to low MBL oligomers (Sorensen et al., 2005). The MBL-MASP-complex seems to be analogous to the C1-immune-complexes (interaction with Fc-part of IgG or IgM), but it differs in function as well as in composition.
In solution MASPs dimerize and this formation is calcium-dependent (Sorensen et al., 2005). MASPs of humans, mice and rats are structurally related proteins.

1.5.4.1.3.1 Human MASP-1

Human MASP-1 was first isolated from human serum complexed with MBL by affinity chromatography. A mannan-sepharose column was used under calcium-containing conditions, based on the fact that MASP dimerization is dependent on the presence of calcium (Sorensen et al., 2005). Human MASP-1 is encoded by a 680aa polypeptide chain and a leader peptide of 19aa. The protein is generated by alternative splicing from a common MASP-1/-3 gene (see Fig. 8). The calculated molecular weight of the MASP-1 zymogen is 76kDa.
MASP-1 in the human system possesses four N-linked glycosylation sites. Regarding to its protease activity, it seems to cleave C2 but not C4. However, it also cleaves C3 even though with lower efficiency, and thereby may activate C3 directly. MASP-1, therefore, also activates the alternative pathway of complement activation. Recombinant human MASP-1 was demonstrated to activate and cleave C3 only marginally (Rossi et al., 2001; Ambrus et al., 2003). Beside this, MASP-1 is able to activate MASP-2 within a MASP-2/MBL-complex (Takahashi et al., 2008). The production of recombinant human MASP-1 from plasma is described by Thielens et al. in 2001 (Thielens et al., 2001).

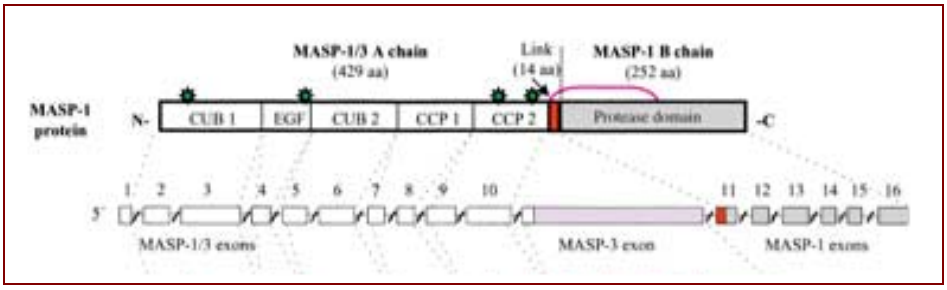

Figure 8.: Exon structure of human MASP-1 protein.

(Modified after Schwaeble et al., 2002)

1.5.4.1.3.2 Human MASP-2

The exon of the second human MASP, MASP-2, encodes a polypeptide of 671aa and a leader peptide of 15aa (see Fig. 9). The only mRNA expression site in human as well as in mice is the liver, like in the case of MAp19 (Knittel et al., 1997). The observed mass in immunoblot analysis is 74kDa, which is consistent to the calculated molecular weight. Compared to MASP-1, human MASP-2 offers no putative N-linked glycosylation site.

In contrast to MASP-1, human MASP-2 cleaves C2 and also C4 (Matsushita et al., 2001) and so performs as a monomer the same function as C1q and C1s in the classical complement pathway (Sorensen et al., 2005; Vorup-Jensen et al., 2000). This proteolytic activity is inhibited by C1-inhibitor (Matsushita et al., 2000; Petersen et al., 2000). So far it seems that only MASP-2 protein is required for activation of the lectin pathway. This is assumed because the lack of both MASP-1 and MASP-3 showed no difference for complement activation in mouse studies (Takahashi et al., 2000). MASP-2 is not only able to cleave C4 but also C2 to form C3 convertase (Rossi et al., 2001). The enzymatic activity for C2 is comparable to C1s, but MASP-2 is 40 times more efficient in cleaving C4. C1s is a component of the classical complement pathway and forms complexes with C1r (Rossi et al., 2001).

Beside this, MASP-2 might also show proteolytic activity within the coagulation system. It is assumed to participate in activation of the blood coagulation system through binding and inducing cleavage of pro-thrombin (Krarup et al., 2007). Nevertheless, MASP-2 also reveals auto-activation (Sorensen et al., 2005). Production of recombinant MASP-2 is described by Cseh et al., 2002; Rossi et al., 2001).

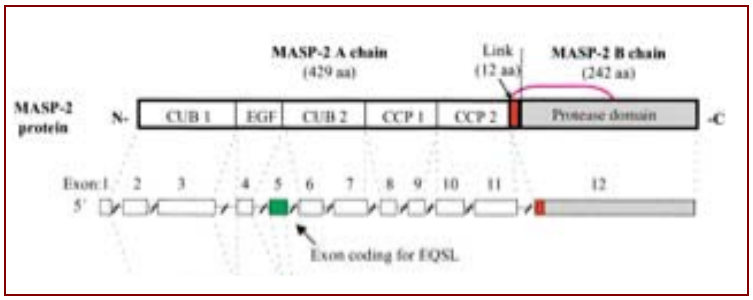

Figure 9: Exon structure of human MASP-2 protein.

(Modified after Schwaeble et al., 2002)

1.5.4.1.3.3 Human MASP-3

The exon of human MASP-3 encodes a polypeptide chain of 709aa, excluding a leader sequence of 19aa (see Fig. 10). The calculated molecular weight of the pro-enzyme is 81kDa. There are seven putative N-linked glycosylation sites in MASP-3. A common MASP-1/-3 gene encodes MASP-3 and the protein is produced by alternative splicing (Dahl et al., 2001; Endo et al., 2003).

Human MASP-3 mRNA expression is found in the liver and in non-hepatic tissues, including spleen, lung, and brain. The question came up, whether human astrocytes are a source of MASP-3 mRNA (Kuraya et al., 2003). Expression is also found in the small intestine and thymus (Lynch et al., 2005). So far, no substrate for human MASP-3 is known and, therefore, a synthetic substrate was used to investigate its proteolytic activity (Zundel et al., 2004). In contrast to the other two MASPs, activated MASP-3 exhibits no proteolytic activity towards either C2, C3, or C4 (Zundel et al., 2004).

Unlike MASP-2 (Vorup-Jensen et al., 2000; Rossi et al., 2001) and also MASP-1 (Zundel et al., 2004), human MASP-3 does not auto-activate spontaneously or self-activates in experiments using recombinant MASP-3 (Zundel et al., 2004). Also in difference to the other MASPs, human MASP-3 is not associated with C1-inhibitor, so its activation and regulation seems to differ from MASP-1 and MASP-2 (Zundel et al., 2004). Interestingly, MASP-3 shows down-regulatory activity for MASP-2 (Dahl et al., 2001). Recombinant, in Insect cells produced human MASP-3 by Zundel et al. (Zundel et al., 2004) exists as a homodimer in the presence of calcium and as a monomer in the presence of EDTA (Zundel et al., 2004).

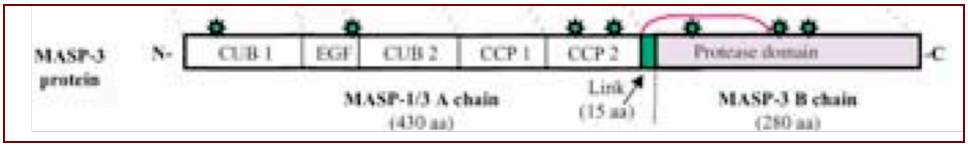

Figure 10: Exon structure of human MASP-3 protein.

(Modified after Schwaeble et al., 2002)

1.5.4.1.3.4 Human MAp19

Human MAp19, also named sMAP (small MBL-associated protein), is a truncated version of MASP-2 and is generated from the MASP-2/-MAp19 gene by alternative splicing (see Fig. 11). The protein polypeptide consists of 185aa with a molecular weight of 19kDA. Expression is found in the liver. The protein is more often found associated with lower-molecular weight MBL (MBL-I), than with higher oligomers (Sorensen et al., 2005).

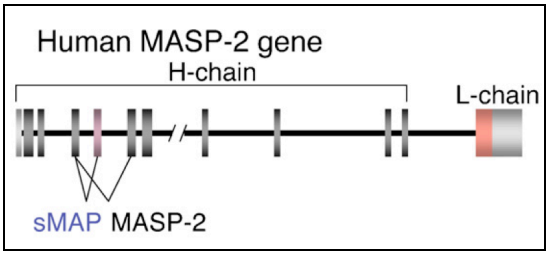

Figure 11: Structure of the common human MAAP-2/Map19 gene.

(Modified after Endo et al., 2011)

1.5.4.1.3.5 Human MAp44

Human MAp44 is, like MASP-3, also a splice variant of the MASP-1/-3 gene (Degn et al., 2010). Expression of MAp44 mRNA is found in high amounts in the heart (Degn et al., 2009). Further mRNA expression is detected in the liver, in bladder, brain, cervix, colon, and prostate (Degn et al., 2009). MAp44 is found to inhibit complement activation via competing with MASP-2 for binding to MBL or ficolins (Degn et al., 2009). So it seems, that MAp44 acts as a control molecule in complement activation.

1.5.4.2 Ficolins

Ficolins are proteins that also serve as soluble pattern recognition molecules. They are able to recognize pathogens and sense danger signals derived from the host (Thielens et al., 2007). The first identified and biochemical characterized ficolin was TGFβ-1 by Ichijo in the year 1991 as a protein from porcine uterus (Ichijo et al., 1991; Ichijo et al., 1993). The Interaction of ficolins with porcine TGF-1 still remains unclear (Endo et al., 2005). In the year 1993 pig ficolin-α and ficolin-β were characterized also by the group of Ichijo (Ichijo et al., 1993). Since 1991 and the first identification and characterization of ficolins (Ichijo et al., 1991), these molecules are under investigation by different groups all over the world. Evolution and genetic aspects of ficolins are discussed by the English group of Peter Garred (Garred et al., 2010). Garlatti et al. discuss structural and functional aspects of ficolins (Garlatti et al., 2010). The same field is also investigated by Matsushita and his group in Japan (Matsushita, 2010). In addition to this, Endo et al. are interested in the interaction of ficolins and MBL within the lectin pathway of the complement system and they also demonstrate interaction of those lectins with both fibrinogen and fibrin (Endo et al., 2010). All ficolins and MBL have their structure and some binding specificities in common, but they are expressed and distributed in different tissues. Due to that fact they may also have tissue-specific functions (Matsushita et al., 2010). Concerning their structure, all ficolins consists of a collagen-like and a fibrinogen-like domain (see Fig. 12). Thereby the collagen region is responsible for association and binding with MASPs. The counterpart to the fibrinogen-like domain in ficolins is the typical C-type lectin domain in MBL (Le et al., 1998; Sugimoto et al., 1998).
All so far tested ficolins bind to acetylated groups such as GlcNAc and GalNAc (Thiel, 2007). These groups are found on microbes.
Beside this, ficolins were demonstrated to bind to apoptotic cells (Karaya et al., 2005; Jensen et al., 2007).

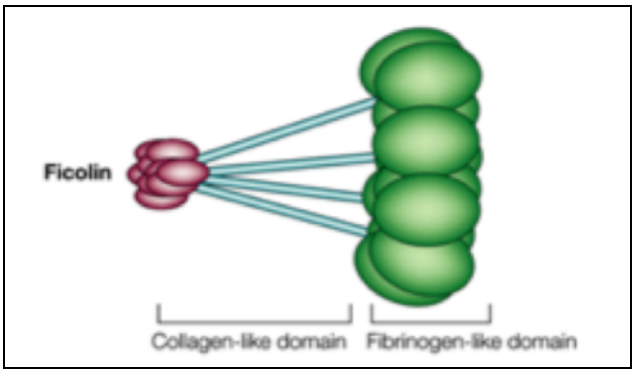

Figure 12: Schematically structure of ficolin.
(Modified after Fujita, 2002).

1.5.4.2.1 Human ficolins

In contrast to other mammalians, in humans three forms of ficolin, instead of two, exists. They are named M-ficolin, L-ficolin and H-ficolin. The last one turned out as a pseudo-gene in other mammalians (see Fig. 13).

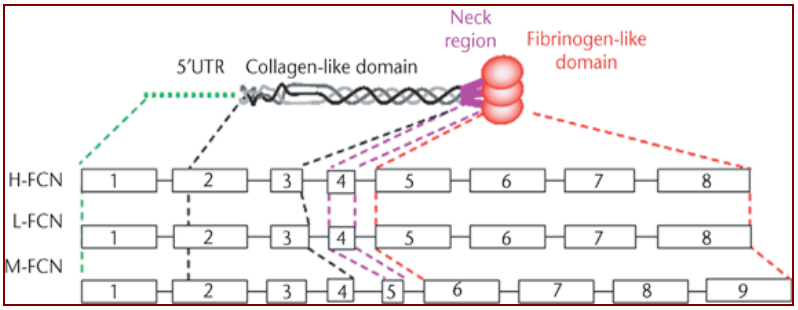

Figure 13: Overview of the exon structure of human ficolins.
(Modified after Takahashi, 2008)

1.5.4.2.1.1 Human M-ficolin

Mainly called M-ficolin this ficolin is also known as ficolin 1 or L-ficolin/p35-related protein. Human M-ficolin was first identified by genomic DNA and cDNA cloning as a by-product during human L-ficolin characterization in 1996 (Endo et al., 1996; Lu et al., 1996a). The gene of M-ficolin is FCN1 and it is located on the same chromosome as the L-ficolin gene (Endo et al., 1996). M-ficolin mRNA expression was found in monocytes, in lung and in spleen (Endo et al., 1996; Lu et al., 1996a; Lu et al.,

1996b; Teh et al., 2000). However, mRNA was not detectable in macrophages (Lu et al., 1996a) or monocytic-derived dendritic cells (Hashimoto et al., 1999).
On the protein level M-ficolin is found on the surface of blood monocytes and pro-monocytic U937 cells (Teh et al., 2000). Tu et al. and Endo et al. confirmed expression in peripheral blood leucocytes in general and additionally demonstrated expression in the lung (Lu et al., 1996a; Endo et al., 1996). Overall, ficolins exists in two forms: a serum-type and a cell-associated form. Recent results showed that this classification is not that strict, especially with regard to human M-ficolin. M-ficolin was believed to be not in the serum. It was found to be a cell-associated lectin (Lu et al., 1996a; Endo et al., 1996). Recently however, it also was found in serum although in lower concentration compared to the „true“ serum-type lectins H- and L-ficolin (Honore et al., 2008; Wittenborn et al., 2010). Nowadays it is possible to identify lower protein concentrations with more specific and more sensitive reagents and instruments.
Liu et al. showed in 2005 that human M-ficolin is also a secretory protein (Liu et al., 2005b). Functionally recombinant human M-ficolin was shown to bind to substrates such as N-acetylglucosamine, N-acetylgalactosamine and sialic acid (Liu et al., 2005b; Frederiksen et al., 2005).
Human M-ficolin recognizes also sialic acid, like its mouse orthologue Ficolin B (Liu et al., 2005b). This is an interesting finding, because sialic acid acts as a receptor for molecules involved in regulation of cellular growth, differentiation, cell-cell-interaction and adhesion (Sotiropoulou et al., 2002; Holmskov et al., 2003).
In 2005, different groups were also able to identify binding of M-ficolin to bacteria such as Group B *Streptococcus* (GBS), *Staphylococcus aureus* or *Staphylococcus typhimurium* (Kjaer et al., 2011; Liu et al., 2005b; Frederiksen et al., 2005). So it is also under investigation whether M-ficolin serves as a lectin-like phagocytic receptor for pathogens (Teh et al., 2000). Responsible for the binding activity of ficolins is their fibrinogen-like domain. The other structural part of ficolin, its collagen like region, is necessary for interactions with MASPs in the complement cascade of the immune system. Human M-ficolin forms complexes with MASP-1 and MASP-2 and thereby activates the lectin pathway (Liu et al., 2005b). M-ficolin is also found associated in complexes with MAp19 (Matsushita et al., 2000; Matsushita et al., 2002; Liu et al., 2005b; Endo et al., 2005).

1.5.4.2.1.2 Human L-ficolin

A typical serum-type ficolin is the human L-ficolin encoded by the FCN2 gene. Other names are Ficolin-2, P35, EBP-37 or hucolin (Edgar, 1995; Endo et al., 2011).
Human L-ficolin was first purified from serum by Matsushita et al. (Matsushita et al., 1996).
As a humoral factor, L-ficolin is synthesized by hepatocytes and secreted into the blood circulation (Sorensen et al., 2005; Matsushita et al., 1996; Akaiwa et al., 1999; Yae et al., 1991). In the liver, L-ficolin has three splicing variants (Endo et al., 1996). Its smallest subunit is the monomer with a molecular weight of 35kDa (Matsushita et al., 1996) and its proposed structure is a tetramer consisting of four triple helices and 12 subunits (Hummelshoj et al., 2007). Each subunit contains a collagen-like strand (N-terminal) consisting of three L-ficolin chains and three C-terminal fibrinogen-like regions. These C-terminal recognition domains are necessary for binding to e.g. acetylated groups on microbial surfaces (Matsushita et al., 2002; Thiel, 2007).
L-ficolin is able to recognize acetylated groups, which is common to all ficolins (Kraruo et al., 2004; Faro et al., 2008; Krarup et al., 2008). More precise, substrates of L-ficolin are GlcNAc (Matsushita et al., 1996), β-1-3-D-glucan (Ma et al., 2004) and DNA (Kuraya et al., 2005; Jensen et al., 2007), both found on bacteria. For this reason bacterial targets for human L-ficolin were investigated. L-ficolin shows binding affinity towards *Salmonella typhimurium* TV119 (contains a lipopolysaccharide with a GlcNAc residue at the non-reducing terminus), *Escherichia coli*, and several serotypes of capsulated *Staphylococci* or *Staphylococcus pneumoniae*. So far it does not detect strains without a capsule, indicating that L-ficolin recognizes a pattern within this structure (Matsushita, 2010). Through its binding to the pathogen surface, L-ficolin leads to the activation of the lectin complement pathway. L-ficolin is also discussed to act as an opsonin to help to destroy invading pathogens (Matsushita et al., 1996).
Additionally to this lectin activity, human L-ficolin has been reported to be able to recognize elastin (Harumiya et al., 1995) and corticosteroids (Edgar, 2005).
L-ficolin shows a broad range of different substrates. Its binding ability is provided by four different binding sites, discovered and characterized by Garlatti et al. (Garlatti et al., 2007). Human L-ficolin forms complexes with MASPs and also MAp19 (Matsushita et al., 2000; Matsushita et al., 2002; Liu et al., 2005b; Endo et al., 2005). Besides activating the lectin pathway of the complement system by complex formation with MASPs, it was shown that human L-ficolin interacts also with C-reactive protein (CRP) and thereby activates the complement via the classical pathway in the absence of immunoglobulins. To do so special conditions of local

infection and inflammations are crucial (Zhang et al., 2009). During the last years studies revealed a correlation of L-ficolin deficiency with premature, low birth weight neonates and prenatal infections (Swierzko et al., 2009).

1.5.4.2.1.3 Human H-ficolin

Additional to the former two ficolins, in humans a third ficolin was characterized, which was found to be a pseudo-gene in other mammalians. Like human L-ficolin it is also a typical serum-type ficolin. H-ficolin was the first ficolin that was recovered already in 1978 by Inaba and Okochi (Inaba and Okochi, 1978). Human H-ficolin is also found under following names: ficolin 3, Hakata-Antigen or β2-thermolabile Macro glycoprotein. This protein was first observed during a reaction with an autoantibody in patients with systemic lupus erythematodes (SLE). Finally, 1998 human H-ficolin was cloned and characterized (Sugimoto et al., 1998).
Like all ficolins, also H-ficolins forms higher oligomers and the smallest subunit, called a monomer, appears at a size of 34kDa. The FCN3 gene encodes the protein and mRNA is expressed in the lung (Akaiwa, 1999) and in the liver (Kuraya, 2003).
Like all the other ficolins H-ficolin binds to GlcNAc, even if this association is very weak compared to the other serum lectin L-ficolin (Garlatti et al., 2007). D-fucose, galactose (Girija et al., 2011) and GalNAc are also substrates, but H-ficolin failed to bind to mannan or lactose (Sugimoto et al., 1998). Interestingly, the only bacterium found to be a target of human H-ficolin is *Aerococcus viridians* (Tsujimura et al., 2002).
Sugimoto et al. could show that H-ficolin is able to agglutinate human erythrocytes coated with lipopolysaccharide from different bacterias e.g. *Staphylococcus typhimurium*, *Salmonella Minnesota* and *Escherichia coli* (O111) (Sugimoto et al., 1998). H-ficolin activates the lectin pathway of complement by forming complexes with MASPs and with MAp19 (Matsushita et al., 2000, Matsushita et al., 2002, Liu et al., 2005b, Endo et al., 2005).

1.5.4.2.2 Mouse ficolins

In the mouse immune system, two ficolins help the body to destroy invading pathogens by the recognition of patterns on the microorganisms. In contrast to humans, in other mammalians only two ficolins exists named ficolin-A and ficolin-B. The orthologue to human H-ficolin was found to be a pseudo-gene.

1.5.4.2.2.1 Mouse ficolin-A

The orthologue to the human L-ficolin is mouse ficolin-A. This protein is also found in the serum and acts as a serum-type lectin, with mRNA expression in spleen and liver, but mainly in liver (Endo et al., 2004). Preferentially mouse ficolin-A protein exists in higher oligomers, mainly tetramers, consisting of 12 subunits (Ohashi and Erickson, 1998). Like all ficolins it has the capacity for binding to acetylated compounds such as GlcNAc (Fujimori et al., 1998, Endo et al., 2004). Due to the assumption that ficolin-A in mice is the counterpart to L-ficolin in humans, it is not remarkable that also the mouse lectin binds to elastin (Ohashi et al., 1998). Two forms of ficolin-A exist: ficolin-A and a variant form that is generated from the ficolin-A gene by alternative splicing. This variant form consists of a shorter collagen-like region and possesses a longer gap sequence (Endo et al., 2005).
Mouse ficolin-A acts as soluble pattern recognition molecule in serum by recognizing structures on pathogens and subsequently forms complexes with MASPs. This combination contributes to the activation of the complement lectin pathway.
Endo et al. demonstrate that also the variant form of mouse ficolin-A binds to MASP-2 and MAp19 (Endo et al., 2005). Endo et al. also shows that also ficolin-A, and its variant form exists in complexes with MASP-2 and MAp19 and is therefore also able to activate the complement system (Endo et al., 2005).

1.5.4.2.2.2 Mouse ficolin-B

The second ficolin in the mouse system is ficolin-B (FcnB). Like its human orthologue FcnB is also a more cell associated than a serum-type lectin. So far it has not been detected in serum.
The main expression site of mouse FcnB is the bone marrow. Even though the site of localization is identified, nearly nothing is known about this lectin and its function in the immune system remains unclear (Endo et al., 2005; Endo et al., 2004). Unlike its human orthologue M-ficolin, mouse FcnB expression is not found in peripheral leucocytes (Ohashi et al., 1998). Beside bone marrow, FcnB mRNA is also detected in the spleen. More precise, Endo et al. showed that FcnB production is mainly found in cells of the myeloid cell lineage in the bone marrow (Liu et al., 2005a). Runza et al. demonstrated in 2006 that FcnB protein is co-localized with LAMP-1 in lysosomes of activated macrophages (Runza et al., 2006).

Recombinant FcnB is able to recognize sialic acid, which it has in common with its human homologue M-ficolin. Only the non-serum-type ficolins (human M-ficolin and ficolin-B in other mammalians, e.g. mouse FcnB) possess this binding specificity.
Furthermore, mouse FcnB was reported to recognize and subsequently aggregate *Staphylococcus aureus* leading to enhanced phagocytosis by macrophages (Matsushita, 2010; Endo et al., 2007).
Even though a common function of all ficolins is their lectin activity and activation of the complement system through complex formation with MASPs, mouse FcnB does not seem to bind to MASP-2 (Endo et al., 2005). Comparing the collagen-like regions of MBL and the ficolins, it seems that mouse FcnB should be able to associate with MASPs. The mouse lectin is endowed with the necessary residues for this binding (Endo et al., 2005) because it has the MASP-2 binding motif. Endo et al. investigate in 2005 possible interactions between recombinant mouse FcnB and MASPs or MAp19. No complexes could be detected in their studies.

1.5.4.2.2.3 Mouse ficolin H

Like in other mammalians, with the exception of humans, the third ficolin turned out to be a pseudo-gene (Endo et al., 2004). The homologue genomic region to human H-ficolin in mouse is located on chromosome 4 (Endo et al., 2004). Until know, a functional protein has only been identified in humans (Endo et al., 2004).

1.3.1.3 Other lectins and collectins

One major group of soluble recognition proteins are the C-type lectins. A subgroup of these lectins are innate immune collectins or collagenous lectins. Collectins consists not only of a collagenous region but also of a carbohydrate-binding domain, which is calcium-dependent (Litvack and Palaniyar, 2010). This carbohydrate-binding domain is also called carbohydrate-recognition domain or CRD. They are further characterized by their multimerization.
Two well-examined collectins, beside MBL, are SP-A and SP-D. From their isolation from lung surfactant they received their names, surfactant protein A and D. Structurally SP-A consists of six units of a homotrimer (each of 35kDa) and thereby generates an octodecamer (“bouquet”). SP-D in contrast is much larger. Four units of homotrimers (43kDa) together form an X- or asterisk-shaped multimer (Litvack and Palaniyar, 2010).

Both surfactant proteins contribute also to the clearance of dying host cells (Wright 2005; Stuart et al., 2006; Thiel, 2007; Palaniyar et al., 2008). More precise: SP-A acts in binding to bacteria, viruses and apoptotic cells and enhances their phagocytosis by lung alveolar macrophages (Schagat et al., 2001; Vandivier et al., 2002; Gardai et al., 2003; Janssen et al., 2008). SP-D binds to apoptotic cells and it was shown that a further ligand of SP-D is genomic DNA. Binding is also observed to fatty acids and phospholipids (Ogasawara et al., 1992; DeSilva et al., 2003). SP-D also connects the innate immunity with the adaptive immunity via binding to the immunoglobulins IgG, IgE, secretory IgA and IgM (Kim et al., 2002; Nadesalingam et al., 2005). Sp-A and SP-D are, in contrast to MBL, not able to activate the complement system (Litvack and Palaniyar, 2010).
Some other collectins that have been studied are CL-P1, CL-L1 and CL-K1 (also named CL-11). They received their names because of their main expression site: in placenta (CL-P1), liver (CL-L1) and kidney (CL-K1) (Keshi et al., 2006, Thiel, 2007, Palaniyar et al., 2008).
Further collectins, typically found in bovine serum, are conglutinin, CL-43 and CL-46 (Thiel, 2007, Litvack and Palaniyar, 2010).

1.6 Aim of work

The aim of this doctoral thesis project was the characterization and functional analysis of mouse FcnB as a pathogen-recognition lectin.
Human ficolins are well characterized and also some functions could be identified. It has been shown that human L- and H-ficolin help in the clearance of dead cells by binding to apoptotic or necrotic cells and the initiation of their removal (Jensen et al., 2007; Kuraya et al., 2005). The most important role of ficolins is the activation of the complement system via the lectin pathway by binding to MASPs (Endo et., 2010; Endo et a., 2011; Matsushita 2010).
Since FcnB has not been studied extensively, the aim of this thesis was to identify the producer cells of FcnB and structurally and functionally characterize the FcnB protein.

2. Material and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Chemical/reagent	Manufacturer
6 x DNA-Loading buffer	New England Biolabs
β-Estradiol	Sigma
β-Mercaptoethanol for Molecularbiology	Sigma
β-Mercaptoethanol for Cell culture	Gibco
Agarose, LE for gel-electrophoresis	Biozym
AcBSA	Sigma
AcLDL	Biomedical Technologies
Biotin, NHS	Sigma
BSA (Fraction V)	Sigma
Chitin beads	New England Biolabs
CpG	Metabion
DAPI	Sigma
DEPC	Sigma
DMSO, steril	Merck
DMSO, non sterile	Sigma
DNA-Molecular weight-Standard 100bp	New England Biolabs
DNA- Molecular weight-Standard 1kb	New England Biolabs
EDTA	Sigma
Ethanol	Roth
Ethidium bromide solution	Promega
FCS	Sigma / PAN
Ficoll-Histopaque 1077	Sigma
Ficoll-Histopaque 1119	Sigma
fMLP	Sigma
Formaldehyd	Sigma
Glacial acid	Merck
Glutamin	PAN
Glycerin	Merck
Glycin	USB
HAT	PAN Biotech GmbH
HT	PAN Biotech GmbH
HEPES	Sigma
Hygromycin	PAN
IgG, rat	Sigma
IgG, mouse	Sigma
Imidazol	Sigma
Immersion Oil	Zeiss
Ionomycin	Sigma
Kaliumhydrogenphosphat	Merck

Chemical/reagent	Manufacturer
Kanamycin	Gibco
Labtek chamber II, Chamber slide	Nalge Nunc International
LPS *Escherichia coli* strain 0127:B8	Sigma
LPS *Salmonella abortus equi*	Prof. M. Freudenberg
Methanol	Merck
Milk powder	Real
Ni-NTA-Agarose	Qiagen
Nitrocellulose membrane 0.45µm (Dot Blot)	BioRad
NOWA solution A and B	MoBiTec
Nuclease-free Water	Marchery Nagel
Opti-MEM® mit GlutaMAX™	Gibco
PCR-Cycler, MyCycler thermal Cycler	BioRad
PCR-Cycler, ICycler thermal Cycler	BioRad
PBS	PAN
Penicillin/ Streptomycin	PAN
Paraformaldehyde	Merck
PMA	Sigma
Ponceau S Concentrate	Sigma
Power Pac 300 (Power Supply)	BioRad
Power Pac 1000 (Power Supply)	BioRad
Power Pac Basis (Power Supply)	BioRad
Precision Plus protein standard marker	BioRad
Protein marker VI	Applichem
Proteinase inhibitor	Roche
Protein-G-Agarose	Roche
RPMI-1640 medium	PAN
Rotiphorese Gel 30 (37;5:1)	Roth
Sodium acid	Merck
Streptavidin-HRP	R&D Systems
Streptavidin-HRP ultra sensitive	Sigma
Tetenal Roentoroll AC	Fischer Solutions
Tetenal Superfix Part 2	Fischer Solutions
Tetenal Superfix MRP	Fischer Solutions
Transfer-membrane Western Blot, 0.45µm	Immobilon miilipore
TRIS base/ultra	Applichem
TRIS-HCl	Merck
Trypan Blue	Sigma
Trypsin	PAN
Tween 20	Fluka

2.1.2 Consumables and equipment

Consumables/ equipment	Manufacturer
Accu-jet pro	Brand
Accuracy weighing machine, Iso 9001 CP2245	Sartorius
Agarose-Gel casting chamber	BioRad
Agarose-Gel-Electrophoresis Chamber Sub cell GT	BioRad
Agarose-Gel-Electrophoresis Chamber Mini Sub cell GT	BioRad
Agarose-Gel-Electrophoresis Chamber Wide Mini Sub cell GT	BioRad
Balance	Mettler PJ400
Balance, Kern EMB 200-1	Kern
Bio Photometer Eppendorf	Eppendorf
Bio Rad Econo System device	BioRad
Cell culture flasks	BD
Cell culture dishes	BD
Cell Counter, AC-12, Assistant	Assistant
Cell Safe Digitalkamera ColorView I	Zeiss
Cell strainer	BD
Cell scraper	Sarstedt
Centrifuge, Eppendorf 5417 R	Eppendorf
Centrifuge, Eppendorf 5415 D	Eppendorf
Centrifuge, Eppendorf, 5418	Eppendorf
Centrifuge, Eppendorf, Cooling, 5810 R	Eppendorf
Color View, Soft Imaging System Leitz, Diaplan, Leica-Microscope	Leitz, Diaplan
Confocal Microscope, FluoView FV1000	Olympus
Cuvette 10x4x45mm, Polystyrol/ Polystyrene	Sarstedt
Cytospin 4, Shandon	Thermo
Digital graphic printer UP-D895	VWR international / Sony
Dry Block Heating Thermostat, Bio TDB-100	Hartenstein
E-cups	Eppendorf
FACS LSR II Flow Cytometer	BD
FACS tubes	BD
Falcon tubes flat bottom 50ml	Sarstedt
Falcon tubes round bottom 50ml	Sarstedt
Falcon tubes 15ml	Sarstedt
Gel-documentation, Bio Imaging System,	Gene Genius Syngene
Microplate Reader	Molecular Devices, MWG-biotech Emax
Image Quant, LAS 4000 mini	GE Healthcare
Incubator, Heraeus BBD 6220	Heraeus
iCycler	BioRad
iQ5 Multicolor Real Time PCR Detection System	BioRad
Laminar Flow, Cell Safe	Integra Bioscience
Laminar Flow, Hera Safe	Thermo
MaxQ shaker	Thermo Scientific

Consumables/ equipment	Manufacturer
Microwave Inverter	Panasonic
Microscope Olympus CK 2, ULW LD 3.0	Olympus
Mithras, Multicolor Plate Reader LB940	Berthold technologies
Multi channel pipette 100µl	Eppendorf
Multi channel pipette 300µl	Eppendorf
MyCycler	BioRad
Nanophotometer	IMPLEN
Needle 20G ½, 0.9x40mm	BD
Needle 27G ¾, 0.4x19mm	BD
Neubauer Zählkammer	HBG
PCR Thermocycler	BioRad
pH-meter	Inolab
Pipette 0.5-10µl	Eppendorf
Pipette 2-20µl	Eppendorf
Pipette 10-100µl	Eppendorf
Pipette 100-1000µl	Eppendorf
Photometer, BioPhotometer	Eppendorf
Pipette tips (with filter)	Biozym
Pipette tips (without filter)	Sarstedt
Pipette 5, 10, 25 ml	Sarstedt
Refrigerator	Liebherr Gastroline
Polyporylen tubes for bacterial cultures	Sarstedt
Power Supply, Power Pack P25 T	Biometra
Power Supply, Microcomputer electrophoresis, Consort E425	Consort
Rotator Mixer, programmable, Multi RS-60	Talron Biotech
Syringe 1ml	BD
Syringe 5ml	BD
Syringe 10ml	BD
Thermal printer DPU-414	Eppendorf
Ultrasonic-waterbath SONOREX	Bandelin
Vortexer REAX 2000	Heidolph
Vortexer, Minishaker MS-2	IKA
Vortexer, Vortex Genie 2	Bender & Hobein AG
Waterbath, mini	Thermo Mix Bk Braun
Waterbath, Julabo TW20	Julabo
Whatman Paper	Hartenstein
X-ray film processor Optimax	Protec Medicine technique

2.1.3 Buffer and solutions

2.1.3.1 Buffer

1x Towbin/ SDS transfer buffer, pH 8.3

Tris	25 mM
SDS	0.02%
Glycine	192 mM
Methanol	20%
H_20	ad 1000 ml

5x Running buffer, pH 8.3

Tris base	120 mM
Glycine	950 mM
SDS	0.5%
H_20	ad 2500 ml

The Running buffer was stored at RT and diluted to 1x Running buffer with Millipore water before running the SDS-PAGE.

5x Sample buffer

Tris-HCl pH 6.8	312.5 mM
SDS	10% (w/v)
DTT	250 mM
Glycerol	50%
Bromophenol blue	0.05% (w/v)

10x TBS buffer, pH 7.3

Tris Base	3g
NaCl	8g
KCl	0.2g
H_20	ad 1000ml

Blocking buffer (Western blot/ Dot blot/ ELISA):

10% skim milk powder was diluted in TBS/ 0.05% Tween 20 with or without 5 mM Ca^{++} according to the experiment.

Blocking buffer (Confocal-microscopy)

Cold water fish gelatin	3%
Goat serum (heat inactivated)	5%
BSA	1%
Tween 20	0.25%

Reagents were diluted final in 1x PBS.

C4 buffer

Veronal Buffer Saline (VBS)	pH 7.4
$CaCl_2$	2 mM
$MgCl_2$	1 mM

Coating buffer pH 9.6

Na_2CO_3	15 mM
$NaHCO_3$	35 mM
H_2O	ad 1000 ml

Enhancement buffer

CH_3COOH	0.57%
Triton X-100	0.1%
PEG-6000	1%
NTA	15 µM
TOPO	50 µM

FACS buffer

1x PBS
2% FCS

Lämmli sample buffer

4x Lämmli buffer	20 ml
Tris-HCl pH 6.8	250 mM
Glycerol	40%
SDS	5%
Bromophenol blue	0.005%
H_2O	1.9 ml

Lämmli sample buffer was stored for long term at -20ºC. Right before use for sample preparation, 10% β-ME was added.

MASP-2 buffer

Tween 20	0.05%
EDTA	10 mM
NaCl	885 mM
Human IgG	100 µg/ml

Dilution was performed in 1x TBS.

Ponceau S red solution

A 5x Ponceau S red stock solution was obtained from Sigma (P7767). The solution was diluted 1:5 with millipore water and stored at RT.

RIPA-cell-lysis-buffer

Tris-HCl pH 7.5	50 mM
NaCl	150 mM
Nonidet P40	1%
Sodiumdesoxycholat	0.5%
SDS	10%

RIPA-buffer for cell-lysis was stored at RT. In general, 1x 10^6 cells are resuspended in 50µl RIPA-buffer. Before storage of the lysates at -80°C, a protease inhibitor was added at a final concentration 1:7.

SDS-PAGE-buffers

1 M Tris-HCl pH 8.8	250 ml	30.28g
1 M Tris-HCl pH 6.8	100 ml	12.11g

Stripping buffer

SDS	10%
Tris pH 6.8	12 M
H_20	ad 500 ml

Streptavidin-Europium buffer

Tween 20	0.05%
EDTA	25 µM

Dilution was performed in 1x TBS.

Veronal buffer Saline (VBS), pH 7.4

Barbital	4 mM
NaCl	145 mM
NaN_3	0.02%
H_20	ad 500 ml

2.1.3.2 Solutions

2x Freezing medium

DMSO	20%
FCS	80%

50-fold HAT

Hypoxanthine	5 mM
Aminopterin	0.02 mM
Thymidine	0.8 mM

50-fold HAT was diluted in RPMI 1640 medium, 10% FCS, 100 U/ml Penicillin/Streptomycin and 50 µM β-ME.

50-fold HT

Hypoxanthine	5 mM
Thymidine	0.8 mM

50-fold HT was diluted in RPMI 1640 medium, 10% FCS, 100 U/ml Penicillin/Streptomycin and 50 µM β-ME.

Coomassie-staining solution

Glacial acetic acid	10%
Methanol	40%
Coomassie Brilliant blue R250	0.2 %
Millipore water	49.8 %

Coomassie-destaining solution

Glacial acetic acid	10%
Ethanol	40%
Millipore water	50%

Trypan-blue solution, pH 7.4

Trypan-blue	0.16% (w/v)
NaCl	150 mM
H_20	ad 1000 ml

2.1.4 Molecular weight markers

(I) Precision Plus Protein All Blue Standard from BioRad. Representative lot of blue pre-stained Precision Plus Protein standard on a 4–20% Gel ™ Tris-HCl Criterion.

(II) Protein marker VI (10-245) pre-stained from Applichem (product code: A8889). Representative lot of dual-color Protein standard is shown on a 4–20% Gel ™ Tris-Glycine, in a Bis-Tris 10% MOPS buffer or in Bis-Tris 10% MES buffer.

2.1.5 Kits

Diff-Quick Differential staining Set (Dade Behring, Eschborn)

Performed for differential morphological cellular analysis by light microscopy (Leica Microscope, Leitz).

DOTAP liposomal transfection reagent (Roche)

Used for liposome-mediated DNA transfer into eukaryotic cells (e.g. insect cells). In this case, it is a transfection mediated by cationic liposomes.

BCA Protein Assay Kit (Pierce/Thermo Scientific)

For determination of protein concentrations after affinity chromatography purification, of monoclonal antibodies or recombinant proteins. For the protein quantification a BSA standard curve was used. This assay is based on the reduction of Cu^{2+} to Cu^{+} in an alkaline environment and the ability of bicinchoninic acid (BCA) to detect Cu^{+} (Smith et al., 1985).

“Pure yield TM Plasmid Miniprep DNA Purification” Kit (Promega)

Used for DNA isolation and purification from *E. coli* cultures.

RNA Isolation-Kit NucleoSpin ® RNA I Kit (Marchery-Nagel)

Used for RNA isolation from Hoxb8-neutrophils, PMN, BMDM, BMDC, and spleen cells. RNA is very sensitive to degradation. This has to be prevented during the isolation process. During the isolation the cells are lysed by incubation in a solution, containing large amounts of chaotropic ions. The advantage of this lysis buffer is that RNases are immediately inactivated. An rDNase digestion removes contaminating DNA, which is bound to the silica membrane. Finally pure RNA is eluted with RNase-free water and the eluate is stored at -80° C for long-term-storage.

2.1.6 Nucleic acids: Oligonucleotides

All primers were purchased from Metabion at a concentration of 100 pmol (100µM), and stored at -20°C in aliquots of 10 pmol until use. The primers used in this work are listed below:

Name	Sequence	Amplicon
RT-PCR FcnB Forward RT-PCR FcnB Reverse	5'- TCA TAC AAG AGG GGC TTT GG-3' 5'- GCC GTC ATT GTC TTG GTC -3'	230 bp
18S Primer Forward 18S Primer Reverse	5'- GTA ACC CGT TGA ACC CCA TT -3' 5'- CCA TCC AAT CGG TAG TAG CG -3'	180 bp
Oligo dT18-Primer	5'- TTT TTT TTT TTT TTT TTT -3'	-

Tab. 1: Overview RT-PCR primers.

2.1.7 Antibodies

2.1.7.1 Primary antibodies for Western blot/ Dot blot/ ELISA

Name	Clone	Species	Modification	Producer
Anti-V5	-	Mouse	Unlabeled	Invitrogen
Anti-V5-HRP	-	Mouse	HRP	Invitrogen
Anti-mouse FcnB moAb	2F1B6B6	Rat	Unlabeled	This work
Anti-mouse FcnB moAb	2F1B6B6	Rat	Biotinylated	This work
Anti-mouse FcnB moAb	2F1B6E2	Rat	Unlabeled	This work
Anti-mouse FcnB moAb	2F1B6E2	Rat	Biotinylated	This work
Anti-mouse FcnB moAb	2F1C7G4	Rat	Unlabeled	This work
Anti-mouse FcnB moAb	2F1C7G4	Rat	Biotinylated	This work
Anti-mouse FcnB moAb	16D3D6	Rat	Unlabeled	This work
Anti-mouse FcnB moAb	16D3D6	Rat	Biotinylated	This work
Anti-mouse FcnB moAb	16G3C2	Rat	Unlabeled	This work
Anti-mouse FcnB moAb	16G3C2	Rat	Biotinylated	This work
Anti-mouse FcnB moAb	11A1A10	Rat	Unlabeled	This work
Anti-mouse FcnB moAb	11A1A10	Rat	Biotinylated	This work
Anti-mouse FcnB moAb	11A1B11	Rat	Unlabeled	This work
Anti-mouse FcnB moAb	11A1B11	Rat	Biotinylated	This work
Anti-mouse FcnB moAb	11A1C7A5	Rat	Unlabeled	This work
Anti-mouse FcnB moAb	11A1C7A5	Rat	Biotinylated	This work
Anti-mouse FcnB moAb	IA4	Rat	Unlabeled	Institute of Immunology Regensburg (Dr. V. Runza)
Anti-mouse FcnB moAb	IA4	Rat	Biotinylated	Dr. V. Runza
Anti-mouse FcnB PoAb	-	Chicken	Unlabeled	Davids Biotech.

Name	Clone	Species	Modification	Producer
Anti-human MASP-2	6G12	Rat	Unlabeled	Hycult
Anti-human C4b	161-1	Mouse	Biotinylated	Immunolex
Anti-human C4b	161-2	Mouse	Biotinylated	Immunolex

2.1.7.2 Primary antibodies for FACS

Name	Clone	Species	Modification	Producer
Anti-mouse CD11b	M1/70	Mouse	PE	BD
Anti-mouse B220	RA3-6B2	Mouse	PerCP	eBioScience
Anti-mouse Ly6C	AL-21	Mouse	FITC	BD
Anti-mouse Ly6G	1A8	Mouse	eFluor450	BD

2.1.7.3 Primary antibodies for confocal microscopy

Name	Species	Modification	Producer
Lamp-1	Mouse	FITC	BD Pharmingen
Isotype-Lamp-1	Mouse	FITC	BD Pharmingen
Phalloidin	Mouse	RED	Invitrogen
Anti-elastase	Rabbit	-	Calbiochem
Anti-nucleosome	Mouse	-	BD Pharmingen
Lysotracker	Mouse	RED	Invitrogen

2.1.7.4 Secondary antibodies

Name	Epitope	Species	Modification	Producer
DyLight 488 anti-rabbit	Rabbit IgG	-	DyLight 488	Jackson ImmunoResearch
DyLight 549 anti-mouse	Mouse IgG	-	DyLight 549	Jackson ImmunoResearch
Goat anti rat IgG (WM)-HRP	Rat IgG	Goat	HRP	Sigma
Goat anti-rat IgG (y-chain)-HRP	Rat IgG	Goat	HRP	Biolegend
Goat anti-mouse IgG-HRP	Mouse IgG	Goat	HRP	Biozol
Sheep anti-rat IgG1	Rat IgG1	Sheep	-	Serotec
Sheep anti-rat IgG2a	Rat IgG2a	Sheep	-	Serotec
Sheep anti-rat IgG2b	Rat IgG2b	Sheep	-	Serotec
Sheep anti-rat IgG2c	Rat IgG2c	Sheep	-	Serotec
Sheep anti-rat IgM-HRP	Rat IgM	Sheep	HRP	Serotec
Goat anti-chicken-HRP	Chicken IgY	Goat	HRP	Abnova

2.1.7.5 Secondary detection reagents

Name	Modification	Producer
Streptavidin-Alexa 488	AF488	Invitrogen
Streptavidin-Alexa 546	AF546	Invitrogen
Streptavidin-HRP	HRP	R&D Systems
Streptavidin-HRP ultrasensitive	HRP	Sigma
Streptavidin-Europium	Eu^{3+}	Perkin Elmer
ToPro3	-	Invitrogen

2.1.7.6 Blocking antibodies

(I) Fc receptor Blocking: Rat anti mouse FcyR II/III (clone 2.4G2)

2.1.8 Bacteria

2.1.8.1 Pseudomonas aeruginosa

Name	Producer
Pseudomonas aeruginosa, PAO-1, GFP-tagged	Kindly provided by Dr. Analia Trevani (Academia Nacional de Medicina, Buenos Aires, Argentinien)

Bacteria were routinely grown at 37°C in Luria-Bertani (LB) broth or on LB agar.

2.1.8.2 Staphylococcus aureus

All *Staphylococcus aureus* strains were grown in Todd-Hewitt broth medium at 37°C for O/N. Bacteria were spun down at 2000g for 15min at 5°C and washed three times with PBS. The bacteria were fixed in 1% PFA for O/N or longer at 4°C. Residual aldehyde groups were blocked for 1h at RT with 0.1 M ethanolamine. Bacteria were again centrifuged at 2000g, 15min at 5°C. Washing was performed three times with TBS and 0.9% NaN_3 and stored at 4°C. Bacterial concentration was determined by reading the optical density at 600 nm. An optical density of 1 corresponds to 1.8 x 10^9 bacteria/ml (Krarup et al., 2005).

Serotype	ATCC number	Producer
T-1	12598	Inst. of Microbiology, University of Aarhus, Denmark
T-2	49519	Inst. of Microbiology, University of Aarhus, Denmark
T-3	12600	Inst. of Microbiology, University of Aarhus, Denmark
T-4	49520	Inst. of Microbiology, University of Aarhus, Denmark
T-5	49521	Inst. of Microbiology, University of Aarhus, Denmark
T-6	12603	Inst. of Microbiology, University of Aarhus, Denmark
T-7	49525	Inst. of Microbiology, University of Aarhus, Denmark
T-8	12604	Inst. of Microbiology, University of Aarhus, Denmark
T-9	12606	Inst. of Microbiology, University of Aarhus, Denmark
T-10	12607	Inst. of Microbiology, University of Aarhus, Denmark
T-11	12608	Inst. of Microbiology, University of Aarhus, Denmark
T-12	12609	Inst. of Microbiology, University of Aarhus, Denmark
T-13	12610	Inst. of Microbiology, University of Aarhus, Denmark

Tab. 2: Overview serotypes of *S. aureus* used for binding studies.

2.1.8.3 Group B *Streptococcus* (GBS or *Streptococcus Agalactiae*)

All Group B *Streptococcus* strains were grown in Todd-Hewitt broth medium at 37°C for O/N. Then the bacteria were spun down at 2000g for 15min at 5°C and washed three times with PBS. The bacteria were fixed in 1% PFA for O/N or longer at 4°C. Residual aldehyde groups were blocked for 1h at RT with 0.1 M ethanolamine. The Bacteria were again centrifuged at 2000g, for 15min at 5°C. Washing was performed three times with TBS and 0.9% NaN_3 and stored at 4°C. Bacterial concentration was determined by reading the optical density at 600 nm. An optical density of 1 corresponds to 1.8 x 10^9 bacteria/ml (Krarup et al., 2005).

Serotype	Strain	Producer
1a	090	Inst. of Microbiology, University of Aarhus, Denmark
1b	ss618	Inst. of Microbiology, University of Aarhus, Denmark
II	18 RS 21	Inst. of Microbiology, University of Aarhus, Denmark
III	3782	Inst. of Microbiology, University of Aarhus, Denmark
IV	3139	Inst. of Microbiology, University of Aarhus, Denmark
V	1169	Inst. of Microbiology, University of Aarhus, Denmark
VI	10214	Inst. of Microbiology, University of Aarhus, Denmark
VII	7271	Inst. of Microbiology, University of Aarhus, Denmark
VIII	22634/86	Inst. of Microbiology, University of Aarhus, Denmark
IX	534/04	Inst. of Microbiology, University of Aarhus, Denmark
(non-capsulated)	B848/64	Inst. of Microbiology, University of Aarhus, Denmark

Tab. 3: Overview serotypes of GBS used for binding studies.

2.1.9 Eukaryotic cell lines

2.1.9.1 Drosophila Schneider-2 (DS-2) cell line

For recombinant expression of mouse FcnB, the Drosophila Schneider 2 cell expression system from Invitrogen was used. This cell line was used for the overexpression of FcnB.

2.1.9.2 SP2/O-Ag14 myeloma cell line

As myeloma cells for the fusion with antibody producing plasma-B-cells, SP2/O-Ag14 myeloma cells were used (ATCC No. CRL 1581).

2.1.9.3 ERHoxb8 cells

This cell line was kindly provided by Dr. H. Häcker (Wang, G. G. et al 2006). The ERHoxb8 cells are progenitor cells when cultivated in estradiol-containing medium. After withdrawal of estradiol (also oestradiol), the cells differentiate within 3-4 days to mouse neutrophils.

2.1.9.4 RAW macrophages 264.7

These RAW cells (ATCC number; TIB-71™) are mouse macrophages which grow adherent in RPMI medium containing 10% FCS.

2.1.10 Animals

C57BL/6 mice and female rats (ACI, Wistar and Fischer 344) both aged 8-12 weeks were also obtained from Charles River. The animals were kept under conventional conditions with food and water *ad libitum* in the animal facilities of the University of Regensburg and treated in accordance with institutional guidelines and the German Federal Regulations of Animal Experimentation.

2.1.11 Software and Database	**Company**
Cell^F Imaging Software	OLYMPUS
FACS software: BD FACSDiva software and FlowJo software	BD
Graph Pad Prism version 4	Graph Pad Prism
iCycler Analysis Software, IQ5TMOptical System Software 2.0	BioRad
Image Quant Tl 7.0 Image Analysis Software	GE Healthcare
Microsoft Office 2008	Microsoft Office
MicroWin 2000 (Mithras)	Berthold
Software Fluoview 1000	OLYMPUS

2.2 Methods

2.2.1 DNA and RNA-based techniques

2.2.1.1 Isolation of genomic DNA from *E. coli*

For DNA isolation and purification “Pure yield TM Plasmid Miniprep System” from Promega was used according to the manufactures instructions.
Briefly, one day before the DNA isolation, bacterial cultures in LB-medium were prepared and inoculated with material of *E. coli* colonies. 600µl of bacterial culture were added to a 1.5ml microcentrifuge tube. To lyse the cells, 100µl of cell lysis buffer was added and the tube was mixed by inverting. Then 350µl of cold neutralization buffer were added and the suspension was centrifuged at maximum speed for 3min. The supernatant was transferred to a “Pure yield TM” mini column. The column was placed into a collection tube and centrifuged at maximum speed for 15sec. The flow-through was discarded and the column was placed back into the same collection tube. Then the column was washed: first with 200µl of Endotoxin Removal Wash (ERW) and centrifuged for 15sec and second with 400µl of Column Wash Solution (CWC) and 30sec centrifuged at maximum speed. For elution of DNA, column was placed into a clean 1.5 ml tube. 30µl of nuclease free water was added directly to the column matrix and incubated for 1min at RT. To finally elute DNA, column was centrifuged for 15 sec at maximum speed. DNA concentration was measured (see 2.2.1.2) and DNA stored at -20°C.

2.2.1.2 Quantification of nucleic acids

Purified DNA samples were measured at a wavelenght of 260nm (“BIO-photometer”, Eppendorf). In general, samples were diluted in a 1:50 ratio, with 2µl of probe to 98µl water. As a blank-control 100µl of water was used. A dilution was prepared in an eppendorf tube and then transferred to a photometer cuvette (10x4x45mm, Sarstedt). The DNA concentration was calculated according to the Lambert-Beer’s law where an optical density of 1 (OD260=1) corresponds to 50 µg of dsDNA/ml. The concentrations of RNA samples were measured at 280nm.

2.2.1.3 Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique used to enzymatically amplify a defined DNA sequence *in vitro* (Mullis, 1990). PCR reactions were carried out in a programmable thermocycler (BioRad iQ5 Thermo Cycler). In order to avoid DNA contamination, the different components of the reaction were pipetted with filtered tips.

2.2.1.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to analyse the PCR products after RT-PCR. In general, 1% agarose gels were prepared by dissolving agarose in 1xTAE buffer. RT-PCR products were diluted in 6x DNA loading buffer and loaded on the gels. Samples were separated according to their size at 100 Volts for 1h. Finally DNA bands were visualized by ethidium bromide staining under a 366 nm UV light.

2.2.1.5 RNA isolation

RNA isolation was performed with the NucleoSpin ® RNA I- Kit from Marchery-Nagel (Düren, Germany) according to the manufacture´s instructions.
The total RNA-isolation protocol in detail is as follows:

(I) Lyse cells by adding 350 µl of Buffer RA1, containing fresh added 3.5µl β-ME to the pellet and gently mix or vortex the tube.

(II) Filtrate lysate by filtration through a NucleoSpin ® Filter (violet ring). The filter is placed in a 2 ml collection tube and centrifuged for 1min at 11.000g.

(III) After centrifugation the filter is discarded and flow through is homogenized by adding 350µl of 70% and mixed by pipetting 5 times up and down.

(IV) Each sample is than loaded to a NucleoSpin ® RNA II Column (light blue ring) and placed in a 2 ml collection Tube.

(V) Columns are centrifuged for 30s at 11.000g. Column is afterwards placed in a new 2 ml collection tube.

(VI) Membrane is desalted by adding 350µl MDB (Membrane Desalting Buffer) and for 1min centrifuged at 11.000g to dry the membrane. Desalting step is important to make the following rDNase digestion step more efficient.

(VII) For DNA digestion first a mixture has to be prepared containing for each sample: 10µl rDNase and 90µl rDNase Reaction Buffer.

(VIII) Solution is mixed and 95µl is directly applied to the center of the silica membrane of the column. Incubation is performed at RT for 15min.

(IX) Membrane washes (3 times): first with 200µl Buffer RA2 to inactivate the rDNAse. Column is centrifuged for 30sec at 11.000x g and placed into a new 2 ml collection Tube.

(X) The second washing step is performed by adding 600µl of Buffer RA3 followed by a centrifugation step for 30s at 11.000g. The flow through is discarded and column is placed back into the collection tube.

(XI) Finally, 250µl Buffer RA3 are applied to the column and centrifuged at 11.000g for 2min. This step is recommended to dry the membrane completely. After centrifugation column is put into a nuclease-free 1.5 ml collection tube.

(XII) Isolated RNA is eluted in 60µl RNase-free water (or 40µl, if higher concentrations are desired) and centrifuged at 11.000g for 1min.

(XIII) RNA is stored at -80°C to keep it stable.

2.2.1.6 cDNA synthesis

Before performing RT-PCR analysis, isolated RNA has to be transcribed into cDNA („complementary DNA“). Reverse transcription is accomplished using the standard first-strand synthesis protocol from Promega (see 2.1.5).

In general, 500ng of RNA were transcribed to single-stranded cDNA. The maximum volume, however, is 15.75µl, in case that the concentration of RNA is not high enough. First appropriate amounts of RNA were mixed with 2µl of Oligo dT primer (Fermentas, Oligo dT 18) and filled up to 17.75µl with water. The samples were heated up for 5min at 70°C (denaturation and primer hybridization/annealing) and then cooled down on ice. 5µl of 5x MLV-buffer (Promega, M531A) were added to the reaction, 1µl of MLV enzyme (200 Units) and 1.25µl of dNTP-Mix (each 10µM). MLV-enzyme is a reverse trancriptase from murine leukemia virus. Samples were incubated for 1h at 40°C (elongation) and the reaction was stopped and enzymes were finally inactivated at 70°C for 15min. Finally, cDNA was placed on ice when directly used for RT-PCR analysis or stored at -20°C for long-term-storage.

2.2.1.7 RT-PCR

Real time PCR (RT-PCR) is a useful technique to amplify a gene of interest without introns. It is a good method to analyze specific mRNA transcript expression in a particular tissue or different cell types. Therefore, first RNA must be reverse transcribed into cDNA ("complementary DNA").
For mRNA expression analysis the thermocycler iQ5 multicolor real-time PCR detection system from Bio-Rad was used.

RT-PCR-reaction:

cDNA 1:5 dilution	5µl
RT-PCR Primer For (100µM)	0.1µl
RT-PCR-Primer Rev (100µM)	0.1µl
2x Sensi Mix*	12.5µl
Millipore water	7.3µl
	25µl

Tab. 4. Overview RT-PCR reaction mix.

* The 2x SensiMix Plus SYBR Kit & Fluorescein Kit from Quantace is a ready to use mix for Real time PCR, including the hot start Taq-polymerase, the fluorescein SYBR green I as an internal reference, dNTPs, magnesium chloride and stabilizers. The SYBR green mix was stored at -20°C. The RT-PCR-program is listed below.

Temperature	Time	Cycles	Procedure
95°C	7min		
95°C	15sec	45x	
60°C	30sec		Realtime
95°C	1min		
55°C	1min		
55°C	30sec		Meltcurve

Tab. 5. Overview RT-PCR program.

2.2.2 Cell culture techniques

2.2.2.1 Eukaryotic cells

2.2.2.1.1 Drosophila Schneider-2 cells (DS-2)

The Drosophila Schneider 2 (DS-2) cell line (Invitrogen; Catalog no. R690-07, Version F) is derived from a primary culture of late stage (20h to 24h) *Drosophila melanogaster* embryos (Schneider, 1972). Cells grow at 28°C without CO_2 in an incubator. In tissue culture flasks they build a semi-adherent monolayer, compared to a growth in suspension in shaking flasks.
DS-2 cells are used to produce recombinant proteins and therefore these cells are a good expression system.
Culture medium for DS-2 cells is commercially available (for example the “Insect Express Prime” from PAA (Catalogue no. E21-895), with 10% FCS and 100µg/ml kanamycin). The selection medium during transfection is as the culture medium, but with additional Hygromycin-B at a concentration of 300µg/ml. The induction medium for protein expression is as the selection medium, with 500µM final concentration of $CuSO_4$, but without FCS.

2.2.2.1.1.1 Thawing of DS-2 cells

When initiating the cell culture of DS-2 cells, a cryotube from the liquid nitrogen tank was quickly thawed and the cells were transferred to a 15 ml falcon tube, containing 9 ml pre-warmed (room temperature) culture medium. Cells were centrifuged at 1000g for 3min, supernatant discarded to remove the DMSO and the cells were resuspended in 5 ml culture medium and plated in a 6 well or a flask.
DS-2 cells were incubated at 28°C for 4 to 5 days, until they were 100% confluent.

2.2.2.1.1.2 Passaging of DS-2 cells

In general, the cell density is between 6x 10^6 to 20x 10^6 cells per ml. When passaging the cells, cells were collected by carefully pipetting them up and down to break up cell clumps and were then centrifuged at 1000g for 3min. Cells were split twice a week in a dilution of 1:5 or depending on cell growth. Hereby, the conditioned

medium was mixed with freshly prepared medium. Cells were always split into new wells or flasks.

2.2.2.1.1.3 Freezing of DS-2 cells

Before starting the freezing of DS-2 cells, cryotubes were kept on ice and labeled. Freezing medium contains 40% conditioned medium, 40% fresh culture medium, 10% DMSO and 10% FCS. In general 1x 10^7 cells are frozen per cryotube. Before freezing, cells were counted and the cell viability was determined. Cells were centrifuged at 1000g for 3min; the pellet was resuspended in fresh culture medium and the cell number was determined. Conditioned medium was saved after the centrifugation to prepare the freezing medium. Appropriate cell numbers were diluted in freezing medium and 1 ml suspension was transferred to each vial.
Cells were first slowly frozen down, by transferring the cryotubes to the -80°C freezer and 2 days later to the liquid nitrogen tank for long-term storage.

2.2.2.1.1.4 Transfection of DS-2 cells

For stable transfection of DS-2 insect cells, the liposomal transfection reagent DOTAP from Roche was used. Transfection of eukaryotic cells was mediated by cationic liposomes. For transfection, the DNA used for transfection should be of high purity and the cells to be transfected should proliferate well. It is recommended to passage cells the day before transfection. The cell density should be around 60-80% at time of transfection. For transfection, 5µg of DNA were diluted in HEPES-buffered-saline (HBS) buffer to a final volume of 50µl. In parallel, 30µl DOTAP were diluted in HBS to a final volume of 100µl. DNA and DOTAP solutions were mixed together and incubated 10-15min at RT. Meanwhile, the DS-2 cells were centrifuged for 3min at 1000g and resuspended in fresh culture medium. Then, the DNA-DOTAP-mixture was added directly to the cells to be transfected and incubated up to 72h. The transfection medium was replaced by the selection medium. The selection medium consists of normal culture medium, containing 0.3mg/ml antibiotic Hygromycin B.

2.2.2.1.1.5 Induction of protein expression

Protein expression was induced with 500µM $CuSO_4$, added to the culture medium. Normally, cells were induced for 5 to 7 days. Supernatants from differently transfected DS-2 cell clones can be tested in Dot blot or ELISA. If recombinant protein is produced, the expression can be detected with antibodies against the respective recombinant protein or against the V5-His-Tag. Also cells can be lysed in lysis buffer and the cell lysates checked in Western blot analysis. For collection of recombinant protein, DS-2 cells were centrifuged at 3000g for 5min and the supernatant was sterile-filtered and stored in flasks for later protein purification.

2.2.2.1.2 SP2/O-Ag14 myeloma cells

Myeloma cells (SP2/O-Ag14, ATCC No. CRL 1581) are used as fusion partner for plasma B cells to generate hybridomas for the production of monoclonal antibodies.
This myeloma cell line is a non-light chain-immunoglobulin-secreting line derived from a cell line created by the fusion of Balb/C mouse spleen cells with the mouse myeloma P3X63Ag8. These cells are resistant to 8-azaguanine (20□g/ml) and do not survive in media containing HAT. B-cells in contrast, are able to grow in HAT-medium, but they are not long-living cells. So, only fused cells with the capacity of an immortal myeloma cell and a cell able to grow in HAT will survive after fusion and during selection in HAT. Myeloma cells were cultured in RPMI medium, containing 10% FCS, 2 mM L-glutamine and were incubated at 37°C and 5% CO_2. For the culture, the cell density should be between $5x10^4$ and $5x10^5$ viable cells/ml. Some cells may attach, but in general SP2/O-Ag14 myeloma cells are suspension cells. Medium was changed every 2 to 4 days, depending on the cell density.

2.2.2.1.3 ERHoxb8 cells

ERHoxb8 cells are kept in a precursor state when cultured in medium containing β-estradiol. After withdrawal of estradiol the cells differentiate within 3 to 4 days to neutrophils. For culture the cells were grown in OPTIMEM medium from Gibco/ Invitrogen, supplemented with heat-inactivated 10% FCS, 1% Penicillin/ Streptomycin (Pen/Strep) and 30µM β-ME. Stem Cell factor was always added fresh (SCF; supernatant from transfected CHO-cells, produced in our laboratory) in a 1:25 dilution containing either 1µM estradiol (precursor cells) or not.

2.2.2.1.3.1 Thawing of ERHoxb8 cells

Before thawing of ERHoxb8 cells, complete medium was prepared and warmed up to 37°C temperature. Cells were thawn quickly in a water bath and cell suspension was transferred to a 15 ml falcon and slowly 9 ml of medium were added dropwise. After centrifugation for 5min at 300g, at 4°C, cells were resuspended in 3 ml medium and placed into a 6 well. On the next day 1 ml fresh medium was added. One day later, the cells were centrifuged again at 300g for 5min at 4°C and resuspended in 6 ml medium and seeded in two 6 wells.

2.2.2.1.3.2 Passaging of ERHoxb8 cells

ERHoxb8 cells were cultivated in 6 well plates and split every second day. The cell number was adjusted to $5x10^5$ cells per well. For differentiation, cells were centrifuged, washed two times in 1x PBS with 10% FCS and resuspended in medium without estradiol. Cells were counted and adjusted to the required cell number for the experiment.

2.2.2.1.3.3 Freezing of ERHoxb8 cells

Before freezing, the ERHoxb8 cells were split the previous day. Cells were centrifuged at 300g, 5min at 4°C, resuspended in medium and placed on ice. In general, $5x10^6$ cells were prepared for one cryotube. Cells were diluted in an equal volume of 2-fold freezing medium (20% DMSO, 80% FCS, SCF 1:25 and 1µM estradiol). Normally cells were first placed at -80°C for at least one day, before storage at -190°C in the liquid nitrogen tank.

2.2.2.1.4 RAW macrophages 264.7

RAW 264.7 cells are a mouse macrophage-like cell line. The culture medium was RPMI medium with 10% FCS and 0.1% gentamicin. RAW macrophages are adherent cells, cultivated at 37°C, 5% CO2 in T75-flasks. Every 2 to 3 days, the cells had been split 1:20 into new T75 flasks. For passaging, the old medium was removed and the cell layer washed once with 10ml cold PBS, by rinsing carefully the cell layer. The PBS was removed and the cells were detached by adding 1ml trypsin directly to the cells. The trypsin step was performed for 1-2min at RT and afterwards the trypsin was removed. Now, the detached cells were resuspended in 20ml RPMI

10% FCS medium. Cells were centrifuged at 300g, 8min at 4°C and the pellet was resuspended in 10ml medium. The cell number was determined and cells were distributed to new flasks when passaging or adjusted to the required cell number for the experiment. If cells were used in experiments, the detachment step with trypsin can be replaced by scraping the cells off.

2.2.2 Primary cells

2.2.2.1 Generation of BMDM

Bone marrow derived macrophages were generated according to the protocol by Lutz and Schuler, 1999 (Lutz and Schuler et al, 1999).
Briefly, mice were anesthetized with ether and killed. The skin was wetted with 70% ethanol and *femura* and *tibia* were isolated and cleaned from tissue. Bones were first incubated for 2min in 70% ethanol and then washed in PBS. The epiphysis was opened on both sides and the bone marrow was flushed out with 10 ml cold and sterile PBS, until the bone marrow was fully washed out (Syringe. Needle: 27G ¾, 0.4x19mm). The cell suspension was transferred to a falcon tube with cold 50 ml medium. Cells were filtered through a cell strainer, centrifuged for 10min at 300g and 4°C and washed once with culture medium (RPMI medium with 10% FCS, 1% Pen/Strep). After resuspension in culture medium, cells were counted and cultured in square plates (BD). In general, $1x10^7$ cells were seeded in one square plate with 20 ml medium.

Culture protocol for BMDM:

(I) **d0**: Cells were seed in a square plate with $1x10^7$ cells in 20 ml medium

(II) **d5**: The medium was removed and exchanged against fresh medium (thereby non-adherent cells were removed)

(III) **d6**: square plates were placed on ice. Medium was collected in a 50 ml falcon. The cell layer was washed with 10ml cold PBS and after a short incubation, the cells were carefully detached with a cell scraper. Cells were resuspended in the medium collected before. Afterwards, the cells were pelleted by centrifugation at 300g, 8min at 4°C. The supernatant was discarded and the pellet was resuspended in RPMI medium without FCS.

(IV) Cell numbers were determined and cells were cultured several days or the cells were prepared for experiments.

2.2.2.2 Generation of BMDC

Bone marrow derived macrophages were generated according to the protocol by Lutz and Schuler, 1999. Bone marrow cells were isolated as described previously.

Culture protocol for BMDC:

(I) **d0:** Cells were seed in a petri dish with $2x10^6$ cells per 10 ml medium

(II) **d3:** 10 ml fresh culture medium were added directly to the dishes

(III) **d6:** The medium was removed and exchanged against fresh medium (thereby non-adherent cells were removed)

(IV) **d8:** The plates were placed on ice and the medium was collected into a 50 ml falcon tube. The cell layer was washed with 10 ml cold PBS and after a short incubation the cells were carefully detached with a cell scraper. Cells were resuspended in the medium collected before. Afterwards, the cells were pelleted by centrifugation at 300g, 8min at 4°C. The supernatant was discarded and the pellet was resuspended in RPMI medium without FCS.

(V) Cell numbers were determined and cells were cultured several days or the cells were prepared for experiments.

2.2.2.3 Generation of peritoneal exudate macrophages (PEC)

Peritoneal exudate cells (PEC) were generated to use them as “feeder cells” after the fusion during the generation of hybridomas. “Feeder cells” were used because they provide conditioned medium for the hybridoma cells. They also phagocytose dead cells and particles after the fusion and stabilizes the hybridoma cells.
In general, the evening before the isolation of PEC, 1 ml sterile PBS was injected into mice with a 1 ml syringe and a needle size 27G ¾, 0.4x19mm. The next morning mice were killed and the peritoneum was flushed with 10ml cold RPMI medium containing 10% FCS pro mouse (needle: 20G ½, 0.9x40mm, yellow). The medium was recovered again with the syringe (needle: 20G ½, 0.9x40mm, yellow) and transferred into a 50 ml tube. Cells were centrifuged for 10min at 300g, washed twice with PBS/10% FCS and resuspended in an appropriate volume of medium. If cells were used for fusion they were resuspended in HAT medium. Cells were counted and the cell numbers were adjusted. For cell analysis, the cells were centrifuged onto a cytospin-slide at 600rpm, for 6min with low acceleration. Normally the yield of

isolated PEC was around 1x 10^6 cells per mouse. The next day sterility was checked before adding the hybridoma cells.

2.2.2.4 Generation of PMN from bone marrow

Neutrophils from mouse bone marrow were isolated according to the protocol by Ermert et al 2009 (Ermert et al 2009).

2.2.2.4.1 Ficoll-purification of PMNs

Polymorphonuclear neutrophils (PMNs) from Balb/c bone marrow were isolated over a Histopaque gradient. Thereby cell types were separated regarding to their size and density.
First, bone marrow cells were isolated and each cell preparation was resuspended in 3 ml RPMI-medium without FCS. In general, bone marrow cells from one mouse were loaded on one gradient.
Gradients were prepared as follows: (I) apply 3 ml of Ficoll-Histopaque (density 1.119; Sigma-Aldrich) into a 15ml tube. Slowly add 3 ml of Ficoll-Histopaque (density 1.077; Sigma-Aldrich) and then 3 ml cell suspension was carefully overlayed on the gradient. The gradient was centrifuged for 30min and 700g (without brake) at 4°C. After centrifugation, cell populations will be separated and distributed in two layers. The upper layer contained the mononuclear-enriched fraction and the lower layer contained the neutrophil-enriched population. This lower layer should be localized in the interface of the two Ficoll-Histopaque layers. The upper layer was aspirated with a glass pipette and the cells in the lower phase were carefully collected and transferred into a new 50 ml tube. Cells were washed two times with 10-20ml RPMI medium and centrifuged for 10min and 400g at 4°C. Afterwards, the cell pellet was resuspended in complete medium, e.g. RPMI medium with 10% FCS, for the experiment. For analysis and determination of purity of the isolated neutrophils, a cytospin and FACS-analysis were performed. Normally nearly 80% pure neutrophils are obtained.

2.2.2.4.2 Percoll-purification of PMNs

For PMN isolation and purification from Balb/c bone marrow, cells were isolated in the same way as with Ficoll-purification, However, the cell suspension was separated over a Percoll gradient. The gradient consisted of 3 layers differing in their Percoll-densities. In brief, cells were resuspended in RPMI medium with 5% FCS

and further given over a cell strainer and resuspended in HBSS (Hanks Balanced Salt Solution) medium. Cell purification was performed with the following gradient:

(I) 3ml of 78% Percoll solution (lowest layer)
(II) 3ml of 69% Percoll solution and /middle layer)
(III) 3ml of 52% Percoll solution (upper layer)

100% Percoll solution was prepared with 18 ml Percoll and 2 ml of 10x PBS. After centrifugation for 30min at 1500g without brake, the cells in the interphase of 69% and 79% Percoll were collected and washed with PBS and again centrifuged for 10min, 400g at 4°C. PMN purity was determined by FACS analysis staining for the neutrophil-specific marker Gr1.

2.2.2.4.3 FACS staining protocol for PMN analysis

Quality and purity of isolated PMNs were determined by FACS-analysis. After isolation, the cell number was determined and adjusted so that between $5x10^5$ to $1x10^6$ cells were transferred into a FACS-tube and pelleted for 5min at 300g at 4°C. Before the specific antibody staining, Fcy-receptors were blocked with rat anti mouse FcyR II/III, clone 2.4G2. One µl of antibody was diluted in 200µl FACS-buffer (1x PBS with 2% FCS). Blocking was performed for 30min, 4°C in the dark. Then the cells were washed once with FACS-buffer and centrifuged for 5min at 300g. Antibodies were diluted in FACS-buffer and staining was done for 30min, 4°C in the dark. After a final washing step, cells were resuspended in 200µl FACS buffer and directly analyzed or fixed with 2% PFA (paraformaldehyde), diluted in FACS-buffer. Fixed cells were stored at 4°C and analyzed within 1 week.

FACS staining protocol for PMN analysis

Antibodies:

CD11b-PE	diluted 1/200
B220-PerCP	diluted 1/600
Ly6 C-FITC	diluted 1/200
Ly6 G-V450	diluted 1/400

Antibodies were diluted in FACS buffer.

2.2.3 Mycoplasma test

Every eukaryotic cell line used in this work was periodically controlled for mycoplasma contamination. Cytospins from cells were stained with DAPI (1:100 dilution) and analyzed under the fluorescence microscope. Mycoplasma appeared under the microscope as small blue-fluorescent dots.

2.2.4 Generation of monoclonal rat anti mouse FcnB antibodies

2.2.4.1 Preparation of rat splenocytes for fusion

For isolation of rat spleen, rats were bled and killed. The spleen was removed under sterile conditions and cut into pieces with a scissor. Then the spleen pieces were gently strained through a metal strainer by using the stamp of a 5ml syringe. Flushing was performed with around 30 ml of RPMI-medium without FCS. After filtration, cells were centrifuged for 5min at 300g at 4°C. After two washing steps with 25ml RPMI medium, the cells were resuspended in RPMI medium and ¼ of the cells was used for the fusion. The rest was kept frozen in liquid nitrogen. In general, the ratio of spleen cells to myeloma cells was 3:1.

2.2.4.2 Fusion

The fusion was done to combine the characteristics of immortal myeloma cells with antibody-producing spleen cells to generate hybridoma cells. During fusion splenocytes were together with myeloma cells in a ratio 3:1. In this thesis, SP2/O-Ag14 myeloma cells (ATCC No. CRL 1581) were used. Both cell types were merged in a 15 ml tube and centrifuged at 300g, for 5min at 4°C. The following fusion steps include:

(I) Supernatant after centrifugation was removed thoroughly by aspiration
(II) Cells were incubated in a 37°C water bath, while rotating
(III) 1 ml of 37°C-prewarmed PEG was added to the cells, while rotating
(IV) Cells were incubated at 37°C for 1min while rotating
(V) 10ml RPMI-medium without FCS was added drop-wise within 5min at RT while gently shaking
(VI) The cell suspension was diluted with RPMI-HAT medium up to 30 ml

(VII) The cell suspension was distributed on 3x 50 ml tubes and filled-up with RPMI HAT medium to 50 ml total volume each

(VIII) Fused cells were carefully transferred to 15x 96 well microtiter plates, containing PEC as “feeder cells”

Additional, one row contained $5x10^4$ SP2/O myeloma cells as a negative control. These cells should die within a few days, because they are not able to grow in HAT-medium.

2.2.4.3 Maintenance and expansion of hybridomas

Hybridomas were tested in ELISA for specific antibody production. Positive clones were sub-cloned and further tested. When clones remained positive in ELISA, they were cultured and transferred from 96-well plates to larger vessels and later to flasks. For antibody production and collection, the FCS percentage in the culture medium was reduced from 10% to 2%. After a few days, the conditioned medium was collected by centrifugation (300g, 8min, 4°C) and sterile-filtered into flasks until the antibody purification on protein G sepharose.

2.2.4.4 Sub-cloning by dilution

This technique was used for the isolation of single cell clones of the hybridomas. Sub-cloning should be repeated several times, until the arising clones are likely to originate from one single antibody-producing cell. Sub-cloning was performed in a 96-well microtiter-plate. First, 100µl of culture medium was transferred in every well, except the first well (A1) by using a multichannel-pipette. Then, 200µl cell suspension of positive clone was pipetted to well A1. In general, the cell number of the hybridomas that should be subcloned was adjusted to about 100 cells. Dilution serie was performed from A1 to H1, while gently pipetting each step log 2. Second log 2 dilution series was performed with an 8-channel-pipette from row 1 to row 12. Finally, each well should contained total volume of 100µl. The plate was kept at 37°C, 5% CO2. On the next day, cells were fed by adding 100µl/well culture medium. After 4 to 5 days, clones growing should be visible under the microscope. Supernatants of clones should be tested for example in ELISA for specific antibody production.
Subcloning should be repeated with positive candidates until monoclonality is reached.

2.2.4.5 Freezing hybridomas

Hybridoma cells and corresponding subclones were frozen for long-time storage in liquid nitrogen. For this, first the cell number was determined by counting them with trypan-blue staining. Then, 0.5 - 1x 10^7 cells/ml were resuspended in fresh medium (RPMI medium with 10% FCS) and diluted 1:2 in 2x freezing medium. Sample preparation was performed on ice.

2.2.5 Bacterial binding studies

To test whether recombinant mouse FcnB binds to bacteria, different serotypes from Group B *Streptococcus* and *Staphylococcus aureus* were tested.
10^9 bacteria per 100µl TBS/ 0.05%Tw/ 5 mM Ca^{++} buffer were incubated with 5µg recombinant mouse FcnB-V5-His protein in a final volume of 200µl. Samples were incubated for 4h at RT while rotating. After centrifugation (10.000g, 10min, 4°C), unbound recombinant FcnB in the supernatants was quantified with a TRIFMA assays. A five-step tenfold dilution of 10^9 to 10^5 bacteria was made for each bacteria type. The bacteria were tested as described above. Additionally, 5µg recombinant FcnB protein was treated in the same way, but without adding bacteria, to serve as a positive control (= 100% unbound recombinant FcnB).

2.2.6 Cytospin

A simple method to analyze cells (for example after isolation from mouse) was the cytospin analysis by "May-Grünwald-Giemsa-staining". Here, a cell suspension was centrifuged onto a slide and further analyzed under the microscope. First, the cell number was adjusted to around 6x10^5 cells/ml suspension in medium containing 50% FCS. For each slide, a total volume of 100µl was used. Slides were centrifuged for 6min at 600 rpm (low acceleration) and than air-dried before staining. Steps of Diff-Quick-staining were performed as following: (I) 5 times in the fixation solution, (II) 8 to 10 times in the eosin solution, (III) 5 times in methylen blue and (IV) slides were briefly and carefully rinsed with water and dried again. Cell analysis was performed on a Leica microscope under immersion oil (Leitz, Diaplan).

2.2.7 Protein-biochemical techniques

2.2.7.1 Protein purification

2.2.7.1.1 Purification of recombinant FcnB by chelating sepharose

In a previous work, mouse FcnB was cloned into a vector for expression in insect cells (pMTA/BiP/V5/His-A) after transfection of DS-2 cells. This vector contains the protein under regulation of a cupper-inducible promotor, in addition also a vector for later selection was co-transfected. After transfection and selection, clones positive for the expression of recombinant FcnB-V5-His protein were cultured. Afterwards, protein expression was induced by addition of copper (0.5 mM $CuSO_4$) to the medium. Induced supernatant, containing the recombinant protein is collected and sterile filtered. The supernatant is then transferred to a chelating sepharose fast flow column (GE Healthcare GmbH). After binding of the protein in the supernatant to the column, washing steps were performed: (I) three times with washing buffer 1 (0.02 M Na_2HPO_4, 0.5 M NaCl, pH 7.5) and (II) three times with washing buffer 2, to remove unspecifically bound proteins (10 mM Imidazol, 0.02 M Na_2HPO_4, 0.5 M NaCl, pH 7.5). Elution of bound protein is performed with increasing concentrations of imidazole (ranging from 10 mM to 250 mM) in the elution buffer. Elution fractions were collected in 0.5 ml aliquots and analyzed by SDS-PAGE for the presence of the recombinant protein. Equivalent fractions were pooled and dialyzed O/N against 1x TBS buffer. Protein concentrations are measured with PIERCE BCA Kit.

2.2.7.1.2 Purification of monoclonal antibodies by protein-G sepharose

In general, antibodies were purified using 2 ml protein-G sepharose, packed into a column and attached to the Bio Rad Econo System device. Before loading the column with the antibody solution, the resin was pre-equilibrated. A four-fold volume of binding buffer was passed through the column with a flow rate of 0.5 ml/min. Then the antibody solution was loaded onto the protein G sepharose column at the same flow rate. Non-specifically bound proteins were washed off with binding buffer until a baseline UV absorbance was reached monitored at 280 nm.
Elution was performed at 0.5 ml/min with elution buffer (0.1 M glycine pH 3.1) and samples were collected in 1.5 ml Eppendorf tubes, in 1 ml fractions. Immediately after elution, samples were neutralized with 1M Tris pH 11. Afterwards, purified antibody fractions were analyzed on SDS-PAGE. Corresponding fractions were

pooled and dialyzed against TBS O/N at 4°C, while swirling. Protein concentrations of dialyzed samples were determined using a PIERCE BCA kit. Aliquots of antibodies were stored for long term at -20°C. To re-use the protein-G sepharose column, the resin was regenerated by washing first with 6 M Urea, washing and flushing with 20% ethanol and stored.

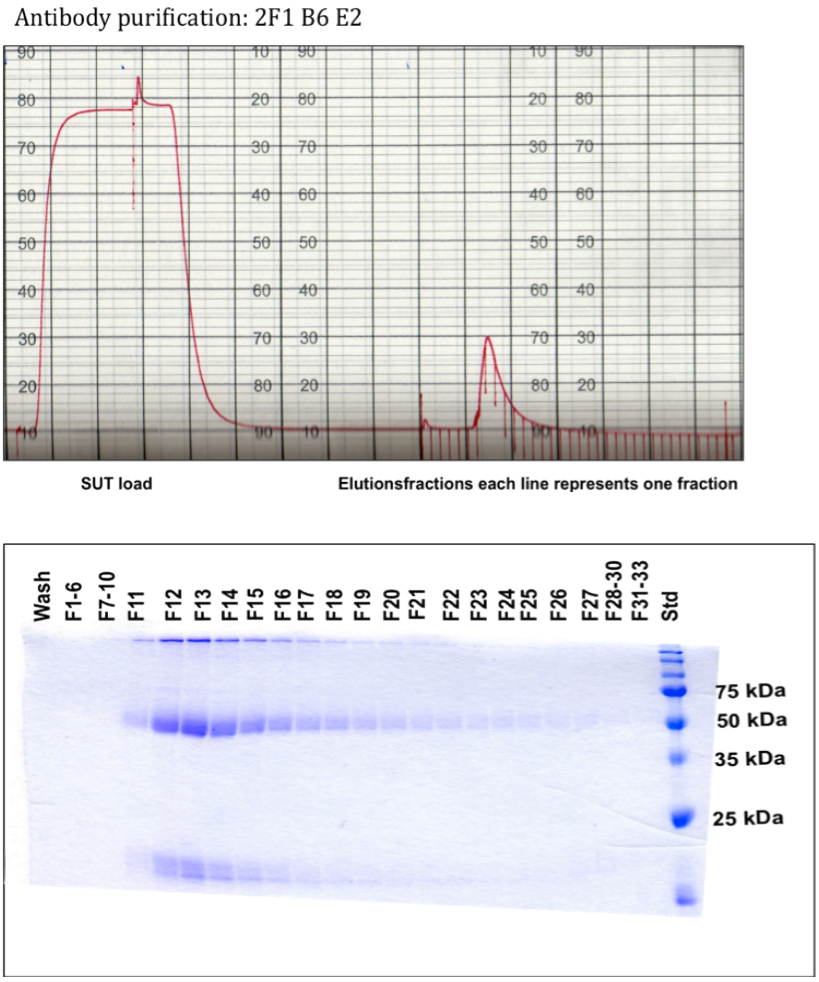

Figure 14: Protein purification of one monoclonal rat-anti-mouse FcnB antibody.
Representative, the elution profile of the purification of the antibody 2F1 B6 E2 is shown. Elution fractions were loaded on a SDS-PAGE gel and stained with Coomassie blue.

2.2.7.2 Preparation of the protein lysates

For preparation of protein lysates from cells, the cells were collected and washed by a centrifugation step at 300g, for 8min at 4°C. The supernatant was discarded and the cells were resuspended in 2 ml PBS. The cell number was determined by trypan blue exclusion staining. The cell suspension was transferred to a microcentrifuge

tube and centrifuged for 5min and 14.000 rpm at 4°C. For cell lysis, $1x10^6$ cells were resuspended in 50µl RIPA buffer (containing protease inhibitor). The lysates were stored at -80 °C. Before application, the protein concentrations of the lysates were determined with Pierce BCA kit.

2.2.7.3 Determination of the protein concentration

For determination of the protein concentrations, the protein Assay Kit "BCA Protein Assay Reagent (bicinchoninic acid)" from Pierce was used. First, 10µl of each standard (BSA standard curve) or unknown sample were pipetted in duplicates into a 96 well microtiter plate. Then 200µL of the WR (working ratio: 50 parts solution A to 1 part solution B) was added to each well and carefully mixed by pipetting before the plate was incubated at 37°C for 30min. After incubation, the plate was cooled down to RT. Finally the absorbance was measured at 562 nm wavelength on a plate reader and the protein concentration was calculated with the BSA standard curve.

2.2.7.4 SDS-PAGE

10% Separation gel (10 ml total: for 2 mini gels, each 1mm or one large gel)

Substance	Volume [ml]
1M Tris-HCl pH 8.8	3.75
30% Acrylamide	3.33
Glycerol	0.574
H_2O	2.2
10% SDS	0.1
10% APS	0.05
Temed	0.01

4% Concentration gel (5 ml total: for 2 mini gels, each 1 mm)

Substance	Volume [ml]
1 M Tris-HCl pH 6.8	0.625
30% Acrylamide	0.65
H_2O	3.65
10% SDS	0.050
10% APS	0.025
Temed	0.005

2.2.7.5 Coomassie staining

SDS-PAGE and membranes after western blotting were stained with brilliant blue Coomassie staining solution. In general, the gel or membrane was incubated in Coomassie-solution for 1h at RT, then de-stained and finally dried under vacuum conditions. This method is useful for checking of the protein loading.

2.2.7.6 Biotinylation of antibodies

To enhance the detection signals of antibodies, the strong binding of biotin to Streptavidin was used. Thereby, biotinylated antibodies are detected with an HRP-coupled-Streptavidin reagent. First, antibodies were diluted in PBS. Around 200µg of the antibodies were diluted in a total amount of 500µl PBS and dialyzed against PBS O/N at 4°C under rotation. Then the antibodies were dialyzed against PBS pH 8.5 (adjusted with 0.4 M KH_2PO_4) to provide binding conditions for biotin. A second dialysis was performed for 2h at RT, while rotating.
The antibody solutions were placed from the dialysis pipe to blocked microcentrifuge tubes. These tubes were normally blocked with 1x TBS/Tw and later washed 3 times before using. In blocked microcentrifuge tubes, the antibodies were incubated with biotin (NHS, Sigma) for 4h at RT. For this purpose 33.4µl NHS-Biotin in DMSO were adjusted to 200µg antibody. After biotinylation, the biotinylated antibodies were dialyzed against 1x TBS with 0.1% (15mM) azide. The first dialysis was performed O/N at 4°C and the next day the dialysis was repeated against TBS two times for shorter time intervals. The concentration of the biotinylated antibodies was determined and then the biotinylated antibodies were stored at 4°C.

2.2.8 Immunoassays

2.2.8.1 Western blot

Blotting:

For Western blot analysis first the proteins were separated according to their molecular sizes on SDS-PAGE. Blotting materials were prepared: **(I)** PVDF membrane 6x8.5 cm, **(II**) 3x3 whatman paper (7x9cm) and **(III)** the stacking gel from SDS-PAGE was cut off and the gel was incubated in transfer buffer. To activate the

membrane, transfer-membrane from Immobilon was incubated for 3min in methanol. The activated membrane, the gel and the whatman papers were soaked in transfer buffer for 10 to 15min at RT. The blotting order was as shown in Fig. 15.

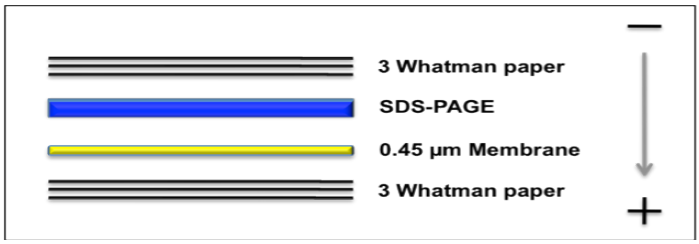

Figure 15: Schematically arrangement for Western blot analysis.

Blot conditions: 1h with 25 Volt constant in 1x transfer buffer. After blotting, efficiency was tested with Ponceau S red staining. Additionally, the SDS-PAGE was stained with Coomassie after blotting to check transfer efficiency.

Ponceau S red staining:

After blotting, the membrane was incubated for 2min in Ponceau red dilution (1:5 in water). For this the protein-covered membrane site was placed upside down into staining solution. Afterwards, the membrane was successively de-stained with distilled water. Protein loading was checked and then the membrane was blocked before incubation with the antibody.

Blocking:

Membrane blocking was normally performed at RT for 1h in 10% skim-milk powder, 1xTBS/ 0.05% Tw/ 5 mM Ca^{++}. Non-specific binding sites were blocked.

Antibody staining:

After blocking, the membrane was exposed to the antibody diluted in 3% skim-milk powder/TBS/Tw/Ca^{++} for 1h at RT or O/N at 4°C. The membrane was washed 3 times with TBS/Tw. Each washing step was performed for 10min. The secondary antibody-HRP was diluted in 3% skim-milk powder/TBS/Tw/Ca^{++} and the membrane was incubated for 45min at RT with the secondary antibody solution. In the case that the primary antibody was biotinylated, the secondary Streptavidin-HRP was diluted in PBS/ 1% BSA and incubated for 30min at RT. Washing steps were performed

between both antibody-exposures and afterwards (TBS/Tw, 3 times, each 10min, at RT). For development, ECL-Substrate from NOWA Solution A and B (MoBiTec) was used at a ratio 1:1. Incubation was performed for 1min at RT, while shaking. Exposure was done for 1-10min, depending on the signal obtained.

Membrane stripping:

For western blot analysis, the membrane can be developed successively with different antibodies. Therefore, antibodies used first have to be removed. This process is called “membrane stripping”.
First, the membrane was incubated in 99% ethanol for 15min under rocking conditions. Then, the membrane was incubated for 35min at 57°C in a water bath in the stripping buffer. Afterwards the membrane was washed 3 times for 5min with TBS/Tw and blocked again with milk powder, before the membrane was incubated with the next antibody.

Membrane staining:

After the western blot was developed, the membrane was stained with brilliant blue Coomassie staining solution. In general, the membrane was incubated in Coomassie-solution for 1h at RT. Then the membrane was de-stained. When successfully de-stained and when the background was clear, the membrane was washed in water before drying it.

2.2.8.2 Dot blot

Dot blot is a method for detection of proteins by antibodies in their native (not denatured) form on a nitrocellulose membrane. For this, the samples were dotted on the membrane and dried. If the protein sample contains the specific antigen, recognition by the antibody results in a detectable signal. For more detailed analysis of the antigen, concerning the size, a western blot analysis is indispensable.

2.2.8.3 Enzyme-linked immunosorbent assay (ELISA)

This method is also based on the binding between antigens and antibodies. The interaction is detected by an enzymatic color reaction. Three different kinds of ELISA-application are known:

(I) Antibody capture assay, where an antibody is coated to „fish“ appropriate antigen from a protein solution
(II) Antigen capture assay, for example to test antibodies regarding to their specificity and quality
(III) Sandwich assay, based on two antibodies: one capture- and one detection antibody, to make the ELISA system more sensitive

In general, ELISAs were performed in 96-well microtiter plates. Samples were directly (coating) or indirectly added to the plate. Unbound protein was washed away and an enzymatic color switch, induced by binding of enzyme-linked secondary antibodies, determined bound protein. Mainly, these secondary antibodies were coupled to horseradish-peroxidase.

2.2.8.3.1 Antigen-capture-ELISA for determination of serum titers and for hybridoma selection

(I) Coating of recombinant mouse FcnB in Coating buffer is performed O/N, 4°C
(II) Unspecific binding sites are blocked with 10% skim-milk powder in TBS/Tw for 1h at RT
(III) Samples (e.g. serum or supernatant) are transferred at different dilutions to the plate and incubated O/N at 4°C
(IV) Wells are washed 3 times with TBS/Tw
(V) Secondary goat anti rat IgG-HRP is added for detection 1h, RT
(VI) Wells are washed 3 times with TBS/Tw
(VII) ECL-solution is added (NOWA solution A and B (ratio 1:1) for development
(VIII) Reaction is stopped with 2N H_2SO_4
(IX) Plates are measured at 450nm

2.2.8.3.2 AcBSA-binding assay

Microtiter wells were coated with 10µg/ml AcBSA in Coating buffer O/N at 4°C. After blocking residual binding sites, serial dilutions of recombinant FcnB diluted in TBS/Tw/Ca or TBS/Tw/EDTA buffer were added and incubated O/N at 4°C. After washing with TBS/Tw, the wells were incubated O/N at 4°C with the monoclonal rat anti-FcnB antibody IA4 (3µg/ml, TBS/Tw/Ca), washed with TBS/Tw, and the secondary antibody goat-anti-rat IgG (Y-chain-specific)-HRP (1:3000 diluted in TBS/Tw) was added for 1 h. After washing, HRP substrate (1:1 diluted, NOWA Solution A and NOWA Solution B from Mo Bi Tec) was added, the reaction was stopped with 2N H_2SO_4, and the absorption was measured at a wavelength of 450 nm.
To assess whether FcnB exhibits calcium-dependent binding to AcBSA, the FcnB samples were diluted in TBS/Tw buffer with 10 mM of calcium chloride or 50 mM EDTA and tested in the ELISA.
To analyze whether binding of recombinant FcnB to AcBSA is inhibited by DNA, the ELISA was performed with FcnB samples pre-incubated with or without genomic DNA from *E. coli* as described above (10 or 25 µg/ml in TBS/ 0.05% Tw/ 5 mM Ca^{++}).

2.2.8.4 Time-resolved immunofluorometric assay (TRIFMA)

The TRIFMA assay is based on europium bound to streptavidin. When the immunometric assay has been completed the europium is dissociated from the streptavidin at low pH (by adding the Enhancement buffer) and measured by time-resolved fluorescence (TRF).
First, microtiter wells were coated with 10µg/ml AcBSA in coating buffer O/N at 4 °C. After blocking residual binding sites, protein samples were diluted in TBS/Tw buffer and incubated O/N at 4 °C. After washing with TBS/Tw, wells were incubated O/N at 4°C with primary antibody. For this, the primary antibody was biotinylated and can be detected with streptavidin-conjugated Europium (1:1000; 100ng/ml). Europium-streptavidin was incubated for 1h at RT, and washing steps follow. Before the measurement, 200µl of the enhancement-solution were added and incubated for 5min at RT, while shaking. Signals were measured with the DELFIA fluorometer multilabel counter (Victor 3 1420, Perkin Elmer) as Counts/sec.

2.2.8.4.1 Sandwich TRIFMA

Microtiter wells were coated with the primary antibody („capture“ antibody) in Coating buffer O/N at 4 °C. After blocking of residual binding sites, the antigen was added in serial dilutions in TBS/Tw buffer and incubated O/N at 4 °C. After washing with TBS/Tw, the wells were incubated O/N at 4°C with another antibody („detection“ antibody). The „detection“ antibody was biotinylated and can be detected with streptavidin-conjugated Europium (1:1000, 100ng/ml). Europium-streptavidin was incubated for 1h at RT and washing steps follow. Before the measurement, 200µl of the enhancement-solution were added and incubated for 5min at RT, while shaking. Signals were measured with a fluorometer (Victor 3 1420 Multilabel Counter, Perkin Elmer) as Counts/sec.

2.2.8.4.2 MASP-2 binding Assay

(I) Coating 10µg/ml AcBSA with 100µl/well of a 96-well microtiter-plate in coating buffer, O/N at 4°C

(II) Blocking was performed with TBS/Tw, 1h at RT

(III) The (recombinant) ficolin was added in TBS/Tw/Ca^{++} to plate and binding to AcBSA was allowed O/N at 4°C

(IV) The plate was washed 3 times with TBS/Tw/Ca^{++}

(V) Recombinant human MASP-2 was added (500ng/ml) and incubated O/N, 4°C

(VI) The plate was washed 3 times with TBS/Tw/Ca^{++}

(VII) The biotinylated anti-MASP-2 antibody (8B5) was added and incubation was performed O/N at 4°C

(VIII) Washing 3 times with TBS/Tw/Ca^{++}

(IX) Streptavidin-Eu^{3+}, diluted 1:1000 in Europium-buffer was added for 1h at RT

(X) The plate was washed 3 times with TBS/Tw/Ca^{++}

(XI) 200µl of the enhancement buffer was pipetted to each well and the plate was shaken 5min at RT

(XII) Measure the plate by TRF (Ex: F340nm, Em: 615nm)

2.2.8.4.3 C4-deposition Assay

Binding of ficolins to MASPs leads to complement activation. Complement activity was measureable by the detection of the complement factor C4b. C4b was only cleaved when complement activity was induced.

C4-deposition Assay:

(I) Coating 10µg/ml AcBSA with 100µl/well of a 96-well microtiter-plate in coating buffer, O/N at 4°C
(II) Blocking was performed with TBS/Tw, 1h at RT
(III) (Recombinant) ficolin/MASP-mixture was added in TBS/Tw/Ca^{++} to plate and binding to AcBSA was allowed O/N at 4°C
(IV) Plate was washed 3 times with TBS/Tw/Ca^{++}
(V) Recombinant human C4 was added (1:1000) and incubated for 2h at 37°C
(VI) Plate was washed 3 times with TBS/Tw/Ca^{++}
(VII) Anti-C4-biotinylated antibody mix was added and incubation is performed for 1h at 37°C
(VIII) Washing 3 times with TBS/Tw/Ca^{++}
(IX) Streptavidin-Eu^{3+} was added 1:1000 in Europium-buffer for 1h at RT
(X) Plate was washed 3 times with TBS/Tw/Ca^{++}
(XI) 200µl enhancement buffer was pipetted to each well and plate was shaken 2min at RT
(XII) Measure the plate by TRF (Ex: F340nm, Em: F615nm)

2.2.9 Confocal microscopy with PMNs

For functional analysis of mouse FcnB, PMNs isolated from Balb/c bone marrow were studied by confocal microscopy. First PMNs were isolated following general an isolation protocol.

2.2.9.1 Pre-treatment of labtek chambers or cover slips with poly-L-lysine

For better attachment of isolated bone marrow-derived PMNs, the wells or slides were covered with a poly-L-lysine layer. The labtek chamber (Nalge Nunc) can directly be covered with 0.01% poly-L-lysine (PLL). 250 µl of the PLL-solution were added and incubated for 20min at RT, and washing steps with 500µl distillated water

follows. The labtek chamber was air-dried, before the cells were added. Drying is arranged for at least 1h under the sterile hood. In the case of the cover slips, first an sterilization is performed. The cover slips were first incubated in 250µl ethanol for 20min at RT. After washing 2 times with 500µl water, 250 µl of PLL were added and incubated for 20min at RT. An additionally washing step was done with 500µl distilled water. The cover slips were dried before the cells were transferred to them.

2.2.9.2 Seeding of PMNs and stimulation

After isolation and FACS analysis for purity, $5x10^5$ PMNs were seeded in 200 µl medium, in a labtek chamber pre-treated with poly-L-lysine for 1h at 37°C. Then, the cells were stimulated. 200µl of RPMI/10% FCS medium was added containing a vehicle (DMSO) or 120ng/ml PMA (PMA is diluted in DMSO suspension). The cells were cultured for 16h at 37°C and 5% CO_2 in an incubator.

2.2.9.3 Preparation of PMNs for confocal microscopy

(I) After the incubation of PMNs for 16h at 37°C, the supernatant was removed and the wells were washed with PBS
(II) Cells were fixed for 30min in 1% (final) PFA, dissolved in PBS
(III) Cells were washed with PBS 3 times for 5min
(IV) Cells were permeabilized with 50µl 0.5 % Triton X-100 (in PBS) for 1min at RT
(V) Cells were washed 3 times in PBS for 1min
(VI) 100 µl of blocking buffer was added and incubated for 30min at 37°C
(VII) Blocking buffer was removed from the wells
(VIII) 50µl primary antibody solution was added, diluted in blocking buffer and incubated for 1h at 37°C
(IX) Washing was performed 3 times for 5min with PBS
(X) 50µl of the secondary antibody diluted in blocking buffer was added for 1h at 37°C
(XI) Cells were washed 3 times in PBS for 1min
(XII) DNA staining with ToPro3 (1:1000 final, light sensitive!) for 15min
(XIII) Washing was performed 2 times for 5min with distilled water
(XIV) 5µl of Fluoromont were added
(XV) Neutrophils were analyzed under the confocal microscope

2.2.10 Animal experiments

Female rats (ACI, Wistar and Fischer 344) were purchased from Charles River. Primary cells were isolated from C57BL/6 mice, aged between 8 and 10 weeks. 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.

2.2.10.1 Immunization of rats

For generation of monoclonal rat-anti-mouse FcnB antibodies, three rats were immunized with the Drosophila Schneider S2 cell-produced-recombinant-mouse FcnB. Three different rat strains were chosen, to enhance the likelihood to generate monoclonal antibodies.
Three female rats (strains: Wistar, ACI and F344) were immunized with 100µg/ml of the purified recombinant FcnB-V5-His protein in CFA s. c. and boosted in four-week intervals each time with 100µg/ml recombinant FcnB-V5-His protein s.c. (first boost with IFA, other boosts without adjuvant). Before the immunization (pre-immune), 1 week after the immunization and 1 week after each boost, the animals were anesthetized (8 mg xylazine + 37 mg ketamine/body weight in 0.2 ml PBS). The blood was drawn from the retroorbital plexus and the antibody titer was determined by ELISA. When a good response was observed, the corresponding animal was boosted for a last time and sacrificed three days afterwards. The spleen cells were used for fusion with SP2/O cells.

Immunization protocol:

For generation of monoclonal rat anti mouse FcnB antibodies, three rats were immunized in parallel with recombinant mouse FcnB produced in DS-2 cells. This recombinant protein is tagged with a V5 Tag and a His-Tag. Both tags enable easy purification over corresponding columns, but also increase the risk to generate antibodies during immunization, raised against a tag. Following, the steps of each rat immunization are listed.

Species	Rat
Strain	Agouti, ACI
Gender	Female
Age at beginning	≈ 7 weeks
Arrival date/ birth date	∗ 15.06.2009, EZ (selfishness)
Immunized antigen	Recombinant DS-2 FcnB-V5-His, Pool B, purification from 19.06.2009, conc. Of 0.710 mg/ml, supplemented with Ectoin for protein stabilization
Start immunization	04.08.2009

Date	**Bleeding**	**Immunization**	**Serum**
04.08.2009	Yes	Immunization i.p. (100µg/ml in TBS + 1.2 ml CFA)	1ml (-20°C)
25.08.2009	No	1. Boost i.p. (100µg/ml + 1.2ml IFA)	
02.09.2009	Yes		1ml (-20°C)
21.09.2009	No	2. Boost i.p. (100µg/ml)	
24.09.2009	Yes	KILL + FUSION with ¼ spleen	2ml (-20°C)

Tab. 6: Overview immunization ACI rat.

100µg/ml of protein was diluted in TBS-buffer and injected i.p. with CFA or later IFA. After second boost and killing of the rat, spleen cells were prepared according to the protocol for fusion. One day before, PEC were seeded as “feeder” cells. For fusion ¼ spleen was fused with SP2/0 myeloma cells in a ratio of 3:1.

Species	Rat
Strain	Wistar
Gender	Female
Age at beginning	≈ 5 weeks
Arrival date/ birth date	∗ 05.07.2003, EZ (selfishness)
Immunized antigen	recombinant DS-2 FcnB-V5-His, Pool B, purification 19.06.2009, 0.710 mg/ml, supplemented with Ectoin
Start immunization	04.08.2009

Date	**Bleeding**	**Immunization**	**Serum**
04.08.2009	Yes	Immunization i.p. (100µg/ml + 1.2 ml CFA)	1ml (-20°C)
25.08.2009	No	1. Boost i.p. (100µg/ml + 1.2 ml IFA)	
02.09.2009	Yes		1ml (-20°C)
21.09.2009	No	2. Boost i.p. (100µg/ml + 1.2 ml IFA)	
29.09.2009	Yes		1ml (-20°C)
26.10.2009	No	3. Boost i.p. (100µg/ml)	
29.10.2009	Yes	KILL + FUSION with 1/5 spleen	3ml (-20°C)

Tab. 7: Overview immunization Wistar rat.

100µg/ml of protein was diluted in TBS-buffer and injected i.p. with CFA or later IFA. After second Boost and killing of the rat, spleen cells were prepared according to the protocol for fusion. One day before, PEC were seeded as “feeder” cells. For fusion 1/5 spleen was fused with SP2/0 myeloma cells in a ratio of 3:1. Spleen size was obviously very big, compared to normal spleen. Also spleen cell number was higher than normally obtained.

Species	Rat
Strain	Fisher 344, F344
Gender	Female
Age at beginning	≈ 7 weeks
Arrival date/ birth date	∗ 13.06.2003, EZ (selfishness)
Immunized antigen	Recombinant DS-2 FcnB-V5-His, Pool B, purification from 19.06.2009, conc. Of 0.710 mg/ml, supplemented with Ectoin for protein stabilization
Start immunization	04.08.2009

Date	**Bleeding**	**Immunization**	**Serum**
04.08.2009	Yes	Immunization (100µg/ml + 1.2ml CFA)	1ml (-20°C)
25.08.2009	No	1. Boost i.p. (100µg/ml + 1.2 ml IFA)	
02.09.2009	Yes		1ml (-20°C)
21.09.2009	No	2. Boost i.p. (100µg/ml + 1.2 ml IFA)	
29.09.2009	Yes		1ml (-20°C)
26.10.2009	No	3. Boost i.p. (100µg/ml + 1.2 ml IFA)	
02.11.2009	Yes		1ml (-20°C)
15.12.2009	No	4. Boost i.p. (100µg/ml + 1.2 ml IFA)	
05.01.2010	Yes		2ml (-20°C)
19.01.2010	Yes	KILL, store spleen at -80°C	2ml (-20°C)

Tab. 8: Overview immunization F344 rat.

Rat was not used for fusion so far, because the titer was not that high, compared to both other immunized rats. Therefore, spleen cells were isolated and stored at -80°C for later fusion, if necessary.

2.2.10.2 Collection of blood and serum preparation

Before and after the immunization and also after the booster injections with recombinant FcnB-V5-His protein, blood from all three rats was collected in a 15 ml falcon and stored O/N at 4°C to allow blood clotting. On the next day, the clotted blood was carefully separated from the wall of the falcon, the samples were centrifuged at 3000g for 10min and afterwards the supernatant was transferred into a new eppendorf tube and centrifuged again. Then, the serum was transferred again to a new tube and stored at -20°C.

2.2.11 Statistical calculations

Experimental data represent mean values ± standard deviation (SD). The statistical analyses were performed using the One-way ANOVA by GraphPad Prism4.
Values with $p < 0.05$ were accepted as statistically significant and indicated with *, values with $p < 0.001$ were indicated with ** and values with $p < 0.0001$ were indicated with ***.

3. Results

3.1 Recombinant FcnB expressed in DS-2 insect cells

For the characterization of FcnB the protein was recombinantly expressed in Drosophila Schneider 2 cells (DS-2). The protein was tagged with a V5-His protein to enable purification and detection. Different fractions after purification were analyzed on SDS-PAGE (Fig. 16, left) and the protein concentrations were determined. Further, 5µg/ml of the recombinant protein were loaded onto SDS-PAGE and blotted to nitrocellulose and characterized using the monoclonal rat anti-mouse FcnB antibody IA4 for detection. In Western blot analysis under non-reducing conditions the FcnB monomer appeared at a molecular weight of ~ 35 kDa, but higher oligomers were also observed (Fig. 16, right).

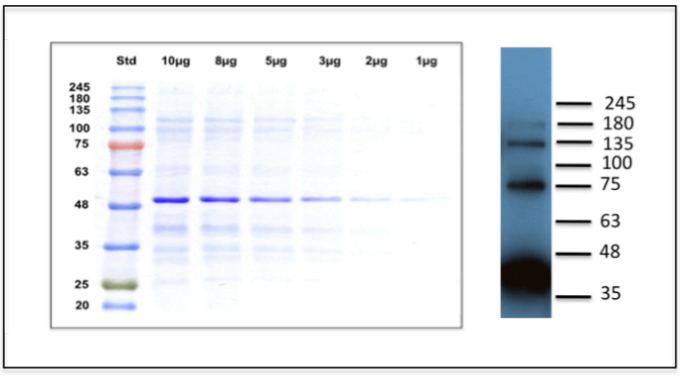

Figure 16: Gel-analysis of recombinant FcnB-V5-His protein.

The protein was loaded at different concentrations on SDS-PAGE, separated under reducing conditions and stained with Coomassie-blue (left). Further, the recombinant protein (5µg/ml) after a SDS-PAGE run was blotted and detected with the monoclonal rat anti-mouse FcnB antibody IA4 (right). The molecular weight standard (Std) is indicated in kDa.

3.2. Expression of FcnB

3.2.1 Expression of FcnB in Hoxb8-neutrophils

RT-PCR analyses were performed to determine sources for mRNA expression of FcnB. The expression was analyzed in a neutrophil-like cell line: The ERHoxb8 cell line was kindly provided by G. and H. Häcker. ERHoxb8 cells provide a good source for the analysis of mouse neutrophils *in vivo* (McDonald et al., 2011). These cells represent progenitor cells of neutrophils and are able to stay in a progenitor status when cultured in estradiol-containing medium. After withdrawal of estradiol, the cells differentiate within 3 to 4 days to neutrophils.
RT-PCR was performed to test whether these cells are a source of FcnB. The expression of FcnB was analyzed using transcribed RNA samples from Hoxb8-neutrophils during their differentiation, starting from the progenitors. Fig. 17 shows the expression of FcnB depending on the day of culture in the absence of estradiol. The expression profile reveals an increase from day 2 (d2) on with a maximum expression level achieved on day 3 (d3). During the cultivation of Hoxb8-neutrophils, the expression decreased to basal expression levels. This demonstrated expression of FcnB without stimulation.

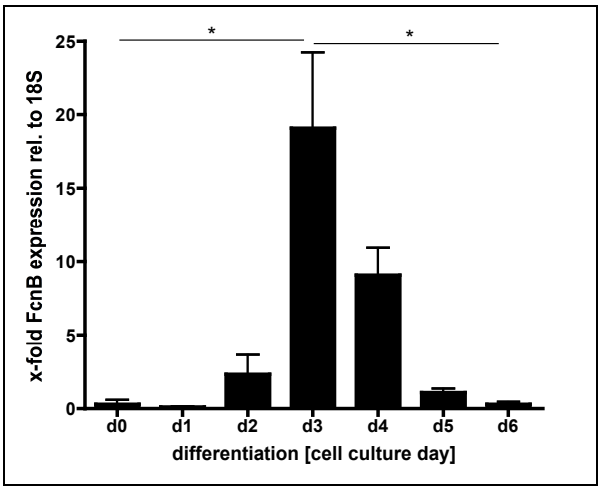

Figure 17: Analysis of FcnB expression in Hoxb8-neutrophils.
Hoxb8-neutrophils were generated from the progenitor cells and analyzed by RT-PCR at indicated time-points during cell culture for FcnB expression. The data represent mean ± SD of 5 independent cultures. The experiment was repeated at least 3 times.

3.2.1.1 Stimulation of Hoxb8-neutrophils with LPS *E. coli*

Besides non-stimulated Hoxb8-neutrophils, the stimulation effect of different reagents was investigated. Therefore, the cells were differentiated and adjusted to a defined cell number before starting the stimulation experiment. The cells were left untreated for 6h after seeding into wells and stimulated afterwards with LPS from *E. coli*. After different time-points the cells were lysed, the RNA was isolated, reverse transcribed, and RT-PCR was performed. As shown in Fig. 18, the FcnB expression of non-stimulated Hoxb8-neutrophils on day 3 (d3) was enhanced by stimulation with LPS. The cells responded to LPS with increased expression levels within 30min. Longer stimulation times did not lead to further enhanced expression and the expression started to declined after 1h of stimulation.

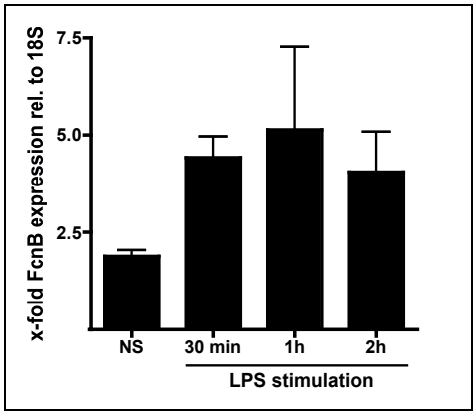

Figure 18: FcnB expression of LPS-stimulated Hoxb8-neutrophils (day 3).
Hoxb8-neutrophils were generated from progenitor cells and stimulated on day 3 of differentiation with LPS from *E. coli* (100ng/ml) for the indicated times and FcnB expression was analyzed by RT-PCR. The results are given as mean ± SD from 6 independent cultures. The experiment has been repeated at least 2 times.

Since longer stimulation times with LPS seemed to reduce FcnB expression stimulation with LPS was performed for 24, 48 and 72h. Strong expression levels in Hoxb8-neutrophils of day 3 of culture without stimulation were observed. Longer incubations with LPS had a reducing effect on FcnB mRNA levels (see Fig. 19).

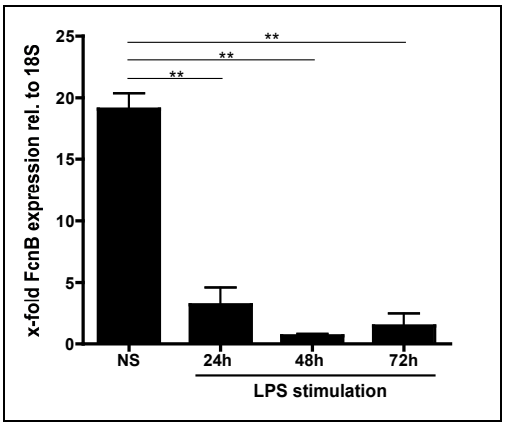

Figure 19: FcnB expression in Hoxb8-neutrophils after long-time exposure to LPS.

Hoxb8-neutrophils were generated from progenitor cells and stimulated on day 3 of culture for indicated times with LPS from *E. coli* (100ng/ml) and FcnB expression was analyzed by RT-PCR. Results are given as mean ± SD from 3 independent cultures.

To investigate whether the induction of expression was possible at a later time point of differentiation, stimulation experiments with LPS from *E. coli* were repeated with Hoxb8-neutrophils on day 5 of differentiation. A similar increase in FcnB expression was obtained within only 30min of stimulation. This shows that FcnB is not only rapidly induced, but also inducible independently of the differentiation state of neutrophils derived from ERHoxb8 cells (see Fig. 20).

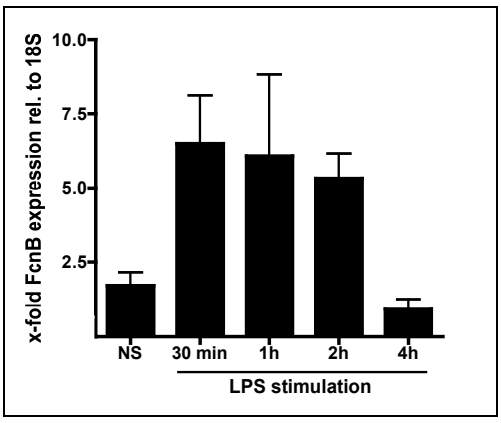

Figure 20: FcnB expression of LPS-stimulated Hoxb8-neutrophils (day 5).

Hoxb8-neutrophils were generated from progenitor cells and stimulated on day 5 of differentiation with LPS from *E. coli* (100ng/ml) for the indicated times and FcnB expression was analyzed by RT-PCR. The results are given as mean ± SD from 4 independent cultures. The experiment has been repeated at least 2 times.

3.2.1.2 Stimulation of Hoxb8-neutrophils with PMA/Ionomycine

Hoxb8-neutrophils were stimulated with a mixture of PMA and Ionomycine for different times. As seen for LPS, mRNA expression showed a rapid increase after 30min of stimulation with PMA and Ionomycine compared to non-stimulated control samples. The expression decreased after 2h of stimulation (see Fig. 21).

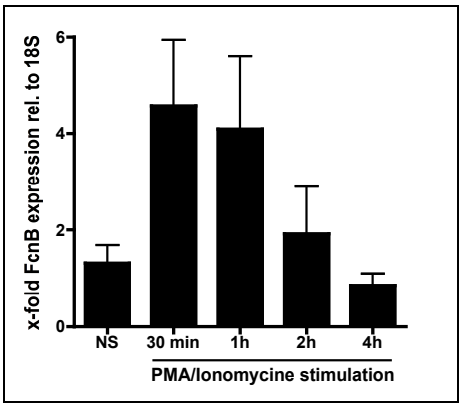

Figure 21: FcnB expression of PMA/Ionomycine-stimulated Hoxb8-neutrophils.
Hoxb8-neutrophils were generated from progenitor cells and stimulated on day 5 of culture for the indicated times with PMA/Ionomycine (PMA: 15ng/ml and Ionomycine: 1µM) and FcnB expression was analyzed by RT-PCR. Results are given as mean and SD from 5 independent cultures.

3.2.1.3 Stimulation of Hoxb8-neutrophils with CpG

Hoxb8-neutrophils were stimulated with CpG for different times. In contrast to LPS and PMA/Ionomycine, CpG had no effect on FcnB mRNA expression. The experiment was repeated 4 times independently with similar results. CpG did neither increase nor affect the expression of FcnB otherwise (see Fig. 22).

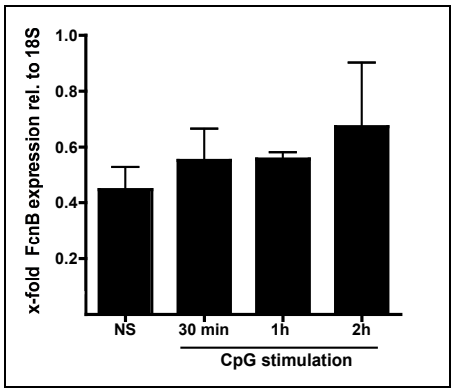

Figure 22: FcnB expression of CpG-stimulated Hoxb8-neutrophils.

Hoxb8-neutrophils on day 5 of differentiation were stimulated for indicated time points with CpG (5µg/ml) and FcnB expression was analyzed by RT-PCR. Data represent mean ± SD from 4 independent cultures.

3.2.1.4 Stimulation of Hoxb8-neutrophils with aggregated IgG

Hoxb8-neutrophils were stimulated with aggregated rat IgG for different times. Aggregated immunoglobulins were used to stimulate Fc-receptors by agglutinating them. Aggregated IgG had no significant effect on mRNA expression of FcnB. Aggregated IgG did neither increase nor affect otherwise the expression of FcnB. The experiment was repeated 5 times independently (see Fig. 23).

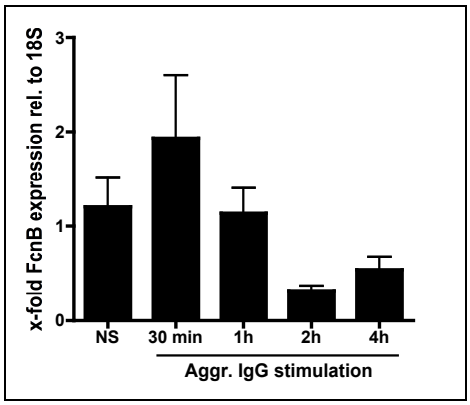

Figure 23: FcnB expression of Hoxb8-neutrophils stimulated with aggregated IgG.

Hoxb8-neutrophils were generated from progenitor cells and stimulated on day 5 for indicated times with aggregated mouse IgG (20µg/ml) and the FcnB expression was analyzed by RT-PCR. Results are given in mean ± SD from 5 independent cultures.

Taken together, FcnB mRNA is expressed by Hoxb8-neutrophils. During the differentiation to neutrophils, the expression peaks around d3 and decreases afterwards to basal levels. RT-PCR analysis and stimulation experiments indicated a rapid mRNA expression after stimulation, performed at d3 and also on d5 of differentiation to neutrophils. LPS and PMA/Ionomycine resulted in an increase of the expression, whereas reagents like CpG or aggregated IgG did not affect the FcnB expression.

3.2.2 Expression of mouse FcnB in primary PMN

3.2.2.1 Stimulation of PMN with LPS *E. coli*

PMN isolated from bone marrow were either left non-stimulated or were stimulated for 30min with LPS. Results demonstrated that PMNs from the bone marrow expressed FcnB and the expression was enhanced up to three-fold within 30min by LPS stimulation (see Fig. 24).

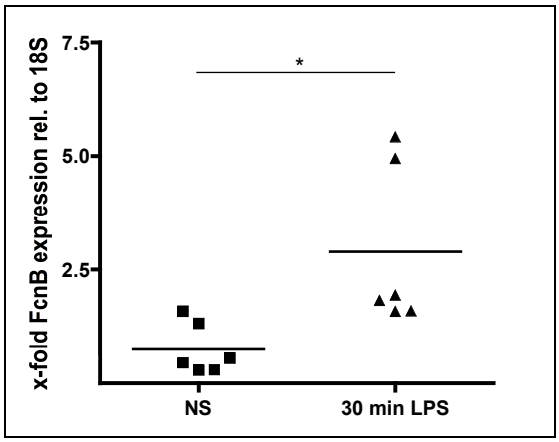

Figure 24: FcnB expression in PMN.

The expression of FcnB mRNA was investigated in PMN isolated from the bone marrow without stimulation or after stimulation with LPS *E. coli* (100ng/ml). Results are given in mean from 6 independent cultures. Statistical analysis was performed using an unpaired t-test.

3.2.2.2 Expression of FcnB in sorted PMN

Primary polymorph nuclear neutrophils (PMN) from bone marrow and spleen were isolated from C57BL/6 mice. To separate the immature PMN subpopulations PMN-MDSC (polymorph nuclear neutrophil-myeloid derived suppressor cells) and MO-MDSC (mononuclear neutrophil-myeloid derived suppressor cells) first a negative depletion by MACS (by anti-CD4, anti-CD8, anti-B220, anti-NK1, and anti CD11c) was performed to enrich CD11b+ cells. Second, PMN-MDSC ($CD11b^+$ $Ly6G^+$ $Ly6C^+$) and MO-MDSC ($CD11b^+$ $Ly6G^-$ $Ly6C^+$) were separated by a positive selection for $Ly6G^+$ cells.

FcnB mRNA was exclusively expressed by PMN-MDSC, which had a purity of around 90 %. Expression of FcnB by PMN was found in the spleen, as well as the in bone marrow (see Fig. 25). In contrast, no expression of FcnB was found in MO-

MDSC, which had a purity of around 60 % when isolated from the spleen and only 31% when isolated from the bone marrow. Cell isolations and MACS experiments were performed by Dominic Schmidt.

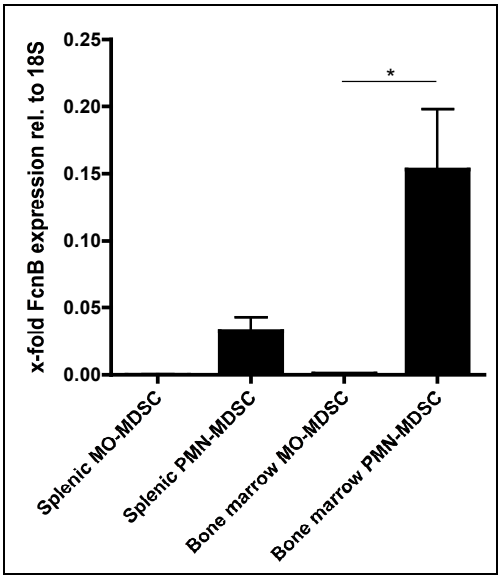

Figure 25: FcnB expression of subpopulations of PMN from bone marrow and spleen.
Purified PMN-MDSC ($CD11b^+$ $Ly6G^+$ $Ly6C^+$) and MO-MDSC ($CD11b^+$ $Ly6G^-$ $Ly6C^+$) from bone marrow and spleen were used for FcnB expression analysis by RT-PCR. Results are given in mean ± SD from 3 independent technical replicates.

3.2.3 Expression of FcnB in bone marrow-derived macrophages (BMDM)

To investigate FcnB expression in macrophages, cells from bone marrow were isolated and cultured in M-CSF containing medium for differentiation into macrophages. At each day of cell culture, cells were isolated and FcnB expression was determined by RT-PCR. The FcnB expression was high during the first days of differentiation in M-CSF-containing medium and decreased during differentiation steadily (see Fig. 26).

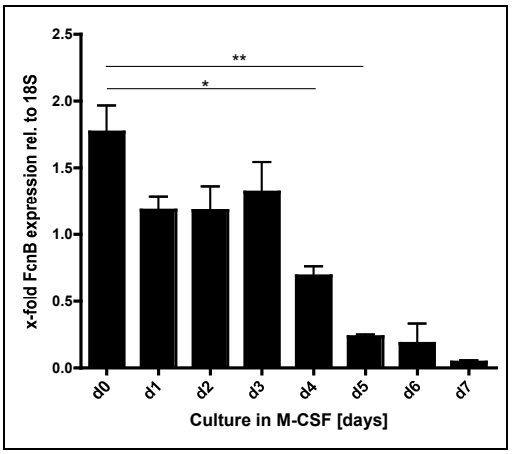

Figure 26: FcnB expression in BMDM.

In vitro generated macrophages from the bone marrow were analyzed at indicated times for FcnB expression by RT-PCR. Data represent mean ± SD from 3 independent cultures. The experiments were repeated at least 3 times.

3.2.4 Expression of FcnB in bone marrow-derived dendritic cells (BMDC)

3.2.4.1 Expression of FcnB in BMDC from wild type mice

To investigate FcnB expression in dendritic cells, bone marrow cells were cultured in GM-CSF-containing medium to differentiate precursor cells into dendritic cells. At each day of cell culture, cells were isolated and the FcnB expression was determined by RT-PCR. During the dendritic cell development, FcnB expression peaked on d4 (see Fig. 27).

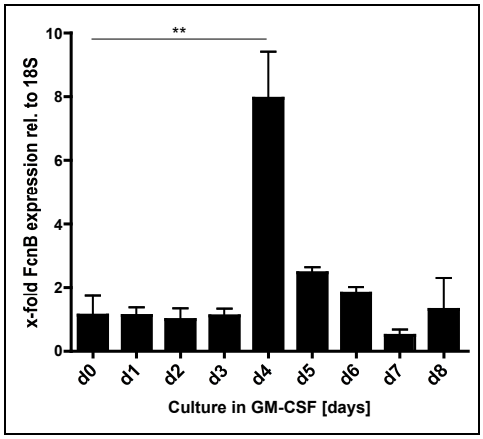

Figure 27: FcnB expression in BMDC.

In vitro generated dendritic cells from the bone marrow were analyzed at indicated times for FcnB expression by RT-PCR. Data represent mean ± SD from 3 independent cultures. The experiments were repeated at least 2 times.

To investigate whether FcnB expression is inducible during differentiation of BMDC, cells were stimulated at different times with a mixture of LPS and IFNγ or left non-stimulated. When BMDC after 4 days of culture in GM-SCF containing-medium were used FcnB was expressed by non-stimulated dendritic cells and the expression was only slightly enhanced when these cells were additionally stimulated with a mixture of LPS and IFNγ (see Fig. 28).

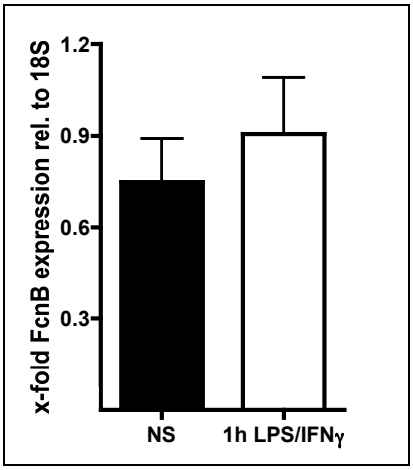

Figure 28: FcnB expression in non-stimulated and stimulated BMDC (d4).

In vitro generated dendritic cells on day 4 of culture were stimulated with LPS from *E. coli* (100ng/ml) and IFNγ (50 ng/ml) for 1h or left untreated. Data represent mean ± SD of 4 independent cultures.

BMDC from d4 of culture in GM-CSF were sorted using the markers $CD11b^+$ $Ly6G^+$ and $Ly6C^{int}$ and these immature BMDC were isolated. These cells were seeded and stimulated with LPS and IFNγ or left non-stimulated for 30min. In contrast to BMDC from d4 of culture in GM-CSF the FcnB expression was significantly higher in the non-stimulated sorted immature BMDC and slightly enhanced after 30min of stimulation (see Fig. 29).

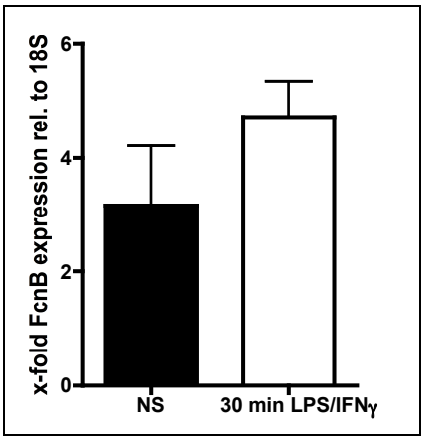

Figure 29: FcnB expression in sorted immature BMDC (d4).

In vitro generated BMDC on day 4 of culture were sorted for $CD11b^+$ $Ly6G^+$ and $Ly6C^{int}$ and stimulated with LPS (100 ng/ml) and IFNγ (50 ng/ml) for 30min or left non-stimulated. FcnB expression was analyzed by RT-PCR. Results are given in mean ± SD from 3 independent cultures.

BMDC taken on day 6 of culture in GM-SCF-containing medium were tested for FcnB expression. The expression of FcnB in non-stimulated BMDC was comparable to the expression seen in BMDC from day 4 of culture. Stimulation with LPS and IFNy had no enhancing but rather a reducing effect in FcnB expression (see Fig. 30).

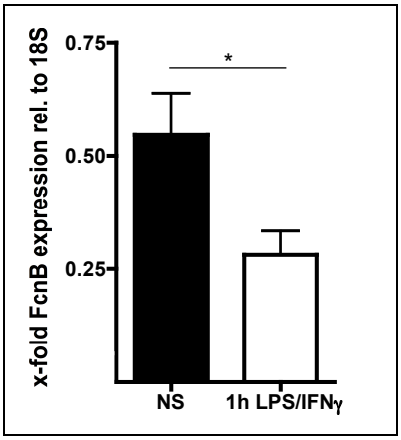

Figure 30: FcnB expression in non-stimulated and stimulated BMDC (d6).

In vitro generated dendritic cells on day 6 of culture were stimulated with LPS from *E. coli* (100ng/ml) and IFNγ (50 ng/ml) for 1h or left untreated. FcnB expression was analyzed by RT-PCR. Data represent mean ± SD of 4 independent cultures.

The expression of FcnB by BMDC from day 8 of cell culture in GM-CSF-containing medium seemed enhanced after 30min stimulation with LPS but unchanged after 1 h of stimulation (see Fig. 31).

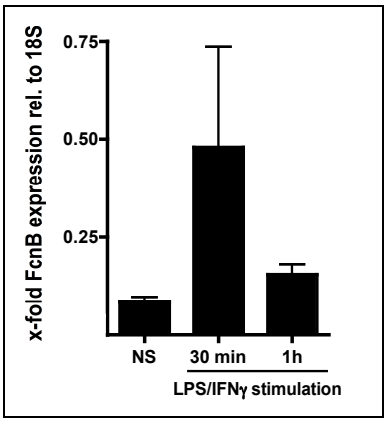

Figure 31: FcnB expression in non-stimulated and stimulated BMDC (d8).

In vitro generated dendritic cells on day 8 of culture were stimulated with LPS from *E. coli* (100ng/ml) and IFNγ (50 ng/ml) for 30min, 1h or left untreated. FcnB expression was analyzed by RT-PCR. Data represent mean ± SD of 3 independent cultures.

FcnB expression by BMDC analyzed on day 10 of culture in GM-CSF-containing medium was lower compared to the expression of cells from day 4, day 6, or day 8 (see Fig. 32).

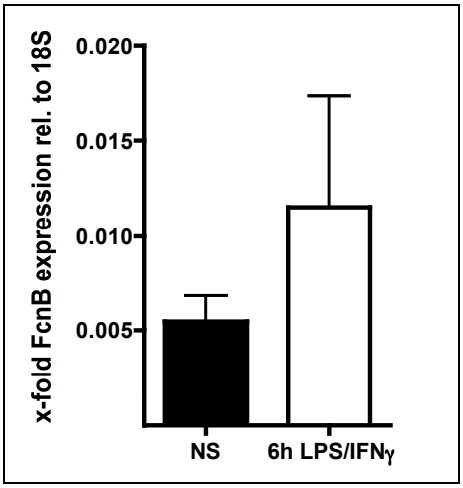

Figure 32: FcnB expression in non-stimulated and stimulated BMDC (d10).
In vitro generated dendritic cells on day 4 of culture were stimulated with LPS from *E. coli* (100ng/ml) and IFNγ (50 ng/ml) for 6h or left untreated. FcnB expression was analyzed by RT-PCR. Data represent mean ± SD of 4 independent cultures.

3.2.4.2 Expression of FcnB in BMDC from wild type and TNFR2-deficient mice

The expression of FcnB by BMDC of wild type mice was compared to FcnB expression by BMDC of TNFR2-deficient mice. Cells of these mice lacking TNFR2, an inhibitor of TNF, experience a continuously inflammatory environment. FcnB expression from non-stimulated BMDC (day 4 of culture) and after 1h stimulation with LPS (*S. abortus equi*) and IFNγ showed slightly increased expression levels in the TNFR2-deficient BMDC compared to wild type BMDC (see Fig. 33).

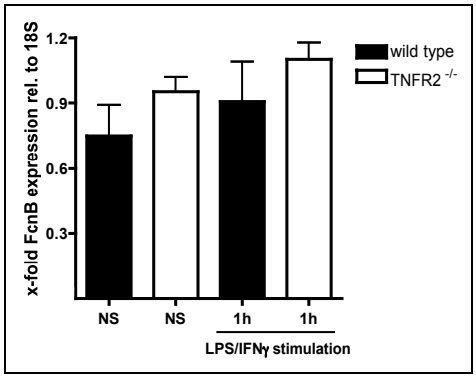

Figure 33: FcnB expression in BMDC (d4) from wild type and TNFR2-deficient mice.

In vitro generated BMDC on day 4 of culture in GM-CSF from wild type (black) and TNFR2-deficient mice (white) were stimulated with LPS from *S. abortus equi* (100 ng/ml) and IFNγ (50 ng/ml) for 1h or left non-stimulated. FcnB expression was analyzed by RT-PCR. Results are given as mean ± SD of 4 independent mice. Results are statistically not significant.

Additional, BMDC from wild type and TNFR2-deficient mice on day 4 of culture were sorted using the markers $CD11b^+$ $Ly6G^+$ and $Ly6C^{int}$. After sorting, the immature BMDC were seeded and stimulated with LPS (*S. abortus equi*) and IFNγ or left non-stimulated for different times. Slightly higher FcnB expression levels were found in non-stimulated immature BMDC as seen before, however, the expression was higher compared to the non-sorted BMDC from day 4 of culture in GM-CSF (see Fig. 34).

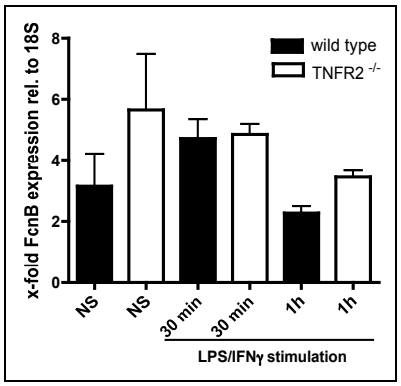

Figure 34: FcnB expression in sorted immature BMDC from wild type and TNFR2-deficient mice.

BMDC were generated from wild type and TNFR2-deficient mice and sorted on day 4 of culture for $CD11b^+$ $Ly6G^+$ $Ly6C^{int}$ and stimulated for different times with LPS from *S. abortus equi* (100ng/ml) and IFNγ (50ng/ml) for 30min, 1h or left non-stimulated. FcnB expression was analyzed by RT-PCR. Results are given as mean ± SD of 3 independent mice.

FcnB mRNA expression from non-stimulated BMDC (day 6 of culture) and BMDC stimulated for 1h with LPS (*S. abortus equi*) and IFNγ showed again slightly increased expression levels in the TNFR2-deficient BMDC compared to BMDC from the wild type BMDC (see Fig. 35).

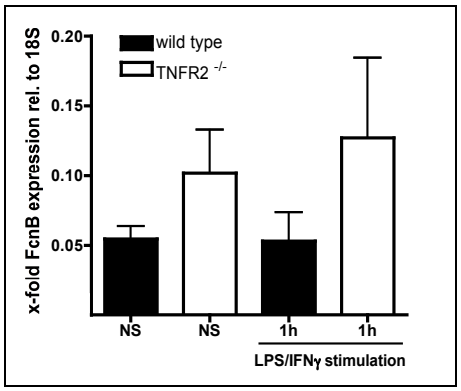

Figure 35: FcnB expression in BMDC (d6) from wild type and TNFR2-deficient mice.
BMDC from wild type (black) and TNFR2-deficient mice (white) were generated in vitro and analyzed on day 6 of culture in GM-CSF for FcnB expression. Cells were stimulated for 1h with LPS from *S. abortus equi* (100ng/ml) and IFNγ (50ng/ml) or left non-stimulated. FcnB expression was analyzed by RT-PCR. by RT-PCR. Data are present as mean ± SD of 4 independent mice.

FcnB expression from non-stimulated BMDC (day 8 of culture) and BMDC stimulated for 30min or 1h with LPS (*S. abortus equi*) and IFNγ showed again slightly increased expression levels in the TNFR2-deficient BMDC compared to wild type BMDC (see Fig. 36).

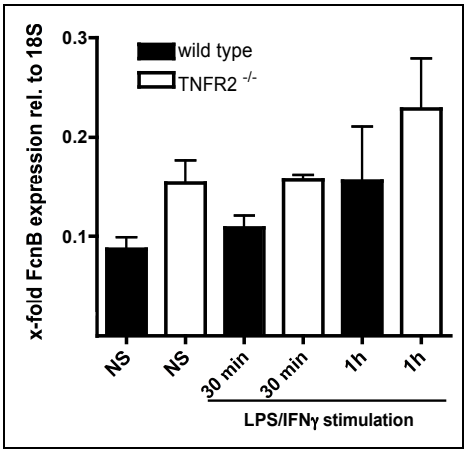

Figure 36: FcnB expression in BMDC (d8) from wild type and TNFR2-deficient mice.
BMDC from wild type (black) and TNFR2-deficient mice (white) were generated *in vitro* and analyzed on day 8 of culture in GM-CSF for FcnB expression. Cells were stimulated with LPS from *S. abortus equi* (100ng/ml) and IFNγ (50ng/ml) for 30min, 1h or left non-stimulated. FcnB expression was analyzed by RT-PCR. Data are present as mean ± SD of 4 independent mice.

BMDC on day 10 of cell culture from TNFR2-deficient mice showed no higher FcnB expression levels compared to BMDC of wild type mice (see Fig. 37).

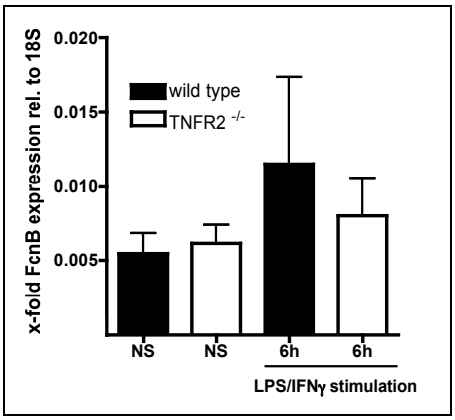

Figure 37: FcnB expression in BMDC (d10) from wild type and TNFR2-deficient mice.
BMDC from wild type (black) and TNFR2-deficient mice (white) were generated *in vitro* and analyzed on day 10 of culture in GM-CSF for FcnB expression. Cells were stimulated for 6h with LPS from *S. abortus equi* (100ng/ml) and IFNγ (50ng/ml) or left non-stimulated. FcnB expression was analyzed by RT-PCR. Data are present as mean ± SD of 4 independent mice.

3.2.4.3 Expression of FcnB in spleen cells from wild type and TNFR2-deficient mice

Spleen cells from wild type and TNFR2-deficient mice were isolated and analyzed for FcnB expression. Splenic cells were stimulated with LPS and IFNγ for different times or left non-stimulated. Spleen cells from TNFR2-deficient mice had significantly higher FcnB expression levels compared to spleen cells from wild type mice and the FcnB expression was further enhanced after stimulation (see Fig. 38).

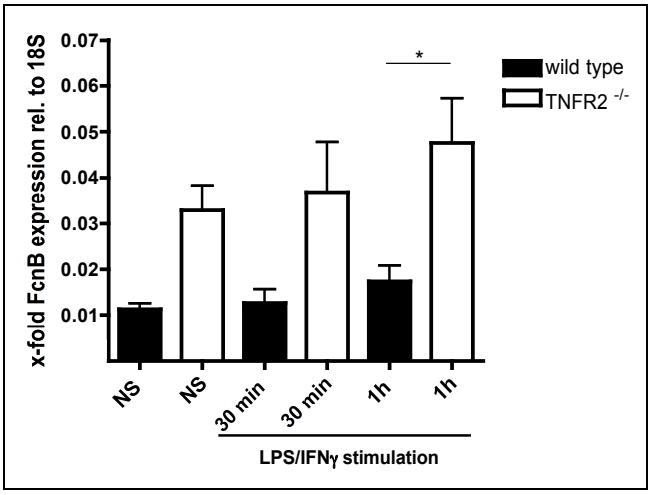

Figure 38: FcnB expression in spleen cells isolated from wild type and TNFR2-deficient mice. Splenic cells from wild type (black) and TNFR2-deficient mice (white) were isolated and stimulated with LPS from *E. coli* and IFNγ for 30min or 1h or left non-stimulated. FcnB expression was analyzed by RT-PCR. Results are given as mean ± SD from 3 independent mice.

In summary a comparison of FcnB expression levels in wild type and TNFR2-deficient mice revealed higher expression levels of FcnB even in non-stimulated BMDC and also in non-stimulated spleen cells isolated from TNFR2-deficient mice. Nevertheless, it has to be considered that the RT-PCR values that were obtained from BMDC on day 4, day 6, day 8 and day 10 isolated from wild type and TNFR2-deficient mice are not comparable among the experiments because they were not measured in the same experiment.

3.3 Binding specificity of recombinant FcnB

3.3.1 Binding to BSA

Binding of recombinant FcnB to BSA was analyzed in ELISA. BSA was coated onto plastic at a concentration of 10 µg/ml and recombinant FcnB was added at different concentrations. The binding of FcnB to BSA was quantified using a specific antibody against FcnB (monoclonal rat anti-mouse FcnB antibody IA4).
Binding signals were all in the same range indicating that there was no binding of recombinant FcnB to BSA (see Fig. 39).

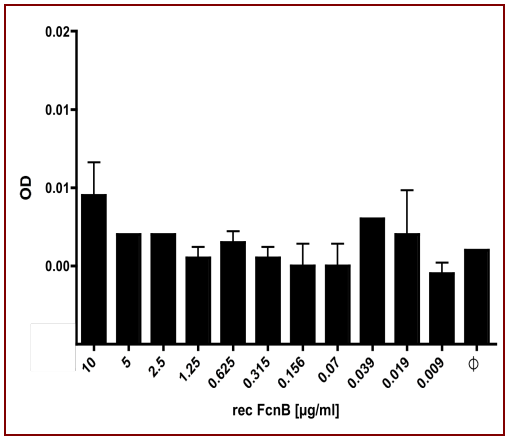

Figure 39: Binding of recombinant FcnB to BSA.
Recombinant FcnB at different concentrations was analyzed for binding to coated BSA using the monoclonal rat ant-mouse FcnB antibody IA4. Data are given was OD measured at 450 nm wavelength and represent mean ± SD of 3 independent technical values. The experiment was repeated more than 5 times.

3.3.2 Binding to AcBSA

To test whether recombinant FcnB binds to acetylated BSA (AcBSA), AcBSA was coated onto plates and binding of FcnB was determined by ELISA. The assay was performed in a calcium-containing buffer. Binding of FcnB to AcBSA was found to be dose-dependent. In addition, binding of FcnB to AcBSA was found to be calcium-

dependent since binding was inhibited by the presence of EDTA in the binding buffer (see Fig. 40).

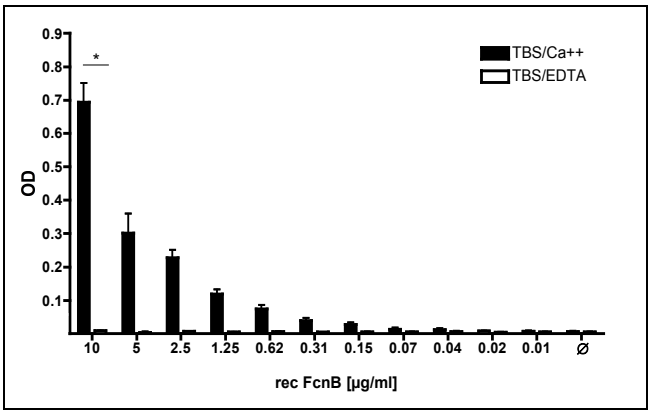

Figure 40: Binding of recombinant FcnB to AcBSA.

Recombinant FcnB at different concentrations was analyzed for the binding to coated AcBSA by using the monoclonal rat anti-mouse FcnB antibody IA4 (black bar). Binding was abolished by the presence of EDTA in the binding buffer (white bar). Data represent OD values measured at 450 nm and are given as mean ± SD of 3 independent technical replicates. The binding experiment was repeated more than 5 times.

To test further whether recombinant FcnB binding to AcBSA is specific AcBSA and BSA as a negative control were coated onto plates. Recombinant FcnB was added at different concentrations and the binding of FcnB was determined by ELISA. The assay was performed in a calcium-containing buffer. Binding of FcnB to AcBSA was found to be dose-dependent. No binding of FcnB to BSA was observed (see Fig. 41).

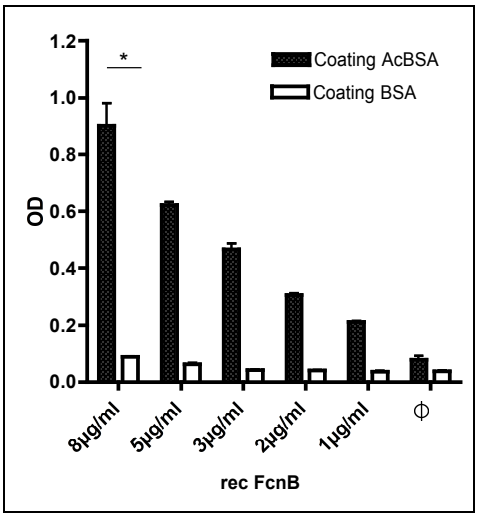

Figure 41: Binding of recombinant FcnB to AcBSA and BSA.

Binding of recombinant FcnB to either BSA or AcBSA coated plastic was compared by ELISA. OD values are measured at 450 nm and data represent mean ± SD of 3 independent technical replicates. Using the unpaired t-test FcnB binding on coated AcBSA compared to BSA was found to be significant with $p < 0.01$.

Binding of recombinant FcnB to AcBSA was also tested in a Dot blot assay. The results demonstrate that recombinant FcnB bound to nitrocellulose-bound-AcBSA, but not to nitrocellulose-bound-BSA (see Fig. 42). These results confirmed AcBSA as a ligand for FcnB, as for all other ficolins.

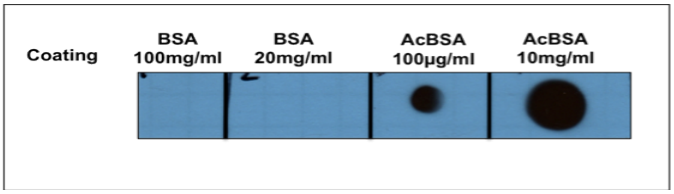

Figure 42: Binding of recombinant FcnB to nitrocellulose-bound AcBSA.

Nitrocellulose-membrane was coated with two different concentrations of either BSA or AcBSA before adding recombinant FcnB (5µg/ml). The binding of FcnB was detected with an anti-mouse V5-tag antibody.

The results shown in Fig. X demonstrate that the binding of recombinant FcnB to AcBSA was clearly inhibited when EDTA was present in the buffer while binding.
To test whether this inhibition of EDTA could be enhanced by the addition of acetate, ELISA was performed in binding-buffer containing either calcium, EDTA, or EDTA

plus acetate. Results in Fig. 43 show that the inhibition of FcnB binding to coated AcBSA was even stronger when additionally acetate was added.

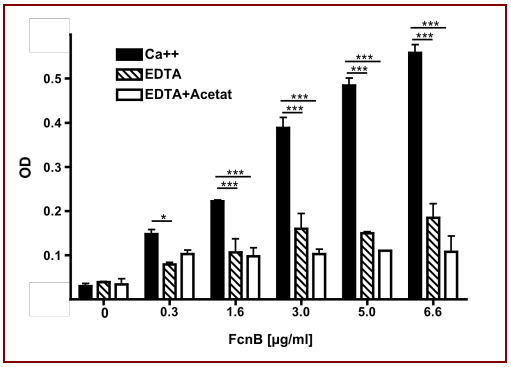

Figure 43: Binding inhibition of recombinant FcnB to AcBSA.

Recombinant FcnB binding to AcBSA was performed in three different buffers, containing either calcium, EDTA, or a mixture of EDTA plus acetate. The binding of FcnB was calcium-dependent and was abolished by the presence of EDTA or EDTA/acetate in the binding buffer.. Results are given as mean ± SD of 3 independent technical replicates.

3.3.3 Binding to acetylated low-density lipoprotein (AcLDL)

Another acetylated ligand was tested for binding to FcnB. AcLDL was used for coating and binding of recombinant FcnB was tested by ELISA. The results demonstrate that recombinant FcnB bound to AcLDL (see Fig. 44). These data revealed AcLDL as another ligand of recombinant mouse FcnB.

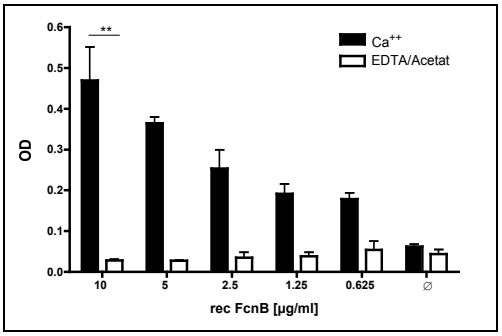

Figure 44: Binding of recombinant FcnB to AcLDL.

Recombinant FcnB at different concentrations was analyzed for the binding to coated AcLDL by using the monoclonal rat anti-mouse FcnB antibody IA4 (black bar). Binding was abolished by the presence of EDTA plus acetate in the binding buffer (white bar). Data are given as mean ± SD of 3 independent technical replicates. Using the unpaired t-test the inhibition by EDTA was found to be significant with $p < 0.001$.

3.3.4 Binding to GlcNAc

Another commonly used ligand of ficolins is N-acetylglucosamine (GlcNAc).The binding of recombinant FcnB to GlcNAc was tested by coating GlcNAc to plastic and determining binding of FcnB by ELISA.
Fig. 45 shows that binding of FcnB to GlcNAc occurred in a calcium-dependent manner. When recombinant FcnB was incubated in TBS buffer in the presence of EDTA the signal was reduced.

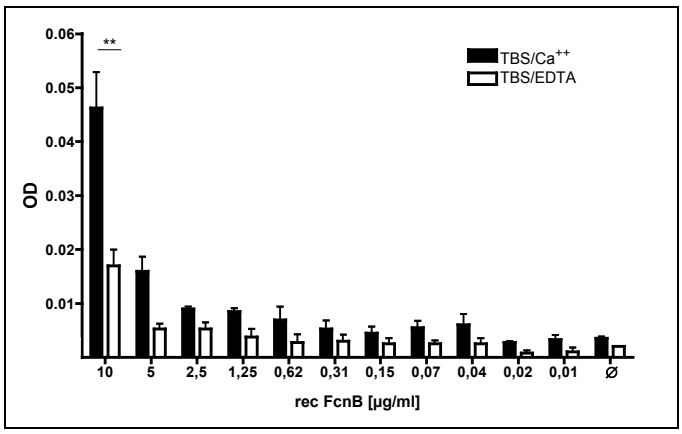

Figure 45: Binding of recombinant FcnB to GlcNAc in a dose-dependent manner.
Recombinant FcnB at different concentrations was analyzed for the binding to coated GlcNAc by using the monoclonal rat anti-mouse FcnB antibody IA4 (black bar). Binding was abolished by the presence of EDTA in the binding buffer (white bar). Data are given as mean ± SD of 3 independent technical replicates. Using the Mann-Whitney-test the inhibition by EDTA was found to be significant with $p < 0.001$.

3.3.5 Binding to fetuin

With regard to the fact that human M-ficolin has been shown to bind to fetuin, also its mouse homologue, FcnB, was tested for binding to fetuin. The assay was performed in the same way as described above.
Fetuin was used for coating of the plastic and binding of recombinant FcnB to fetuin was determined by ELISA. Recombinant FcnB was also able to bound to fetuin. However, signals for the binding were reduced, when FcnB was incubated in a TBS buffer containing EDTA (see Fig. 46).

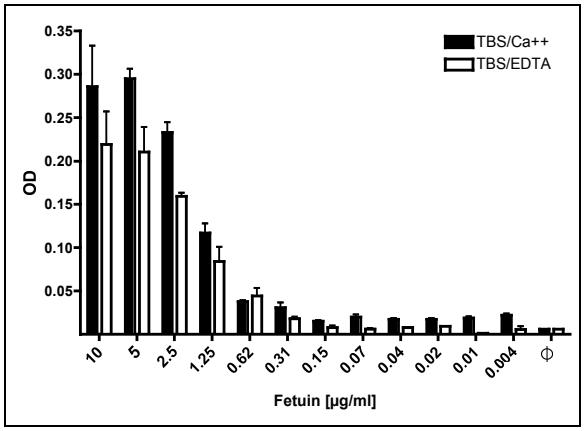

Figure 46: Binding of recombinant FcnB to fetuin.

Recombinant FcnB at different concentrations was analyzed for the binding to coated fetuin by using the monoclonal rat anti-mouse FcnB antibody IA4 (black bar). The binding of FcnB to fetuin was not found to be abolished, but reduced by the presence of EDTA in the binding buffer (white bar). Data are given as mean ± SD of 3 independent technical replicates.

3.3.6 Binding to chitin

Ficolins serve in the innate immune system as pattern recognition molecules by binding to pathogen surfaces. One type of attacking pathogens are fungi. A molecular constituent of the fungal cell wall is chitin. Therefore, recombinant FcnB was tested for binding to chitin-coated beads. The assay was performed in the same way as described above. Chitin-coated beads were used and the binding of recombinant FcnB was determined by ELISA. The data confirmed again the binding of FcnB to AcBSA, but not to BSA (see Fig. 47). Recombinant FcnB bound to chitin-coated beads, revealing chitin as an additional ligand for FcnB.

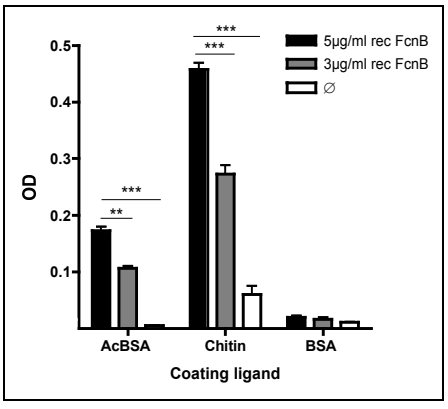

Figure 47: Binding of recombinant FcnB to chitin.

Recombinant FcnB at different concentrations was analyzed for the binding to coated AcBSA, chitin or BSA by using the monoclonal rat anti-mouse FcnB antibody IA4. Data are given as mean ± SD of 3 independent technical replicates.

3.3.7 Binding to DNA from *E. coli*

During the last years experiments demonstrated binding of ficolins to apoptotic cells (recently also published in Schmid, Hunold et al. 2011 by our group for FcnB) and help in the clearance of dying cells. One assumed ligand in binding to these apoptotic cells is DNA. Therefore, DNA was prepared from *E. coli* and used as ligand in binding assays.

The assay was performed in the same way as described above. DNA from *E. coli* was coated in a final concentration of 10µg/ml and FcnB binding was determined by ELISA. The data reveal binding of FcnB to AcBSA in a dose-dependent manner. BSA served as a negative control (see Fig. 48).

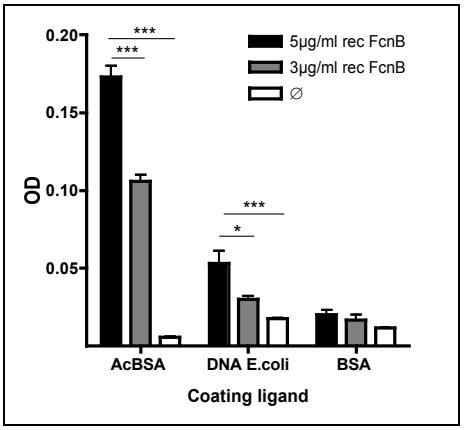

Figure 48: Binding of recombinant FcnB to AcBSA and to DNA from *E. coli*.

Recombinant FcnB at different concentrations was analyzed for the binding to coated AcBSA, DNA from *E. coli* (10µg/ml) or BSA by using the monoclonal rat anti-mouse FcnB antibody IA4. Data are given as mean ± SD of 3 independent technical replicates.

3.3.7.1 Inhibition of FcnB binding to AcBSA by DNA

Ficolins have been shown to bind to dying host cells. It is thought that DNA serves as a possible ligand. To test whether recombinant FcnB binds to DNA, inhibition assays were performed with DNA. The ELISA was performed using AcBSA for

coating. Recombinant FcnB was pre-incubated for 4h with buffer containing DNA from *E. coli*. Pre-incubation with DNA was shown to inhibit the binding of FcnB to AcBSA in the ELISA in a dose-dependent manner. The results also demonstrate again that binding of FcnB to AcBSA was inhibited in the presence of EDTA plus acetate. This leads also to the conclusion that DNA is a ligand for recombinant FcnB and competes with the binding of recombinant FcnB to AcBSA (see Fig. 49).

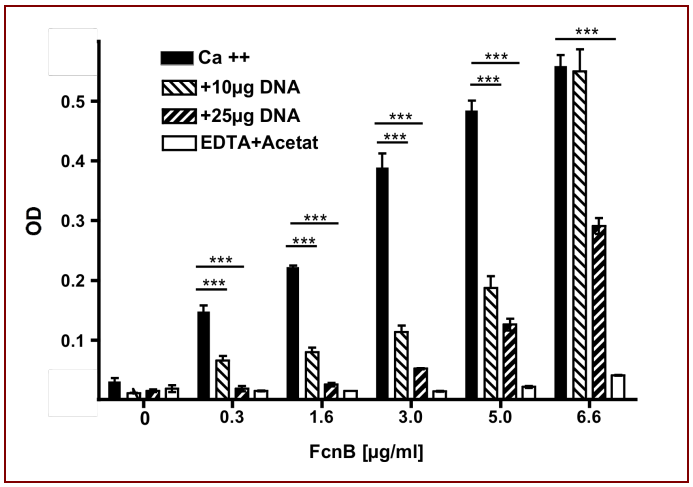

Figure 49: Binding inhibition of recombinant FcnB to AcBSA by DNA.

Recombinant FcnB was either pre-incubated with different concentrations of DNA (*E. coli*) or directly added to the plate either in a calcium-containing buffer or in the presence of EDTA/acetate. DNA reduced the binding of recombinant FcnB to AcBSA dose-dependently. Binding of FcnB to AcBSA was found to be calcium-dependent. Results are given as mean ± SD of 3 independent technical replicates.

3.3.8 Binding to bacteria

3.3.8.1 Binding to *S. aureus*

Direct binding of FcnB to bacteria was tested by adding a constant amount of FcnB to different strains of *S. aureus* to allow for binding. After removal of the bacteria the amount of FcnB left in the supernatant was determined by TRIFMA using biotinylated antibodies specific for FcnB. Only *S. aureus* strain T-5 reproducibly absorbed FcnB from the supernatant (see Fig. 50). 15 % of 5µg/ml recombinant FcnB used for binding was absorbed by the bacteria. This means that around 0.75 µg FcnB were bound by 10^8 *S. aureus* T-5.

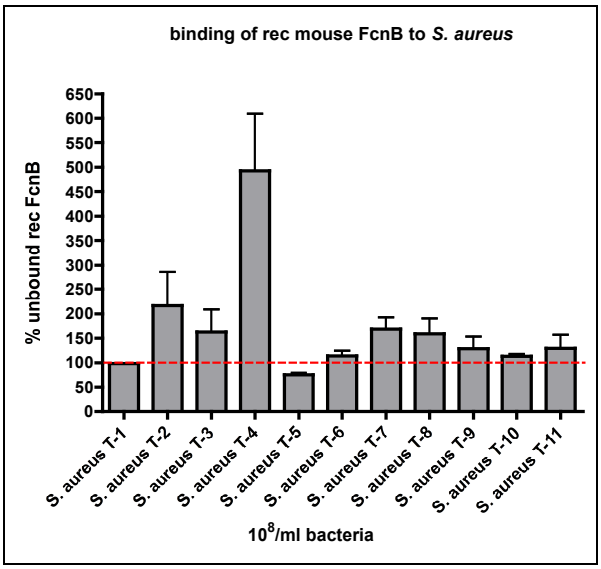

Figure: 50: Binding of recombinant FcnB to various strains of *S. aureus*.

A constant amount of recombinant FcnB (5µg/ml) was used for the absorption by different strains of *S. aureus*. Each bar represents the mean percentage of unbound FcnB from independent single experiments. The amount of recombinant FcnB without bacteria was set to 100%.

3.3.8.2 Binding to Group B *Streptococcus* (GBS)

As with *S. aureus*, it was tested whether different serotypes of Group B *Streptococcus* would absorb FcnB from the medium.
The binding experiments revealed binding of FcnB to nearly all tested GBS strains, except GBS B848/64 and GBS VIII. The binding of recombinant FcnB to GBS serotype B848/64 was expected to be negative, because this strain is the only tested non-capsulated GBS strain. It has been found previously that also other ficolins did not bind to capsulated strains. With the exception of GBS B848/64 and VIII all other Streptococci removed about 20% of the offered FcnB, which means that about 1µg FcnB was bound by 10^8 GBS (see Fig. 51).

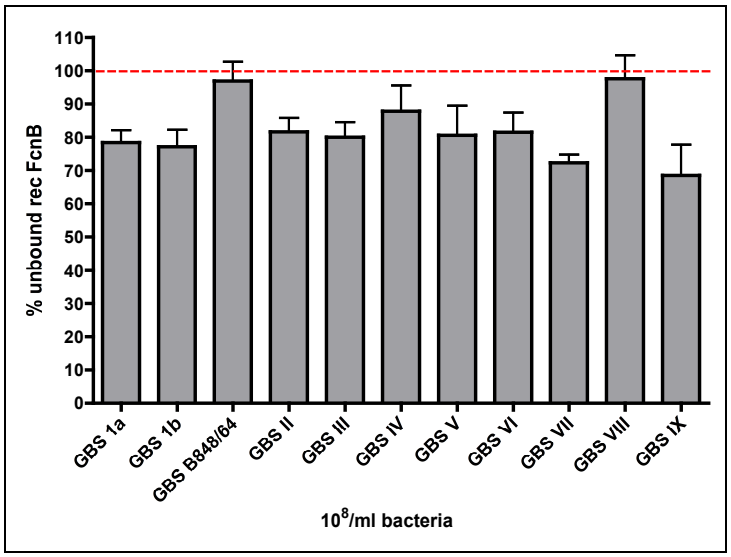

Figure: 51: Binding of recombinant FcnB to various strains of GBS.

A constant amount of recombinant FcnB (5µg/ml) was used for the absorption by different strains of GBS. Each bar represents the mean percentage of unbound FcnB from one independent single experiment. The amount of recombinant FcnB without bacteria was set to 100%.

3.4 Recombinant FcnB and complement activation

To investigate the capacity of recombinant FcnB to activate the lectin pathway, FcnB was tested for the interaction with recombinant human MASP-2 and the ability to lead to C4-deposition due to the activation of MASP-2.

3.4.1 Interaction of FcnB with MASP-2

Recombinantly FcnB, bound to AcBSA, was tested for the interaction with recombinant human MASP-2 (kindly provided by Prof. Jensenius, Aarhus, Denmark). The data revealed binding of FcnB to recombinant human MASP-2 in a dose-dependent manner. The signals were lower compared to those observed with recombinant human M-Ficolin. Recombinant mouse Ficolin A (FcnA) bound to recombinant human MASP-2 with a similar signal intensity as recombinant FcnB. Binding of all ficolins to AcBSA and subsequent binding of MASP-2 were calcium-dependent (see Fig. 52).

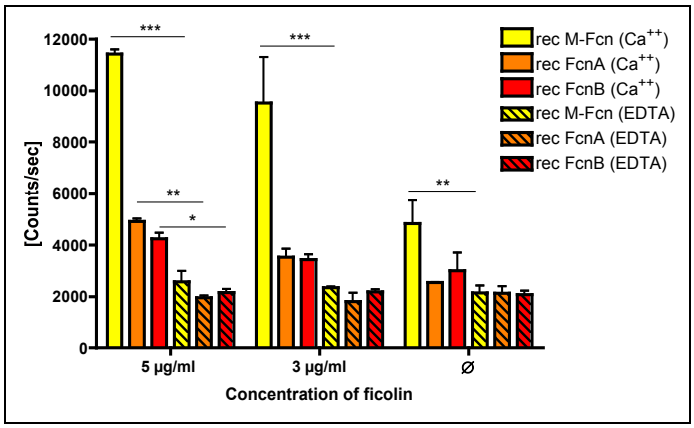

Figure 52: Binding of human and mouse ficolins to MASP-2.

Recombinant human M-ficolin and recombinant mouse ficolins (FcnA and FcnB) were tested for binding to recombinant human MASP-2 in TRIFMA analysis. The binding of MASP-2 to the ficolin was either performed in a calcium-containing buffer or in the presence of EDTA. All tested ficolins were found to bind to MASP-2 in a ficolin-dose-dependent manner. Further, the interaction between MASP-2 and ficolin was found to be calcium-dependent. The interaction between the ficolin and MASP-2 is abolished when EDTA is present in the binding buffer (striped bars). Results are given as mean ± SD of 3 technical replicates. This experiment was repeated at least 2 times. Statistical analyses were performed using Two-way ANOVA.

3.4.2 Activation of the lectin pathway by FcnB

MASP-2 cleaves complement factors C4 and C2 to form the complement convertase C3 (complex of C4bC2a). and generates the cleavage products C4a and C4b. These by-products can be measured by the C4-deposition assays to verify complement activation. To investigate, if FcnB is able to activate the lectin pathway of the complement system, C4 deposition assays were performed. Recombinant human M-ficolin or recombinant mouse FcnB were added to plates coated with AcBSA before recombinant human MASP-2 was added. The addition of recombinant C4 protein leads to cleavage of C4 when MASP-2 is activated by the interaction with the corresponding ficolin and C4-deposition can be measured. Results of the C4-deposition measured by TRIFMA revealed that both ficolins, recombinant human M-ficolin and recombinant mouse FcnB, were able to lead to C4 deposition (see Fig. 52 A). In the two experiments performed recombinant human M-ficolin seemed to be more effective compared to recombinant mouse FcnB. No C4-deposition was observed, when ficolins were added to BSA-coated plates (see Fig. 52 B).

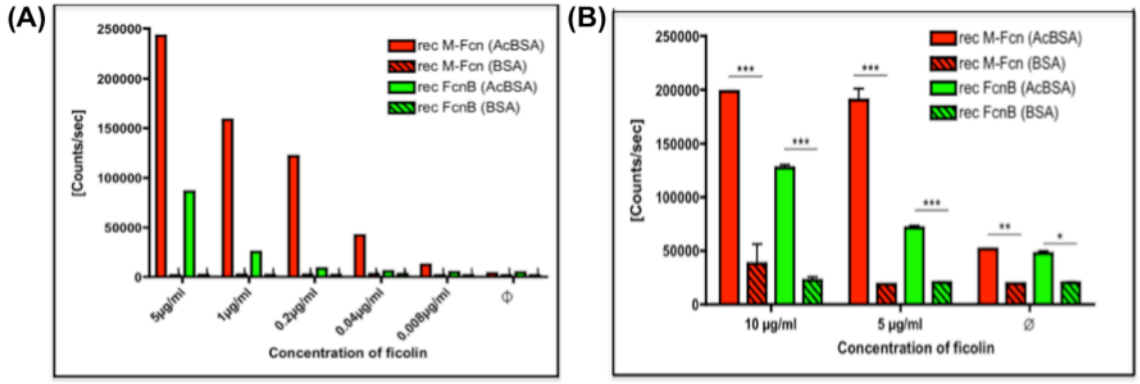

Figure 53: C4-deposition after the binding of ficolin and MASP-2.

Recombinant human M-Ficolin or mouse FcnB (at the indicated concentrations), recombinant human MASP-2 SUT (500ng/ml), and C4 (1.6µg/ml) were added to the AcBSA or BSA coated plastic. C4-deposition was measured by TRIFMA. Data in (A) represent single values of one experiment with one replicate and in (B) data are given as mean ± SD of 3 independent technical replicates.

To verify binding and activation of recombinant human MASP-2 to recombinant mouse FcnB in TRIFMA, it was analyzed whether the binding can be inhibited. Formation of the complex of MASP-2 and FcnB on AcBSA coated plastic and the subsequent cleavage of C4 was calcium-dependent (see Fig. 54).

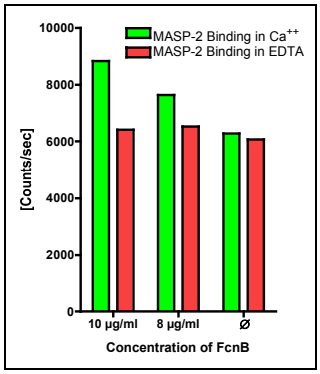

Figure 54: Calcium-dependency of C4-deposition by FcnB.

Recombinant mouse FcnB (at the indicated concentrations), recombinant human MASP-2 SUT (500ng/ml), and C4 (1.6µg/ml) were added to AcBSA coated plastic. C4-deposition was measured by TRIFMA. The experiment was performed either in calcium-containing buffer (green bars) or in the presence of EDTA (red bars). The data represent one single experiment with one replicate.

Since recombinant FcnB was demonstrated to bind to *S. aureus* serotype T-5, it was tested whether FcnB bound to *S. aureus* T-5 can also induce complement activation. For this purpose, the bacteria were used to allow for the binding of recombinant FcnB and the MASP-2 interaction and C4-deposition was tested by TRIFMA.

The complex of recombinant FcnB with recombinant human MASP-2 was significantly shown to form complexes on *S. aureus* serotype T-5 and enabled C4-deposition. In contrast, no C4-deposition was observed on *S. aureus* serotype T-3 or T-9. Recombinant FcnB was found not to bind to these serotypes as already seen in previous TRIFMA experiments (see Fig. 55).

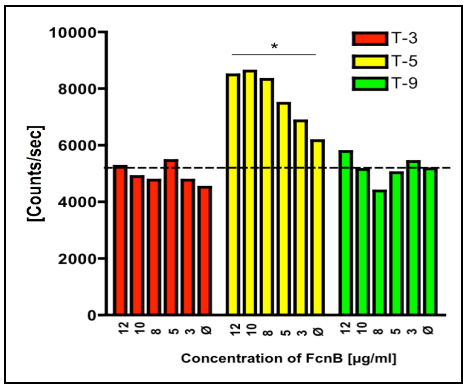

Figure 55: C4-deposition on different *S. aureus* serotypes.

Recombinant mouse FcnB (at different concentrations), recombinant human MASP-2 SUT (1µg/ml), and C4 (1.6µg/ml) were added to coated *S. aureus* serotype T-5 (green bars), T-3 (yellow bars) or T-9 (red bars) and the C4-deposition was measured by TRIFMA. Bacteria were coated at a concentration of 10^8 bacteria/ml. Data represent one single experiment.

In previous experiments, FcnB has been shown to bind to different strains of Group B *Streptococcus*. Therefore it was investigated whether FcnB is able to induce C4 deposition on these bacteria. A selection of strains, which has been shown to strongly bind to FcnB, was analyzed in a C4-deposition assay. Even though the signals were not very high, it seemed that FcnB bound to GBS strain II, strain III and strain IV and initiated C4-deposition, however, the background signals when the assay was performed with GBS strain 1a were very high (see Fig. 56).

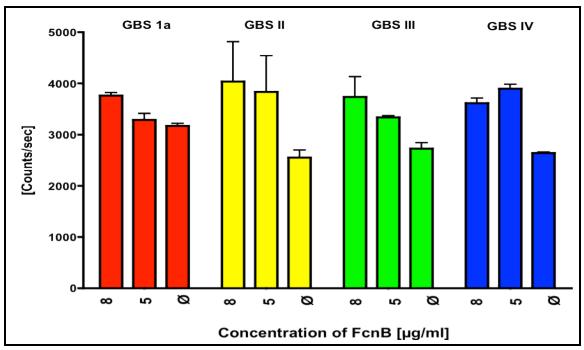

Figure 56: C4-deposition on different GBS serotypes.

Recombinant mouse FcnB (at indicated concentrations), recombinant human MASP-2 SUT (1µg/ml), and C4 (1.6µg/ml) were added to coated GBS serotype 1a, serotype II, serotype III and serotype IV and C4-deposition was measured by TRIFMA. The bacteria were coated in a concentration of 10^8 bacteria/ml. Data represent mean ± SD of 3 independent technical replicates.

3.5 Generation of monoclonal rat anti-mouse FcnB antibodies

3.5.1 Immunization of rats

For the generation of rat anti-mouse FcnB antibodies, three rats with different genetic background were immunized in parallel with recombinant mouse FcnB produced in DS-2 cells. The recombinant protein was tagged with a V5-His-tag. This tag enabled an easy purification over corresponding affinity columns, but at the same time increased the risk to generate antibodies against the tag.

The sera of the three rats were tested by ELISA for their specific production of IgG after immunization against recombinant mouse FcnB. The ELISA demonstrated that the IgG production of all three rat sera was specific for the immunized antigen. The pre-immunization-sera did not show any signal when no antigen but coating buffer alone was present in the well. All three sera showed similar OD signals against the coated recombinant mouse FcnB protein when tested at the same dilution demonstrating a similar immune response by the three rats (see Fig. 57).

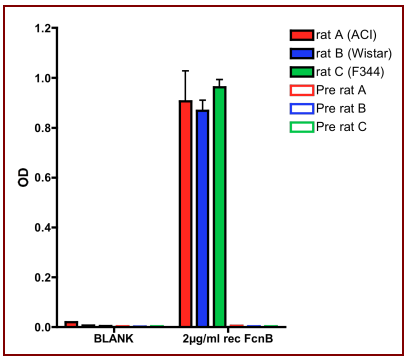

Figure 57: Antigen-specific titer after the first immunization.
Recombinant FcnB (2µg/ml) was coated to plastic and the antisera of the rats (at a 1:1000 dilution) were tested for their interaction with FcnB by ELISA. The BLANK indicates that no FcnB was applied. Results are given as mean ± SD from 3 independent technical replicates with sera from rat ACI (red bar), rat Wistar (blue bar) and rat F344 (green bar).

When different concentrations of FcnB had been applied to the membrane, the anti-sera revealed a very weak non-specific binding of the pre-immunization-sera to FcnB in a dot blot. From the three antisera generated, the antiserum of rat ACI was found

to be best in recognizing FcnB in the dot blot assay. It showed detection of FcnB in a dose-dependent manner detecting 2.5 ng of FcnB at a 1:10.000 dilution of the anti-serum. The antisera did not bind to BSA, which served as a negative control (see Fig. 58).

The antiserum of rat Wistar showed detection of the antigen in a dose-dependent manner detecting 20 ng of FcnB at a 1:10.000 dilution of the antiserum. Also, as seen for the rat ACI, the antiserum from rat Wistar did not non-specific bind to BSA (see Fig. 58).

The antiserum of rat F344 showed the weakest detection signals to FcnB, compared to the other antisera. The antiserum from rat F344 recognized only 20 ng/ml of FcnB at a 1:10.000 dilution of the anti-serum (see Fig. 58).

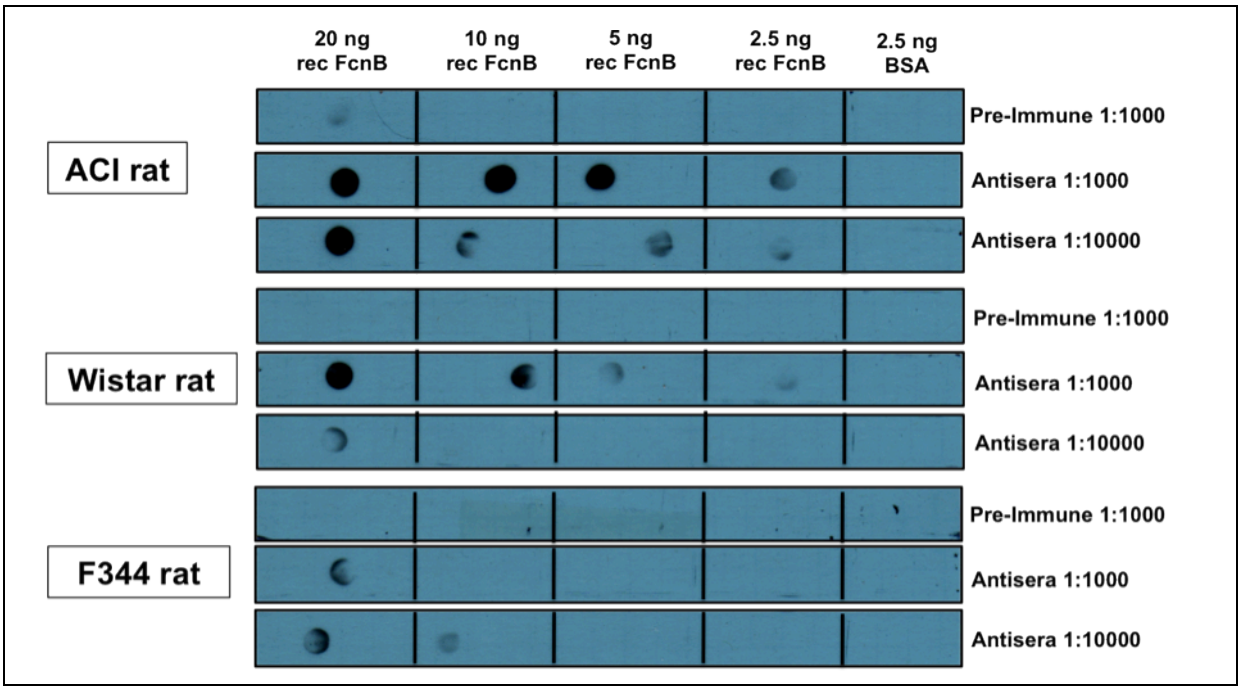

Figure 58: Detection of FcnB by antisera in a dot blot assay.

Different amounts (as indicated) of FcnB were spotted onto nitrocellulose-membrane and exposed to different dilutions (as indicated) of the pre-immune- and antisera of a ACI (A), Wistar (B), and F344 (C) rat, respectively.

To further determine the specificity of the antisera, recognition of recombinant FcnB was analyzed in Western Blot analysis. The Western blot showed that the antisera of the Wistar and ACI rat recognized the recombinant protein (see Fig. 59). At least they detected the monomer of FcnB, running at 35 kDa. The antiserum of the F344 rat did not recognize FcnB in the Western blot.

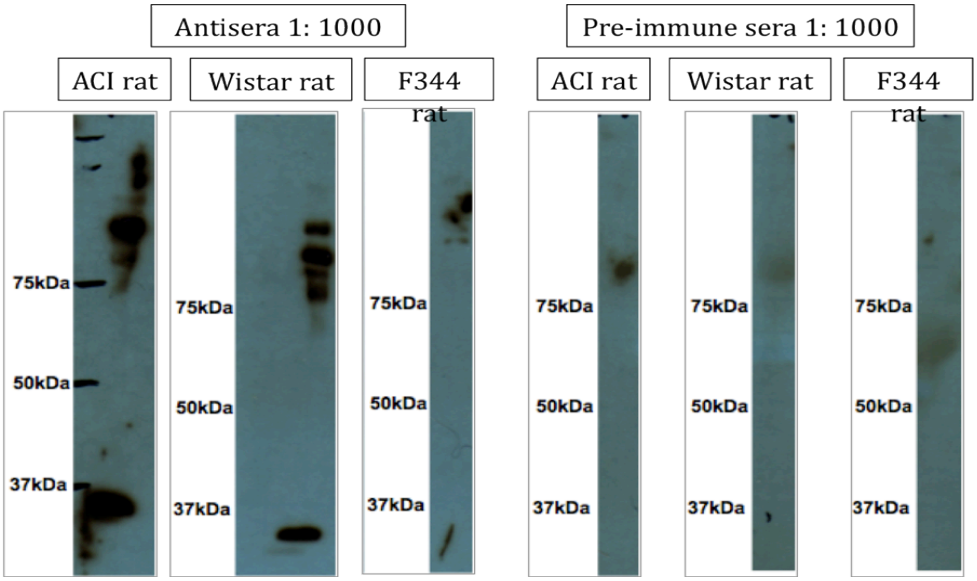

Figure 59: Detection of FcnB in a Western blot assay.

Recombinant FcnB (5µg/ml) was applied to a 10% SDS PAGE and the protein was separated under non-reducing conditions. After blotting the membrane was exposed to the pre-immune- and the antisera of the different rats.

When the antisera were tested after the first boost by ELISA, the data revealed a strong, specific response for all three antisera. The antiserum of the ACI rat showed nearly identical signals as directly after the immunization. The responses of the Wistar rat and the F344 rat showed higher signals after the boost. The antiserum of the ACI rat was positive at a dilution 1:64.000 (see Fig. 60).

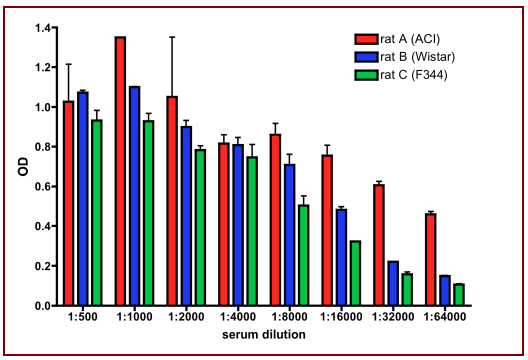

Figure 60: Anti-FcnB titer after the first boost.

Recombinant FcnB (2µg/ml) was coated to plastic and the antisera of the rats (diluted in a logarithmic manner) were tested for their interaction with FcnB by ELISA. Results are given as mean ± SD from 3 independent technical replicates with sera from rat ACI (red bar), rat Wistar (blue bar) and rat F344 (green bar).

After the second boost the antibody response of the ACI and the Wistar rat were further enhanced. The reaction of the antiserum of the F344 rat was not changed after the third injection with the antigen. Strong antibody titers were now obtained with the antisera of the ACI and the Wistar rat (see Fig. 61).

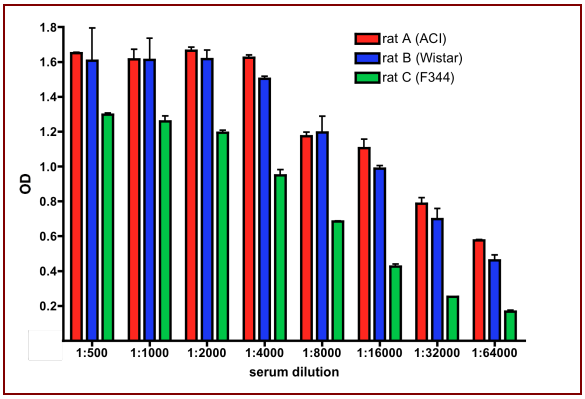

Figure 61: Anti-FcnB titer after the second boost.

Recombinant FcnB (2µg/ml) was coated to plastic and the antisera of the rats (diluted in a logarithmic manner) were tested for their interaction with FcnB by ELISA. Results are given as mean ± SD from 3 independent technical replicates with sera from rat ACI (red bar), rat Wistar (blue bar) and rat F344 (green bar).

Although all three rats had responded to the immunization with recombinant FcnB, the ACI and the Wistar rats had higher antibody titers after the first and the second boost; a third boost did not significantly improve the antibody titer in the F344 rat (see Fig. 62).

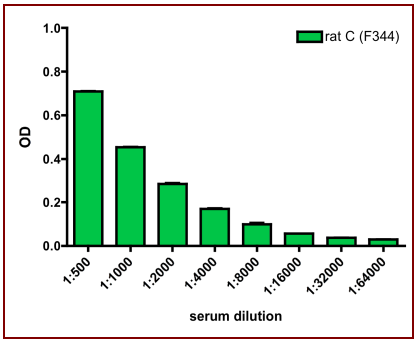

Figure 62: Anti-FcnB titer after the third boost.

Recombinant FcnB (2µg/ml) was coated to plastic and the antisera of the F344 rat (diluted in a logarithmic manner) was tested for the interaction with FcnB by ELISA. Data present single values of one experiment.

The specificity of the antigen response of all three rat anti-sera were tested to determine whether their reaction was raised against the FcnB or against the V5-His-tag. Due to the fact that the rats were immunized with the recombinant protein carrying a highly immunogenic tag, it was necessary to exclude such reactions. Therefore ELISA plates were coated with another protein, which also contained the same tag. In this case the protein consisted of the tagged extracellular domain of the TNF receptor type 2 (TNFR2). The antisera from all three rats reacted strongly with the FcnB-V5-His-tag. The antiserum of the F344 rat showed a lower FcnB response. The antisera of both the ACI and the Wistar rat showed cross-reactivity with the V5-His-tagged control protein. This demonstrated that both anti-sera contain also antibodies raised against the tag. But still the response towards the recombinant mouse FcnB was more than three-fold higher (see Fig. 63).

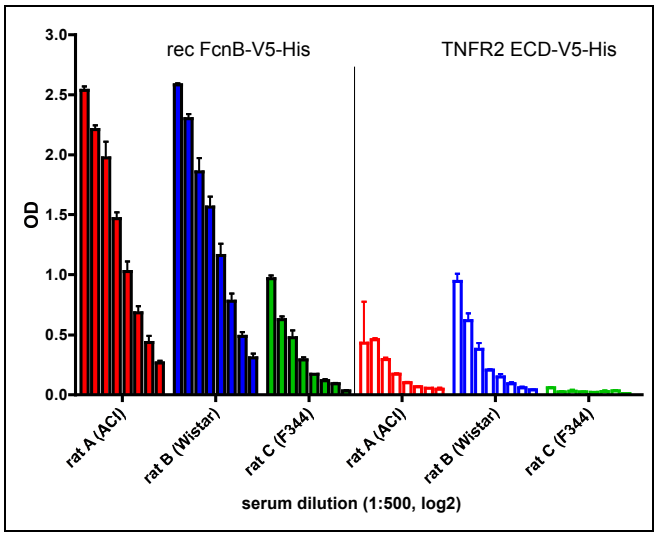

Figure 63: ELISA for cross-reaction of the anti-sera with the V5-His-tag.

Recombinant FcnB (2µg/ml) or the control protein (2µg/ml TNFR2 ECD-V5-His) were coated on plastic and the antisera (1:500. Log 2 diluted) of the rats were applied in the ELISA for the detection of antigen-specific antibodies. Data are given as mean ± SD of three independent technical replicates.

In order to further test for cross-reaction all three antisera were tested in a dot blot assay for recognition of recombinant mouse FcnA. The anti-sera of the ACI rat and the Wistar rat showed a specific response only to FcnB, but no cross-reaction with FcnA (see Fig. 64). The anti-serum of the F344 rat was not tested because it had only a weak ELISA titer.

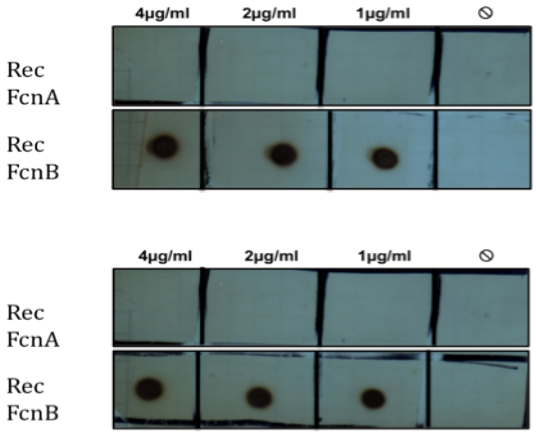

Figure 64: Cross-reaction of the anti-FcnB antisera with FcnA by Dot blot analysis.
Recombinant FcnA and FcnB (at indicated concentrations) were coated to nitrocellulose and the antisera of ACI rat or Wistar rat (diluted 1:1000) were tested for their interaction with FcnA and FcnB by Dot blot analysis.

3.5.2 Generation of monoclonal rat anti-mouse FcnB antibodies

For the generation of monoclonal rat anti-mouse FcnB antibodies spleen cells of the corresponding rat immunized with recombinant FcnB-V5-His-tagged protein were fused with myeloma cells. The resulting clones were tested for their ability to recognize recombinant FcnB. Positive clones were subcloned and further analyzed. All generated monoclonal rat anti-mouse FcnB antibodies are listed in the table below.

Antibody	Rat	Isotype	Fusion
2F1 B6 B6	Wistar	IgG2a	Oct 2009
2F1 B6 E2	Wistar	IgG2a	Oct 2009
2F1 C7 G4	Wistar	IgG2a	Oct 2009
16D3 D6	Wistar	IgG2a	Oct 2009
16G3 C2	Wistar	IgG2a	Oct 2009
11A1 A10	ACI	IgG2a	Sept 2009
11A1 B11	ACI	IgG2a	Sept 2009
11A1 C7 A5	ACI	IgG2a	Sept 2009

Tab. 9: Overview of generated monoclonal rat anti-mouse FcnB antibodies.

3.5.3 Isotype determination of the monoclonal rat anti-mouse FcnB antibodies

In order to determine to which antibody subclass the respective monoclonal rat anti-mouse FcnB antibody belongs, an isotype-ELISA was performed. The ELISA data demonstrate that all generated monoclonal anti FcnB antibodies were of the IgG2b isotype (see Fig. 65).

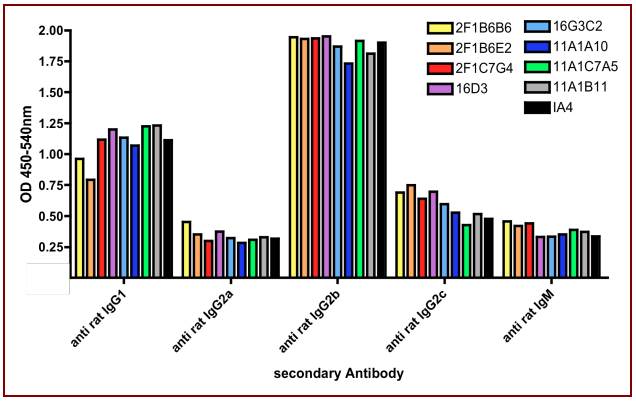

Figure 65: Isotype determination of the monoclonal rat anti-mouse FcnB antibodies.
Recombinant FcnB (5µg/ml) was coated to plastic and anti-mouse FcnB antibodies were added for the detection of FcnB by ELISA. Different secondary goat-anti rat IgG and IgM (as indicated) antibodies were used for the determination of the isotype of the corresponding anti-FcnB antibody. Data are given as values from one single experiment with one replicate.

3.5.4 Establishment of a sandwich-ELISA

By testing each antibody against the others the epitope of the respective antibody should be identified. In this way antibodies should be determined that are useful to act as “capture” or as ”detection” reagent. Therefore, all monoclonal antibodies needed to be biotinylated. Non-biotinylated antibodies were used as “capture” antibodies and biotinylated antibodies were used for ”detection” of bound FcnB (see Fig. 66). Signals-to-noise ratios were calculated to identify proper antibody combinations. After analysis of all combinations of unlabeled and biotinylated rat anti-mouse FcnB monoclonal antibodies, 2F1C7 G4 and 16G3 C2 were selected as antibodies for coating (”capture” antibodies) and 11A1C7A5 and 11A1B11B8 were selected for ”detection”. Also a good choice for detection was the 16D3 antibody even if it has to be kept in mind that the background signals are very high when using this antibody as a ”capture” antibody.

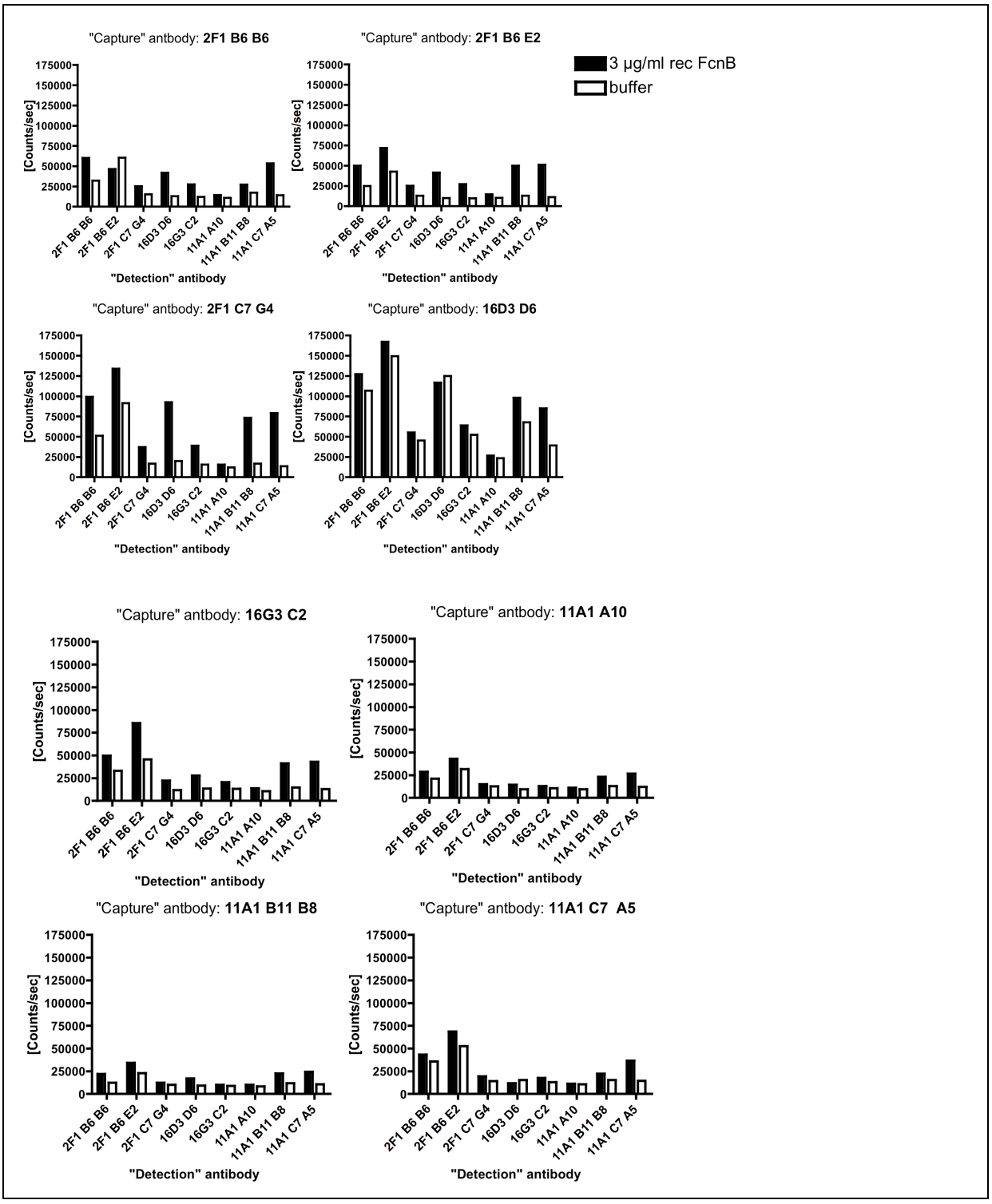

Figure 66: Sandwich-ELISA establishment.

All combinations of unlabeled and biotinylated rat anti-mouse FcnB monoclonal antibodies were tested against FcnB. Thereby the unlabeled rat anti-mouse FcnB antibodies (1µg/ml) were coated to plastic, FcnB (2µg/ml) was added and the detection of FcnB was performed using the corresponding biotinylated rat anti-mouse FcnB antibodies (1µg/ml).

3.5.5 Detection of recombinant mouse FcnB in Western Blot

To test whether the generated monoclonal rat anti-mouse FcnB antibodies detect the recombinant protein in Western blot analysis and to further characterize the anti-FcnB monoclonal antibodies, Western blot analyses were performed.
As seen in the following figures (Fig. 67 an 68), all anti-FcnB monoclonal antibodies recognized at least the monomer of FcnB (35 kDa) when separated on the gel under reducing conditions but also higher oligomeric forms especially when separated under non-reducing conditions. The monoclonal antibodies differed in their recognition pattern with some detecting preferentially the lower oligomers whereas others recognized higher oligomeric forms of FcnB.

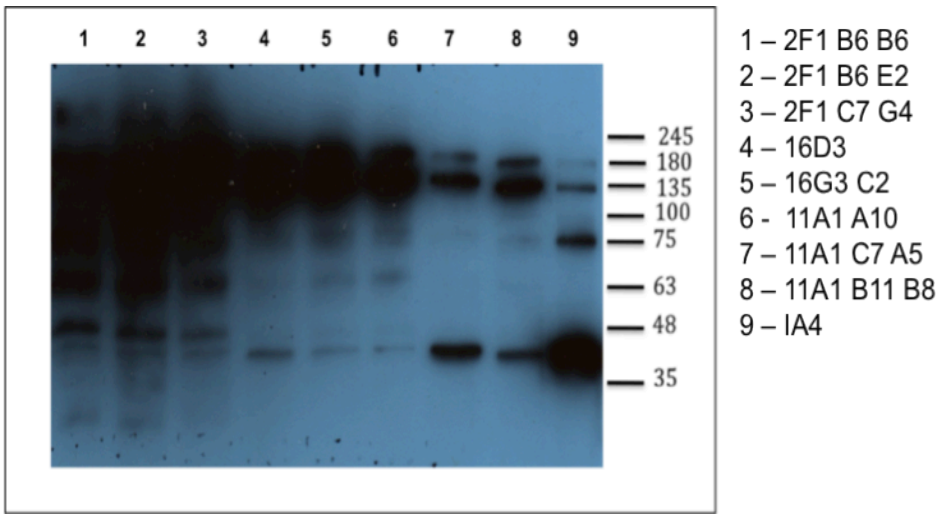

Figure 67: Western Blot analysis for recombinant FcnB under non-reducing conditions.
Recombinant FcnB (5µg/ml) was loaded onto a 10% SDS-PAGE and the run was performed under non-reducing conditions. After the run the protein was blotted onto nitrocellulose and FcnB was detected using the monoclonal rat anti-mouse FcnB antibodies as indicated (5µg/ml).

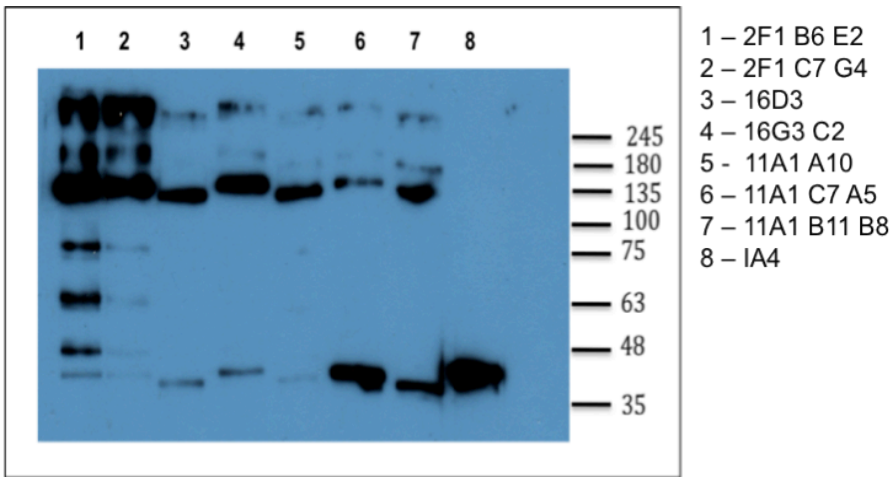

Figure 68: Western Blot analysis for recombinant FcnB under reducing conditions.

Recombinant FcnB (5µg/ml) was loaded onto a 10% SDS-PAGE and the run was performed under reducing conditions. After the run the protein was blotted onto nitrocellulose and FcnB was detected using the monoclonal rat anti-mouse FcnB antibodies as indicated (5µg/ml).

3.6 Detection of native FcnB

3.6.1 Detection of mouse FcnB in Western blot

After having determined the mRNA expression levels of FcnB in different cell types, the respective cell lysates were analyzed in Western blot assays to test for FcnB on the protein level by detection with the monoclonal antibodies.
BMDM were generated and cell lysates of LPS-stimulated or non-stimulated cells were prepared. Native FcnB was detected at an apparent molecular weight of about 55 kDa. FcnB seemed to be constitutively expressed in BMDM. Stimulation with LPS did not further enhance the expression (mice 1, see Fig. 69 A). In contrast, FcnB expression in BMDM of another mice seemed to be induced by stimulation with LPS (mice 2, see Fig. 69 B).

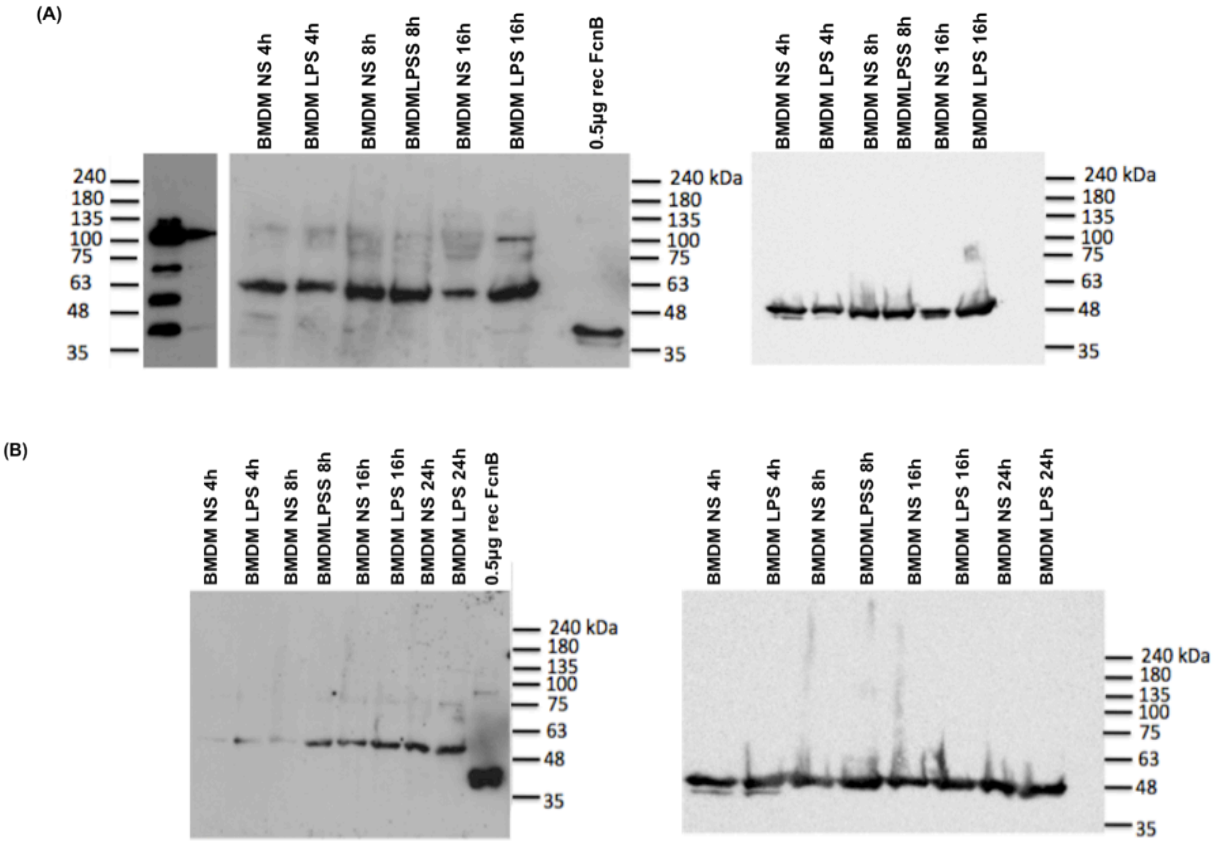

Figure 69: Detection of native FcnB in protein lysates from BMDM.
BMDM were either stimulated with LPS (*E. coli*, 100ng/ml) for indicated times or left non-stimulated and cell lysates were prepared. The cell lysates from two different mice (A and B) were separated onto a 10% SDS-PAGE under reducing conditions and after blotting exposed to the monoclonal rat anti-mouse FcnB antibody (A) (IA4, 5µg/ml). β-actin (B) was used as a control for equal loading.

Lysates of PMN isolated from the bone marrow were also analyzed by Western blot. Also in lysates of primary PMN native FcnB was detected by the specific anti-FcnB antibody IA4. However, the apparent molecular weight after separation on a SDS-PAGE under reducing conditions was about 35 kDa similar to the molecular weight seen with the recombinant FcnB (see Fig. 70).

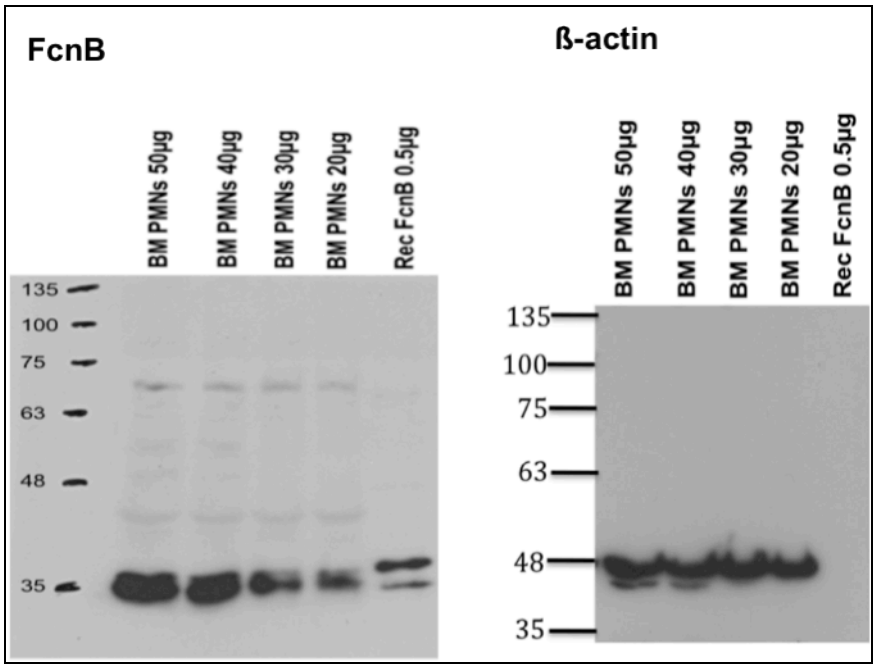

Figure 70: Detection of native FcnB in protein lysates from PMN.

PMN were isolated from the bone marrow and protein lysates were prepared. The cell lysates were loaded at different concentrations (as indicated) onto a 10% SDS-PAGE and separated under reducing conditions. After blotting the samples were exposed to the monoclonal rat anti-mouse FcnB antibody (IA4, 5µg/ml). β-actin was used as a control for equal loading.

Protein lysates from PMN were analyzed by using all generated anti-FcnB monoclonal antibodies for detection of native FcnB in Western blot. All monoclonal antibodies detected the monomer of FcnB. Therefore, all monoclonal antibodies are useful for the detection of native FcnB (see Fig. 71).

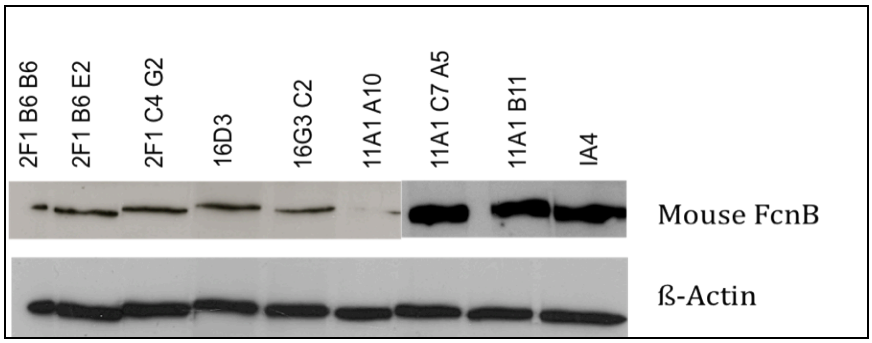

Figure 71: Detection of native FcnB in PMN (by all monoclonal anti-FcnB antibodies).

PMN isolated from the bone marrow were separated on 10% SDS-PAGE under reducing conditions and exposed to all anti-FcnB monoclonal antibodies for detection. Staining for β-actin was used to control equal loading.

3.6.2 Detection of mouse FcnB in serum

To investigate whether mouse FcnB is present in serum, serum samples from different mouse strains were analyzed by TRIFMA (the samples were obtained from Prof. Jensenius laboratory, Aarhus, Denmark). With the positive control of recombinant mouse FcnB-V5-His protein a nice dilution curve was obtained. Serum of Balb/c mice seemed to contain a high concentration of FcnB. In contrast, no FcnB was detected in the sera of all other tested mouse strains (see Fig. 72).

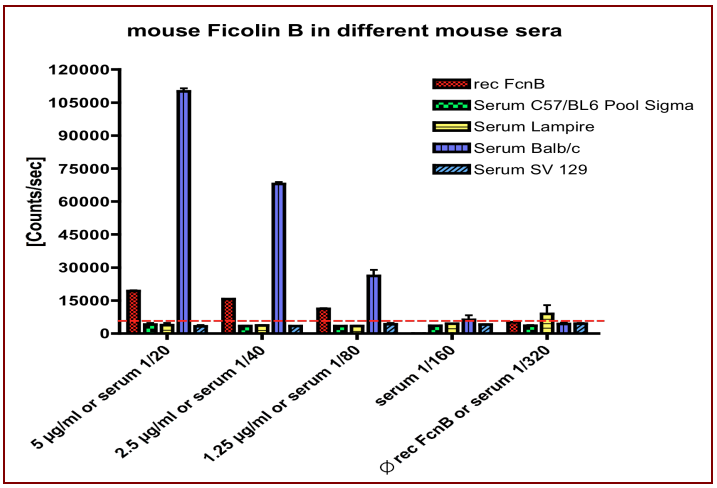

Figure 72: Detection of mouse FcnB in serum.

Sera from different mouse strains were analyzed for FcnB by TRIFMA. Sera (at indicated dilutions) were added to AcBSA coated plates and bound FcnB was detected using a biotinylated monoclonal rat anti-FcnB antibody (1µg/ml 11A1 C7-Bio). Recombinant FcnB (at indicated concentrations) was used as a positive control. Data are given as mean ± SD of 3 independent technical replicates.

To confirm and further characterize this finding, the TRIFMA was performed with the serum from Balb/c mice in different buffers.
The results verified the previous finding, that FcnB was detectable in serum of Balb/c mice. When binding was performed in EDTA-containing buffer, the signals were significantly reduced, demonstrating that the binding was calcium-dependent (see Fig. 73).

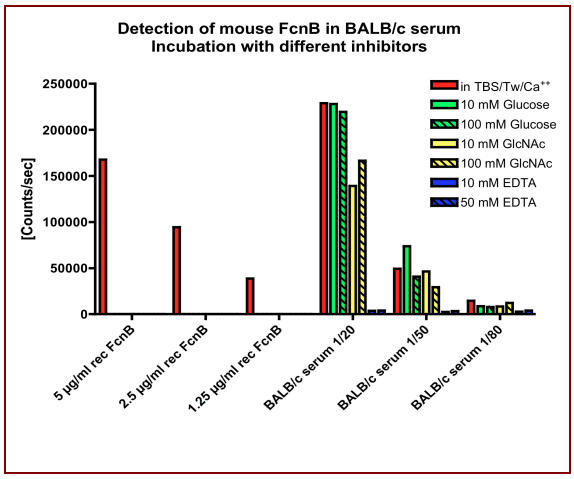

Figure 73: Calcium-dependent binding of serum-FcnB to AcBSA by TRIFMA.

Serum from Balb/c mice was analyzed for FcnB by TRIFMA. The serum (at indicated dilutions) was added to AcBSA coated plastic and incubated either in calcium-containing buffer, in glucose-containing buffer, in GlcNAc-containing buffer, or in the presence of EDTA. Bound FcnB was detected using a biotinylated monoclonal rat anti-FcnB antibody (1µg/ml 11A1 C7-Bio). Recombinant FcnB (at indicated concentrations) was used as a positive control. Data are given as single values of one experiment.

3.7 Localization of FcnB in primary cells

RT-PCR analysis had demonstrated that cells of the myeloid linage expressed FcnB and Western blot analysis had shown that FcnB protein was present in the cell lysates. For the role of FcnB in the innate immune system it is not only necessary to identify the producers of the lectin but also to know its localization.

For these studies PMN isolated from bone marrow were analyzed by confocal microscopy. The experiments were done in the Institute of Medical Immunology, Buenos Aires, Argentina (in collaboration with Dr. Analiá Trevani and Dr. Gabriela Salamoné).

The PMN were stimulated with PMA to induce NET formation. The NET structures were stained with specific antibodies against mouse elastase, mouse nucleosome and DAPI was used to identify DNA. FcnB was detected with the biotinylated monoclonal rat anti-FcnB antibody 11A1 C7. Non-stimulated cells served as a negative control, since NET were formed only after stimulation. Confocal microscopy demonstrated that NET were induced by PMA stimulation after 16h (see Fig. 74). NET were characterized by positive staining with the anti nucleosome antibody. Overlay of the anti-nucleosome staining and the DAPI staining revealed that DNA and nucleosomes co-localized, which is typical for NET structures. Specific staining for FcnB showed the presence of FcnB within some PMN, but was not detected on the NET structures. The staining with the monoclonal rat anti-mouse FcnB antibody 11A1 C7 was specific. No signals were obtained with the corresponding isotype control.

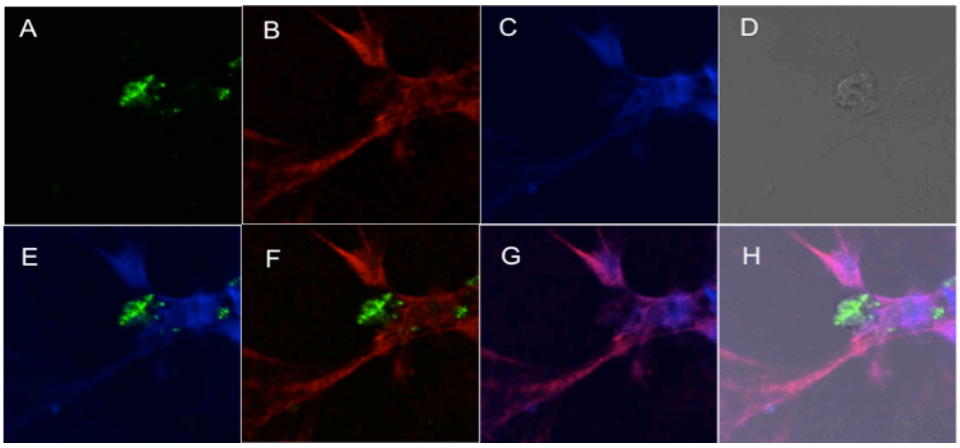

Figure 74: NET formation by PMN.

PMN were isolated from Balb/c bone marrow and stimulated for 16h with PMA. The staining was performed using antibodies against mouse nucleosome and against FcnB (1µg/ml 11A1 C7-bio). ToPro3 was used for DNA staining. Single stainings and overlays are shown. (A) Anti-FcnB 11A1C7-Bio, (B) anti-mouse nucleosome, (C) DNA-staining with ToPro3, (D) White field, (E) Overlay of DNA and FcnB (F) Overlay of nucleosome and FcnB G) Overlay of nucleosome and DNA, and (H) the overlay of nucleosome, DNA and FcnB. Magnification: 40x.

To further investigate the localization of FcnB, PMN were analyzed by confocal microscopy for the presence of FcnB inside of the cells. The results demonstrate the presence of FcnB inside stimulated PMN (see Fig. 75).

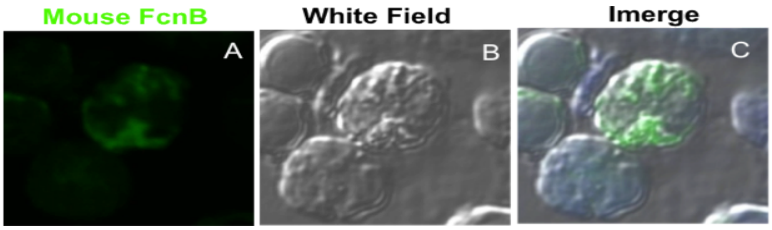

Figure 75: Detection of FcnB inside of PMN.

PMN were isolated from Balb/c bone marrow and stimulated for 16h with PMA. The staining was performed using the anti-FcnB 11A1 C7-bio (1µg/ml). ToPro3 was used for DNA staining. Single stainings and overlays are shown. (A) Anti-FcnB 11A1C7-Bio, (B) White field, (C) Overlay of DNA-staining with ToPro3 and FcnB. Magnification: 40x.

Co-localization studies with different lysosomal-specific antibodies were performed. FcnB was found to co-localize with structures recognized by a LysoRed antibody.

This antibody stains lysosomal structures. Thus, the FcnB protein seems to co-localize with the lysosomes (see Fig. 76).

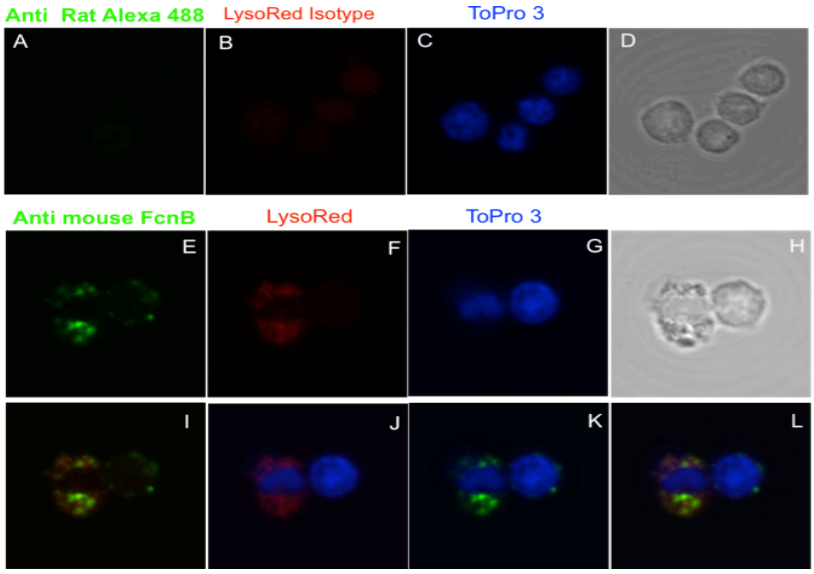

Figure 76: Co-localization of FcnB with LysoRED inside of PMN.

PMN were isolated from the bone marrow of Balb/c mice and stimulated with PMA for 16h. FcnB was stained by the monoclonal rat anti-mouse FcnB antibody 11A1 C7-biotinylated (1µg/ml). DNA was stained with ToPro3 and LysoRED was used for the detection of the lysosomal structures. Corresponding isotype controls are shown in (A) for FcnB and in (B) for LysoRED. DNA staining is shown in (C) and (G). Single stainings are shown in (E) for FcnB and in (F) for LysoRED. The corresponding overlays are shown in (I) for FcnB and LysoRED, (J) for DNA and LysoRED, (K) for FcnB and DNA and (L) for FcnB, DNA and LysoRED. White field pictures of the PMN are shown in (D) and (H). Magnification: 40x.

To support the localization of FcnB in lysosomal structures inside of PMN, additional confocal microscopy analysis was performed using an antibody for the detection of the lysosomes. In this case, a FITC-labeled LAMP-1 antibody was used to identify lysosomal structures. Again, the results showed that FcnB was found inside of stimulated bone marrow-derived PMN. FcnB was identified in lysosomes and stained together with LAMP-1-FITC, indicating the presence of FcnB in lysosomal structures (see Fig. 77). This suggests a possible role of FcnB inside PMN.

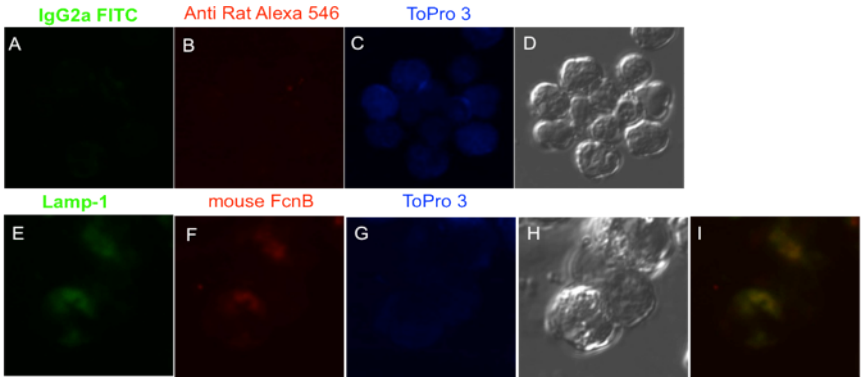

Figure 77: Co-localization of FcnB with LAMP-1 inside of PMN.

PMN were isolated from the bone marrow of Balb/c mice and stimulated with PMA for 16h. Corresponding isotype controls are shown in (A) for Lamp-1-FITC, (B) for FcnB. Further are shown in (C) DNA staining with ToPro3, in (D) Overview white field, in (E) LAMP-1-FITC, in (F) FcnB (1µg/ml 11A1 C7-biotinylated), in (G) DNA staining, in (H) Overview white field, and in (I) overlay of LAMP-1-FITC and FcnB. Magnification: 40x.

To study the role of FcnB in PMN stimulated with living *Pseudomonas aeruginosa* (FITC-labeled) additional confocal-microscopy experiments were performed. The results demonstrated again FcnB inside PMN. Interestingly, FcnB-positive cells seemed to be surrounded by the FITC-labeled bacteria (see Fig. 78). However, no NET formation was induced by *P. aeruginosa*.

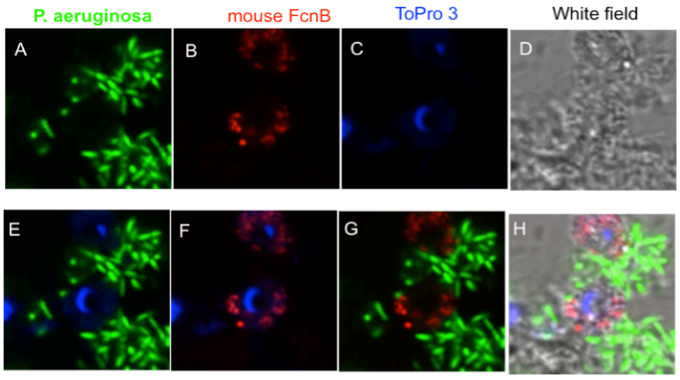

Figure 78: Detection of FcnB inside of PMN stimulated with *P. aeruginosa*.

PMN were isolated from the bone marrow of Balb/c mice and stimulated with *P. aeruginosa* for 16h. Shown are in (A) FITC-labeled bacteria *Pseudomonas aeruginosa*, in (B) FcnB (1µg/ml 11A1 C7-biotinylated), in (C) DNA staining by ToPro3, in (D) Overview white field, in (E) Overlay of DNA and FITC-labeled *P. aeruginosa*, in (F) Overlay of DNA and FcnB, in (G) Overlay of FcnB and FITC-labeled *P. aeruginosa* and in (H) Overlay of FITC-labeled P. *aeruginosa,* FcnB and DNA. Magnification: 40x.

4. Discussion

The innate immune system contributes a first line of defense mechanisms by which it recognizes invading pathogens and initiates inflammatory responses to eliminate them (Janeway and Medzhitov, 2002). Therefore receptors and recognition molecules are necessary to sense dangerous signals and to detect pathogen-associated-molecular-patterns (PAMPs). Up to now, several families of pattern-recognition molecules are identified and studied. Part of these families are the well-characterized TLRs, but also the lectins and collectins (Holmskov et al 2003). After binding to various pattern-associated structures these molecules help to trigger anti-microbial responses. One potent constituent of the innate immunity is contributed by the complement system. This system consists of a cascade of proteolytic enzymes that finally leads to the formation of a membrane attack complex that in turn results in the lysis of the pathogen. Three pathways are known to initiate the complement system: the classical, the alternative and the lectin pathway. Pattern-recognition molecules such as the collectin MBL or the ficolins activate the lectin pathway. The aim of the present work was to characterize the pattern recognition molecule FcnB of the mouse.

4.1. Expression of FcnB

The first ficolins that were described were isolated from the porcine uterus and named ficolin α and ficolin β (Ichijo et al., 1993). Ficolin α was found to be present in serum whereas ficolin β was identified to be expressed in the bone marrow (Ohashi and Erickson,1998) and to be secreted by PMNs (Brooks et al., 2003). This led to the classification of serum-type ficolins and non-serum-type ficolins. Many expression studies were done to identify the producer cells of the human ficolins.
During this work the expression of mouse FcnB was analyzed using RT-PCR analysis. Previous studies by Ohashi and Erickson in the year 1998 demonstrated the expression of mouse FcnB in the bone marrow and in the spleen (Ohashi and Erickson et al., 1998). It was shown that the cells expressing the mouse FcnB mRNA belong to the myeloid cell lineage (Liu et al., 2005a; Endo et al., 2010). These findings were confirmed by this thesis. To investigate the role of mouse Ficolin B in the immune system, it was necessary to analyze and determine the cell type of the mRNA expression. The identification of mouse FcnB producer cells or the general identification of its sources are possible hints for its function.

Results from this thesis demonstrate that bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMDM), as well as PMN were found to express FcnB. RT-PCR analysis demonstrated high expression levels of FcnB mRNA within the first days of cell culture of BMDM and BMDC. The expression decreased during differentiation leads to the assumption that the protein is expressed in immature cells and down-regulated during differentiation into mature macrophages. The expression of FcnB during the *in vitro* differentiation of BMDC from progenitor cells to mature dendritic cells peaked strongly around day 4 in non-stimulated cells and similarly declined during further differentiation. Neutrophils, generated *in vitro* from ERHoxb8 progenitor cells (Wang et al., 2006) facilitated the investigation of FcnB expression in PMN. RT- PCR results revealed that FcnB was present during the differentiation in Hoxb8-neutrophils peaking around day 3 of culture.

Due to the assumption that ficolins serve as pattern recognition molecule the expression was suspected to be enhanced after stimulation. Stimulation studies with various stimuli revealed that LPS as well as PMA/Ionomycine were potent stimulators for FcnB expression by Hoxb8-neutrophils. Also, the results lead to the conclusion that FcnB belongs to a group of proteins, where a rapid induction of the expression is important, due to the fact that the expression was increased within only 30min of stimulation. CpG and heat-aggregated IgG had no effect on FcnB expression.
LPS, as a component of the gram-negative bacteria cell wall, is a potent stimulus and often used for induction of mRNA expression. Therefore, LPS was expected to trigger FcnB expression, due to the fact that LPS is present in the cell wall of all Gram-negative bacteria and Ficolins serve as pattern recognition molecules for the detection of bacteria. CpG was used to analyze whether stimulation of the TLR-9 affects FcnB expression. Aggregated IgG was chosen for stimulation, because it is known to bind to Fc-receptors. Myeloid cells contain Fc-receptors on their surface. Therefore, it was assumed that a challenge of these Fc-receptors leads to induction of FcnB expression. PMA/Ionomycine is generally used for stimulation of PMN. In native PMN stimulation with LPS revealed a further increase of the FcnB expression while no reproducible and significant enhancement of FcnB expression was induced in neither BMDM nor BMDC. This can be explained by the fact that bone marrow progenitor cells are fully differentiated macrophages or dendritic cells, respectively, at the end of the culture. Additional activation stimulates these cells but does not change their maturation, thus, does not affect their differentiation status.

In vitro generated PMN, isolated from the bone marrow were investigated for their FcnB expression. RT-PCR experiments demonstrated expression of FcnB exclusively by $CD11b^+$ $Ly6C^+$ $Ly6G^+$ PMN. In contrast, no expression was observed within the immature monocytic cell population, characterized by $CD11b^+$ $Ly6C^-$ $Ly6G^+$. Thus, these data identified native PMN as source of FcnB among immature myeloid cells. The same expression patterns as seen for PMN from the bone marrow were also seen for PMN isolated from the spleen.

The expression of FcnB was investigated in mice, showing a permanent cellular environment of inflammation. These mice are deficient for the TNF receptor type 2 (TNFR2), which functions by the neutralization of soluble TNF (Tumor-necrosis-factor). Therefore, the lack of this receptor leads to constantly higher amounts of TNF within the body.
A comparison of FcnB expression levels in wild type and TNFR2-deficient mice revealed higher expression levels of FcnB even in non-stimulated BMDC from TNFR2-deficient mice. The analysis of BMDC subpopulations on day 4 clearly demonstrated that mainly immature myeloid cells ($CD11b^+$ $Ly6G^+$ $Ly6C^+$) produced FcnB mRNA. The higher FcnB expression levels found in mice with a constant inflammation suggests a role of FcnB under inflammatory conditions.
The mRNA of the human orthologue to mouse FcnB, M-ficolin, was found to be expressed by monocytes from the peripheral blood, in the lung, in the spleen, and also by type II alveolar epithelial cells (Liu et al., 2005; Liu et al., 2005b). In this thesis the data showed that immature macrophages and dendritic cells as well as PMN express mouse FcnB. While inflammatory stimuli can clearly enhance the FcnB expression in PMN no further enhancement was observed in mature macrophages or dendritic cells from bone marrow cultures of wild type mice. However, enhanced FcnB expression was also seen in dendritic cells derived from mice that lack TNFR2.
To sum up, immature myeloid cells expressed FcnB mRNA as already shown by Liu et al. (Liu et al., 2005). They also found that mouse FcnB is expressed in the bone marrow, the spleen, and also in the embryonic liver (Liu et al., 2005).

4.2 Detection of native FcnB

Monoclonal rat anti-mouse FcnB antibodies were generated by immunization of three rats with different genetic background with DS-2-expressed recombinant mouse FcnB-V5-His. Since the antiserum of the ACI rat and the Wistar rat showed the strongest antigen-specific-responses the spleen cells of these rats were used for fusion with myeloma cells. Eight monoclonal antibodies specific for FcnB were generated, all of which were of the IgG2b isotype. The generated monoclonal antibodies were functionally tested for detection of ligand-bound FcnB. Also, for a second assay system (sandwich-ELISA) different monoclonal antibodies were identified for “catching” and for “detection”. All generated monoclonal antibodies recognized FcnB in ELISA, in TRIFMA, in dot blot and in Western blot. Additionally, all eight monoclonal antibodies recognized the FcnB monomeric form appearing at around 35 kDa on Western blot under reducing conditions as well as higher oligomeric forms under non-reduced conditions. Interestingly, it was found, that one monoclonal antibody, generated in a previous immunization (rat anti-mouse FcnB antibody IA4; generated by Dr. Valeria Runza) seemed to recognize preferently the lower oligomers of FcnB (monomer, dimer, trimer), whereas all new monoclonal antibodies were able to detect also higher oligomers including pentamers, hexamers and heptamers. None of the monoclonal antibodies showed cross-reactivity with recombinant mouse FcnA.

Since mouse FcnB mRNA was identified to be expressed by myeloid cells, or more precise predominantly by immature cells of the myeloid lineage, lysates from various cell subpopulations of the myeloid cell lineage were analyzed by Western blot. Native FcnB was identified on the protein level in lysates of PMN as well as of BMDM from day 7 of differentiation. Differences between lysates from different mice became obvious: sometimes FcnB protein expression seemed to be inducible by LPS stimulation, whereas in cells from other mice, the protein production of FcnB seemed to be constant even in non-stimulated cells. A possible explanation for this may be that there was an ongoing inflammation in some mice. Also in BMDC generated from a mouse line showing a persistent inflammatory cellular environment (TNFR2-deficient mice) the expression of FcnB was higher compared to BMDC generated from wild type mice.

Like its human homologue M-ficolin, the mouse FcnB was also demonstrated to be associated with cells of the myeloid cell lineage (Liu et al., 2005). Human M-ficolin was shown to be expressed on the surface and in granules of monocytes and granulocytes (The et al., 2000; Frederiksen et al., 2005; Rorvig et al., 2009; Zhang et al., 2010). Therefore, it was discussed whether M-ficolin serves as a phagocytic

receptor (The et al., 2000). In 2006 Runza et al. analyzed peritoneal exudate macrophages and found that FcnB was expressed in mature macrophages which is similar to the finding of Liu et al. in 2005 (Runza et al., 2006). Intracellular immuno-stainings in permeabilized peritoneal macrophages revealed that FcnB is localized inside of these cells, that the amount enhanced after LPS stimulation, and that FcnB is co-localized with Lamp-1, which is associated with lysosomal membranes (Runza et al., 2006). The results from the present work that immature myeloid cells predominantly express the mouse lectin supplements these findings. In the present work another cell type of the myeloid cell lineage – the PMN - were investigated for the presence of native mouse FcnB by confocal microscopy. Using the FcnB-specific monoclonal antibodies native mouse FcnB was detected inside primary PMN and co-localized with the lysosomal markers Lamp-1 and LysoRed. The human M-ficolin has also been found to be localized inside of the cytoplasm of PMN (Liu et al. 2005a). These findings further indicate a role of FcnB inside of these cells and could indicate that these lectins might act as intracellular scavenger receptors (Runza et al., 2008). A new interesting finding was the ability of PMN to generate extracellular traps to catch and destroy bacteria, the so-called NET (neutrophil extracellular traps). Thereby also complement could be involved. When the question came up, whether FcnB is present within these NET structures. Investigations of PMN by confocal microscopy revealed the presence of FcnB inside PMN after stimulation with PMA but FcnB was not found to be involved in the NET structures.

Another question whether mouse FcnB is stored within these cells and whether it is released was tackled by further experiments. For a long time, there was a strict separation between serum-type and non-serum-type ficolins. In the case of the human M-ficolin the assumption existed that M-ficolin is not found in serum (Liu et al., 2005b) until new approaches with higher sensitivity revealed that M-ficolin is also found as a soluble protein in serum (Wittenborn et al., 2010; Honoré et al., 2008; Kjaer et al., 2011). Mouse FcnB was also investigated for its presence in serum but it was not detected (Ohashi et al., 1998; Liu et al., 2005a). When sera from different mouse strains were tested in this work by TRIFMA using specific monoclonal antibodies FcnB was detected in serum from Balb/c mice. The binding of this native FcnB from Balb/c serum to AcBSA was shown to be calcium-dependent. Sera of all other tested mouse strains were found to be negative. Interestingly, the demonstration of FcnB protein by confocal microscopy inside PMN was also successfully performed with cells from Balb/c mice. A possible explanation might be that this mouse strain produces exceptionally high amounts of FcnB. This might explain why no FcnB had been detected in serum in the studies performed by Ohashi et al. in 1998. Those experiments had been done with serum of C57BL/6

mice, which was also found to be negative for FcnB in this thesis. The reason why Balb/c mice seem to differ to C57BL/6 mice regarding their FcnB presence remains unclear to this date. Remarkable is that also the levels of MBL-A are higher in Balb/c mice compared to C57BL/6 mice whereas MBL-C levels are equal (Liu et al., 2001).

On protein level human M-ficolin was detected in peripheral blood monocytes (Lu et al., 1996b; Endo et al., 1996; The et al., 2000) localized inside of secretory, cytoplasmic granules of PMN and monocytes (Liu et al., 2005). The porcine ficolin β was also identified to be membrane-associated and to be present in the cytoplasmic compounds of PMN (Brooks et al., 2003). Using the newly generated monoclonal antibodies specific for mouse FcnB previous results from our group (Runza et al., 2006) 2006) were supported by demonstrating the presence of FcnB in lysates of PMN and bone marrow-derived macrophages and dendritic cells. Similar to mRNA expression, inflammatory stimulation seemed to increase the protein production. In native PMN from Balb/c mice FcnB was localized in lysosomal granules similarly as previously found for peritoneal macrophages (Runza et al., 2006). In addition, FcnB was detected in serum from Balb/c mice.

4.3. Binding-specificities of FcnB

Ficolins are known to bind as pattern recognition molecules to various compounds. Their fibrinogen-like domain affords the binding specificity to different ligands, preferently N-acetylated compounds (Endo et al., 2010). Many carbohydrate binding-specificities of ficolins were demonstrated to be calcium-dependent due to the presence of a calcium-binding site within fibrinogen-like domain. Common ligands found for all ficolins are AcBSA and GlcNAc. Using the newly generated anti-FcnB monoclonal antibodies also mouse FcnB was found during this thesis to bind to these ligands in a calcium-dependent manner. Controversial studies were reported for the human L-ficolin whether this interaction with GlcNAc is calcium-dependent (Matsushita et al., 1996; Endo et al., 2006) or calcium-independent (Ohashi and Erickson, 1997; Le et al., 1997).

One binding specificity of mouse FcnB demonstrated here is the recognition of fetuin, which has sialic acid as a constituent. This is in common with its human orthologue M-Ficolin, (Endo et al., 2006). This interaction appeared only marginally to be calcium-dependent but still could be reduced by EDTA in case of the mouse FcnB. Liu et al., reported in 2011 that M-ficolin bound to fetuin but there was no further investigation whether this interaction is calcium-dependent (Liu et al., 2005; Endo et al., 2006; Liu et al., 2011).

Recent data described that the binding of M-ficolin to Pentraxin 3, another pattern recognition molecule, is calcium-independent (Gout et al., 2011; Ma et al., 2009).

Ficolins and MBL function in the recognition of dying host cells and facilitate thereby their clearance. This has recently been described for mouse FcnB by our group (Schmid, Hunold et al., 2011).

In this regard, human L-ficolin was described to bind to DNA (Jensen et al 2006). During this thesis this ability was also verified for mouse FcnB. The interaction of recombinant mouse FcnB with DNA was further demonstrated by inhibition of the binding of FcnB to AcBSA by DNA in a dose-dependent manner.

For the first time it was demonstrated in the present work that recombinant mouse FcnB was able to bind to chitin. Chitin is a highly acetylated polymer of GlcNAc and is mainly found in the cell walls of fungi. The ability of mouse FcnB to recognize chitin leads to the assumption that the mouse FcnB might serve as a pattern recognition molecule during fungal infections. Binding to chitin was also demonstrated for the human M-ficolin (Jepsen et al., 2010, Danish Society of Immunology Annual Meeting, May 25, 2010, unpublished data).

In addition, recombinant mouse FcnB was also tested by TRIFMA for its binding ability towards various serotypes of *Staphylococcus aureus* and *Streptococcus*

Agalactiae (Group B *Streptococcus*, GBS). Bacteria of the Group B *Streptococcus* are pathogenic causing infections. They are also Gram-positive, coccal microorganisms. Surface patterns as exhibited by this group of bacteria are recognized by various recognition molecules, among others by ficolins.

FcnB was demonstrated to bind to nearly all tested serotypes of GBS (11 out of 13) except the serotypes GBS B848/64 and GBS VIII. An expectation was that FcnB might not bind to the non-capsulated serotype B848/64, due to the fact that ficolins are predicted to recognize structures within the capsule.

Also the human M-ficolin was demonstrated to bind to GBS with the only exception of the non-capsulated strain GBS B848/64 (Kjaer et al., 2011). That human M-ficolin as well as its mouse orthologue FcnB were able to recognize nearly all tested GBS serotypes might be explained by the fact that sialic acid is a terminal side-chain residue within the capsule of the GBS (Wessels et al., 1997; Miyake et al., 2004; Kjaer et al., 2011). Both ficolins were demonstrated to bind this ligand.

The observation that FcnB was not able to bind to the GBS serotype VIII was also interesting because also the human M-ficolin exhibited a lower binding ability to this strain compared to all other investigated serotypes. This was argued by a different accessibility of the terminal sialic acid residue in this serotype (Kjaer et al., 2011).

Interactions between the FcnB and bacteria of *Staphylococcus aureus* strains were also analyzed. *S. aureus*, is a microorganism with a coccal form. It belongs to the group of Gram-positive bacteria. Infections with this specific type of *staphyloccocus* often lead to diseases, ranging from being harmful to life threatening.

Only binding of FcnB to the *S. aureus* serotype T-5, but no binding to any other *S. aureus* serotype was observed in this thesis. Among all capsular serotypes that have been described so far, most isolates of *S. aureus* belong to the capsule type 5 or 8 (Karakawa and Vann, 1982; Arbeit et al., 1984; Hochkeppel et al., 1987; Sompolinsky et al., 1985; Thakker et al., 1998). Thereby the capsule type 5 *S. aureus* is also more virulent for mice compared to type 8 *S. aureus* due to a higher production of capsular polysaccharide (Thakker et al., 1998; Watts et al., 2005). Also the *S. aureus* serotyp 5 capsular polysaccaride (T-5) showed a greater degree of N-acetylation and this might be a ligand for FcnB (Watts et al., 2005).

No binding to any tested *S. aureus* serotype was observed for the human M-Ficolin (Kjaer et al., 2011). Liu et al. demonstrated binding of M-ficolin to a clinical *S. aureus* isolate (Liu et al., 2005). However, it is necessary to mention that the M-ficolin binding studies differed in the way of the bacterial preparation and also in the type of the *S. aureus* that was used for the experiments. Thus, FcnB might be able to serve as a pattern recognition molecule during *S. aureus* infection.

Summarizing, mouse FcnB shares many binding properties with its human and rat orthologues (Girija et al 2011; Gout et al 2010) and might act as a potent pattern recognition molecule by recognizing sialic acid (present in the glycoprotein fetuin) DNA (potentially a target in dying cells), and chitin (potentially a target in fungi). The fact that FcnB binds to DNA might be of importance concerning the finding that PMN have stored FcnB and produce NET structures upon stimulation which contain DNA. Furthermore, recombinant FcnB bound to various strains of GBS and to serotype T-5 of *S. aureus*.

4.4 Complement activation by mouse FcnB

Ficolins trigger the complement activation via the lectin pathway due to complex formation with MASPs (Matsushita and Fujita, 1992; Matshushita et al., 2000; Liu et al., 2005a; Endo et al., 2010; Girija et al., 2007).
While various studies were published concerning complement activation by the human ficolins, mouse FcnB and its role in the lectin pathway is only poorly characterized (Girija et al., 2007). The only investigations in this regard were done by the Japanese group around Fujita and Endo (Endo et al., 2005).
Therefore, one aim of this work was to investigate the role of mouse FcnB in complement activation using newly generated tools.
Complex formations of recombinant mouse FcnA with recombinant mouse MASP-2 was demonstrated by Endo et al. 2005, while previous studies by the same group had shown that recombinant mouse FcnB, also produced by insect cells, failed to form complexes with MASP-2 or MAp19 (Endo et al., 2005). In contrast, rat FcnB was shown to activate MASP-2 and subsequently initiate the lectin pathway of the complement system (Girija et al., 2011). Since mouse FcnB failed to activate the lectin pathway of the complement system (Endo et al., 2005) the putative MASP-binding sites were analyzed in human and in other mammalian ficolins (Wallis et al., 2011). The results revealed that all ficolins except the mouse FcnB contain an aliphatic/hydrophobic amino acid residue within the putative MASP-2 binding site, which is located inside the collagen-like region. In contrast, in mouse FcnB this amino acid is replaced by a glutamate residue (see Fig. 79) (Wallis et al., 2011). In substitution experiments with recombinant rat FcnB by Wallis et al. the mouse FcnB residue in this position was replaced by the rat FcnB amino acid which led again to activation of the complement system supporting the importance of this amino acid shift in the mouse FcnB for complement activation (Wallis et al., 2011).

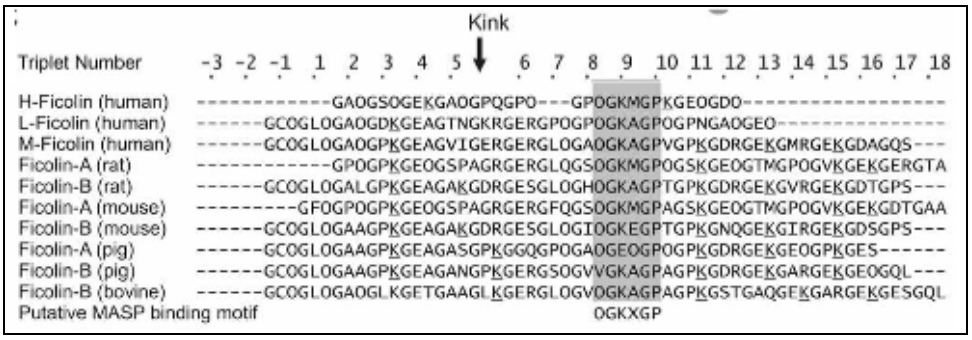

Figure 79: Sequence alignments of the collagen-like domains of ficolins.

The putative MASP-binding motif (grey shaded) is included within the collagen-like region of the ficolin. (Modified after Girija et al. 2007).

However, Endo et al. reasoned that also mouse FcnB should be able to activate the lectin pathway because also mouse FcnB seems to be endowed with the required amino acid residues for the interaction with MASP-2 (Endo et al. 2005). This is conflicting with their former mentioned finding that their recombinant mouse FcnB failed to form complexes with MASP-2 (Endo et al. 2005). In this thesis it was clearly demonstrated that our S2-expressed mouse FcnB not only bound to recombinant human MASP-2 but was also able to activate the lectin pathway of the complement system by cleaving human C4 to C4b in a C4 deposition assay by TRIFMA. This was shown when FcnB was bound to AcBSA or when it was bound to various bacteria. These controversial findings concerning the ability of mouse FcnB to activate the complement system by our group and the group of Endo is potentially due to the multimeric structure of the used protein. In literature it is discussed that the oligomerization of the ficolin is required to be functionally active (Endo et al. 2005). Endo et al reported that their recombinant mouse FcnB only forms trimers (Endo et al. 2005). We demonstrate in this work clearly that our recombinant protein when freshly produced by insect cells exist in oligomers up to 200 kDa and higher assuming the formation of hexamers (210 kDa), heptamers (245 kDa) and octamers (280 kDa). In Fig. 80 a comparison of the DS-2-expressed mouse FcnB from our group as well as FcnB published by the Japanese group is shown after gel separation.

We observed with our recombinant FcnB that it was not stable over time and degraded. Higher oligomers of ficolin, however, are necessary for the function of those molecules.

A) B)

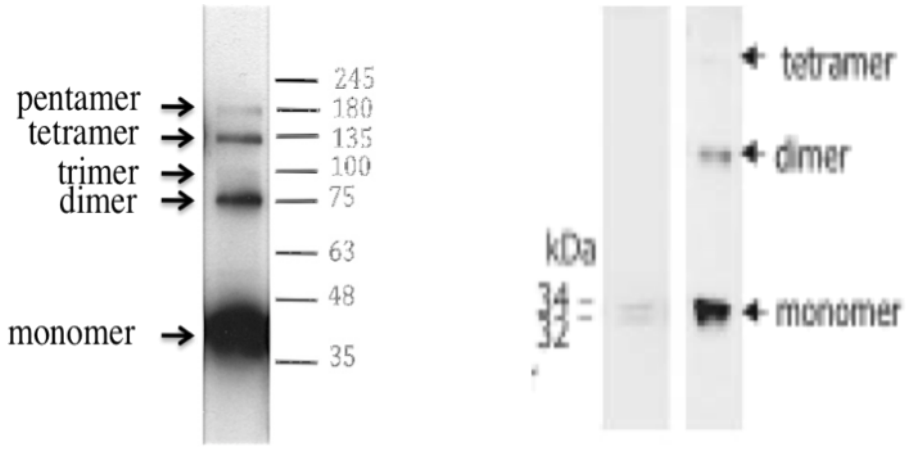

Figure 80: Oligomeric structure comparison of DS-2-expressed recombinant mouse FcnB.
Multimerization of recombinant in insect cells produced mouse FcnB (A) under non-reduced conditions by our group and (B) under reduced conditions (left) and under non-reduced conditions (right) by the group of Endo (Endo et al. 2005).

In summary in this thesis it was demonstrated for the first time that mouse FcnB is able to form complexes with MASP-2 and subsequently cleaves C4. Contradictory results regarding to the role of mouse FcnB within the complement system are probably due to the different oligomerization forms of the used FcnB. Further it was remarkable that recombinant mouse FcnB was able to interact with recombinant human MASP-2 leading to the assumption that assays using recombinant mouse MASP-2 instead of the human orthologue might result in even more efficient C4-deposition. Sequence homologies between the human and the mouse MASP-2 revealed at least 83% similarity in their amino acid sequences.
Recently also a mutated FcnB was produced in our group. Thereby the corresponding glutamic amino acid within the putative MASP-2 binding site was changed to a alanine, like it is found in rat FcnB. Further experiments with the wild type and the mutant FcnB are necessary to determine if this mutant FcnB is more effective in complement activation or if the lower efficiency in activating the lectin pathway by mouse FcnB compared to its human and rat orthologue is possible due to a different role of FcnB in the innate immune system, beside the complement activation.

5. Summary

The innate immune system contributes day by day to the elimination of invading pathogens. The lectin pathway of the complement system is initiated when mannose-binding-lectin (MBL) or ficolins interact with MASP-2.
The aim of the present work was to characterize the mouse FcnB and define its function in the innate immune system and especially its role in the complement activation.
Results from this thesis revealed that immature macrophages and dendritic cells as well as PMN express mouse FcnB (immature myeloid cells). While inflammatory stimuli can clearly enhance the FcnB expression in PMN no further enhancement was observed in mature macrophages or dendritic cells from bone marrow cultures of wild type mice, but was also seen in dendritic cells derived from mice that lack TNFR2.
Previous results from our group (Runza et al., 2006) were supported by demonstrating the presence of FcnB on protein level in lysates of PMN, bone marrow-derived macrophages and dendritic cells, using newly generated monoclonal antibodies specific for mouse FcnB. In native PMN from Balb/c mice FcnB was localized in lysosomal granules similarly as previously found for peritoneal macrophages (Runza et al., 2006). In addition, in serum from Balb/c mice FcnB was detected.
Further, binding properties of FcnB to various ligands were investiagted in this thesis. Our results indicate that FcnB might act as a potent pattern recognition molecule by recognizing sialic acid (present in the glycoprotein fetuin), DNA (potentially a target in dying cells) and chitin (potentially a target in fungi). The fact that FcnB binds to DNA shown in this thesis might be of importance concerning the finding that PMN have stored FcnB and produce NET structures upon stimulation, which contain DNA. Furthermore, recombinant FcnB bound to various strains of GBS and to serotype T-5 of *S. aureus*.
One major function of ficolins is their interaction with MASP-2 what subsequently leads to the activation of the lectin pathway. In this thesis it was demonstrated for the first time that also mouse FcnB is able to activate the lectin pathway. Contradictory results regarding the role of mouse FcnB within the complement system are probably due to different oligomerization forms of the used FcnB.

All results obtained during this work lead to the conclusion that mouse FcnB plays a role in the innate immune system, serving as a pattern recognition molecule for the detection of microorganisms and activating the complement system by binding to MASP-2, like the other ficolins or MBL.

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V. Appendix

List of publications:

Krausse D, Hunold K, Kusian B, Lenz O, Stülke J, Bowien B, Deutscher J., (2009). Essential role of the hprK gene in Ralstonia eutropha H16. *J. Mol. Microbiol. Biotechnol.*, 2009;17(3):146-52. Epub 2009 Aug 6.

Schmid*, M., Hunold*, K., Weber-Steffens, D., Männel, D. N., (2011). Ficolin-B marks apoptotic and necrotic cells. *Immunobiology,* (in Press, November, 2011).

Poster presentations:

9/2011:

Conference „ 41th Jahrestagung der Deutschen Gesellschaft f. Immunologie (DGfI) und der italienischen Gesellschaft f. Immunologie, Klinische Immunologie und Allergologie (SIICA), Riccione, Italien, 28 Sept - 1 Okt 2011.

08/2011:

Conference „13th European Meeting on Complement in Human Disease“, Leiden, Niederlande, 21 Aug - 24 Aug 2011.

09/2010:

Conference „40th Jahrestagung der Deutschen Gesellschaft für Immunologie (DGfI), Leipzig, Deutschland, 22 Sep - 25 Sep 2010.